The role of TRPM2 channels in oxidative stress-induced liver damage

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List of Abbreviations

aa  amino acid
Ab  antibody
ACA anthranilic acid
ADP adenosine diphosphate
ADPR adenosine diphosphate ribose
ALT alcoholic liver disease
AMAP acetyl-m-aminophenol
AMP adenosine monophosphate
ANOVA analysis of variance
AP apurinic/apyrimidinic
APECED autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
AST aspartate aminotransferase
ATP adenosine triphosphate
BER base excision repair
BSA bovine serum albumin
cADPR cyclic-ADPR
CaM calmodulin
CATs catalases
CNS central nervous system
COPD chronic obstructive pulmonary disease
COX cyclooxygenase
DAG diacylglycerol
DCDPC dichlorodiphenylamine-2-carboxylic acid
DDW double distilled water
DM diabetes mellitus
DMEM Dulbecco’s Modified Eagle Medium
DMSO dimethyl sulfoxide
DPQ 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone imine</td>
</tr>
<tr>
<td>NFW</td>
<td>nuclear free water</td>
</tr>
<tr>
<td>NIF</td>
<td>niflumic acid</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-Methyl-D-glucamin</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthases</td>
</tr>
<tr>
<td>NUDT9-H</td>
<td>Nudix-type motif 9 homology</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>OTRPC</td>
<td>Osm TRP Channels</td>
</tr>
<tr>
<td>PBP</td>
<td>para-bromophenacyl bromide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGs</td>
<td>prostaglandins</td>
</tr>
<tr>
<td>PMN</td>
<td>poly-morphonuclear</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SPP</td>
<td>short-patch pathway</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplastic reticulum</td>
</tr>
<tr>
<td>SSB</td>
<td>single-strand break</td>
</tr>
<tr>
<td>STRPC</td>
<td>short TRP channels</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TRPML</td>
<td>transient receptor potential mucolipin</td>
</tr>
<tr>
<td>TRPP</td>
<td>transient receptor potential polycystin</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Abstract

The increased production of highly reactive oxygen and nitrogen species plays a significant role in development of a number of liver disorders associated with hepatocellular death and impaired cell regeneration. Liver injury induced by drug toxicity, ischemia-reperfusion, excessive alcohol consumption and different types of viral hepatitis is in large part mediated by oxidative stress. Liver damage due to oxidative stress induced by drugs, including acetaminophen, accounts for 5% of all hospital admissions and for almost half of all acute liver failures.

One of the features of hepatocellular death mediated by oxidative stress is Ca\(^{2+}\) overload due to its release from intracellular organelles and activation of ion channels on the plasma membrane. Ca\(^{2+}\) is fundamental for normal cellular functioning. Ca\(^{2+}\) signalling, mediated by the rise in free cytoplasmic Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_c\)), regulates many cellular events. However, a sustained rise in \([\text{Ca}^{2+}]_c\) can be detrimental, leading to mitochondrial dysfunction and cell death through apoptosis and necrosis. Although it is well recognised that Ca\(^{2+}\) plays a significant role in oxidative stress-induced liver damage, the molecular identities of the ion channels that provide a pathway for Ca\(^{2+}\) entry in hepatocytes remain unidentified.

One of the potential candidates that could be responsible for such Ca\(^{2+}\) entry pathway in hepatocytes is Transient Receptor Potential Melastatin 2 (TRPM2) channel. TRPM2 is a non-selective cation channel permeable to Na\(^{+}\) and Ca\(^{2+}\). The main physiological activator of TRPM2 channel is ADP-ribose, which binding to NUDT9-H motif in the TRPM2 C-terminus leads to the opening of the channel pore. It is known that oxidative stress promotes generation and release of ADPR from mitochondria and nuclei into the cytoplasmic space, thus promoting activation of TRPM2-mediated Ca\(^{2+}\) entry.

In this thesis, we hypothesised that oxidative stress-induced Ca\(^{2+}\) entry in hepatocytes is mediated by TRPM2 channels, and used acetaminophen overdose as a model of oxidative stress-induced liver damage. We show that hepatocytes express long isoform of TRPM2, which mediates ADPR- and H\(_2\)O\(_2\)-induced Ca\(^{2+}\) entry and the cation current in these cells. Furthermore, we show that TRPM2 channels are activated in hepatocytes treated with high concentrations of acetaminophen and are responsible for Ca\(^{2+}\) overload in acetaminophen-induced liver toxicity. Experiments using TRPM2 KO mice provide first evidence of a pivotal role of TRPM2 channels in acetaminophen-induced
liver injury, showing that lack of TRPM2 expression largely protects liver from acetaminophen overdose.

An important finding that TRPM2 channels translocate from intracellular compartments to the plasma membrane provides explanation for a slow development of Ca\(^{2+}\) entry in response to H\(_2\)O\(_2\) and acetaminophen.

Finally, we show that substances previously known to protect liver from acetaminophen-induced damage are, in fact, inhibitors of TRPM2 current. Chlorpromazine, an antipsychotic drug, reversibly blocks TRPM2 channel pore, and curcumin, a chemical found in common spice, potently blocks activation of TRPM2 current by ADPR.

The results presented in this thesis provide a fundamental knowledge about the role of TRPM2 channels in oxidative stress-induced liver injury, but also open a new chapter in search for the new drugs and drug targets for the treatment of a number of oxidative stress-related liver pathologies.
Declaration of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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AUTHOR STATEMENTS

Chapter 2 & 3:

“The Role of the TRPM2 Channel in Acetaminophen-induced Hepatocellular Damage”

“The Lack of TRPM2 Channel–prevented Acetaminophen-induced Hepatocellular Damage in Mice”

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The main results of these chapter were published as a part of manuscript: “TRPM2 channels mediate acetaminophen-induced liver damage” Proceedings of the National Academy of Sciences of the USA, vol. 111, pp. 3176-3181 (Appendix).

The authors’ responsibilities were as follows:

Ehsan Kheradpezhouh was responsible for the conception and design of the study, collection and assembly of data, data analysis and interpretation, and writing and preparation of the manuscript.

Linlin Ma contributed to design and collection western blot and PCR blot data, and data analysis and interpretation.

Arthur Morphett contributed to design of histopathologic examination of liver tissue samples.
Greg Barritt contributed to the conception and design of the study, data interpretation and preparation of the manuscript.

Grigori Rychkov was responsible for the conception and design of the study, collection of data, data analysis and interpretation, writing and preparation of the manuscript, and acted as the corresponding author.

Authors Signatures:
I agree with the author contributions for the manuscript “TRPM2 channels mediate acetaminophen-induced liver damage”, and give permission for the use of this manuscript in the thesis.

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Arthur Morphett .................................................................

Greg Barritt .........................................................................

Grigori Rychkov ................................................................
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To Mana for her unconditional love and support
Chapter 1: Research Background

1.1 Introduction

Calcium signalling regulates a vast assortment of functions in all cells, ranging from cell proliferation and differentiation to apoptosis and irreversible cell damage. Calcium signalling occurs through a rise in the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)), which is usually maintained around 100 nM. This rise in [Ca\(^{2+}\)]\(_c\) is mediated by the entry of extracellular Ca\(^{2+}\) through various channels, or the release of stored Ca\(^{2+}\) from the endoplasmic/sarcoplasmic reticulum (ER/SR), Golgi apparatus and mitochondria. Cells usually tightly control [Ca\(^{2+}\)]\(_c\) using a variety of different channels, pumps and transporters to regulate the movement of Ca\(^{2+}\) across the plasma membrane or between the aforementioned intracellular organelles. Failure of these mechanisms can result in uncontrolled elevation of [Ca\(^{2+}\)]\(_c\), which can lead to irreversible cell damage and death.

One process in which it is well recognised that Ca\(^{2+}\) signalling and high [Ca\(^{2+}\)]\(_c\) have a fundamental role is that of cell damage mediated by oxidative stress. Oxidative stress is a result of the disruption of the balance between the production of oxidative compounds and natural cellular antioxidant activity. Cellular oxidative compounds can be divided into two main groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are produced naturally during mitochondrial respiration, and are mainly detoxified by cellular antioxidants, such as superoxide dismutase and glutathione. ROS, such as the superoxide anion (O\(_2^-\)), a highly reactive compound), are generated during energy production and can generate hydrogen peroxide (H\(_2\)O\(_2\), less toxic than O\(_2^-\)) or react with nitric oxide (NO) to produce RNS, especially peroxynitrite. During oxidative stress, ROS and RNS accumulate in the cell and, through various mechanisms, disrupt cellular functioning.

Rises in [Ca\(^{2+}\)]\(_c\) is one of the essential processes occurring during oxidative stress. It leads to an increase in free Ca\(^{2+}\) in the mitochondria and nuclei. Accumulation of free Ca\(^{2+}\) in the mitochondria disrupts mitochondrial function, especially the electron transport system, resulting in generation of more ROS, and placing the affected cell in a vicious cycle that results in cell death. High [Ca\(^{2+}\)]\(_c\) can by itself activate cell-destructive enzymes, including caspases, particularly caspase 8, leading to irreversible damage and death. While release of Ca\(^{2+}\) from intracellular stores contributes to ROS-
mediated rise of $[\text{Ca}^{2+}]_c$, the main source is the inward movement of free $\text{Ca}^{2+}$ from extracellular fluid through various $\text{Ca}^{2+}$ channels on the plasma membrane (Persoon-Rothert et al. 1994). Transient receptor potential (TRP) channels have been found to play a significant role in ROS-induced $[\text{Ca}^{2+}]_c$ increases (Miller 2006).

The superfamily of TRP channels is a diverse group of non-selective cation channels permeable to $\text{Ca}^{2+}$. These channels have been categorised into six subfamilies, designated C (canonical), V (vanilloid), M (melastatin), A (ANKTM), P (poly-cystin) and ML (mucolipin). TRP channels activated by ROS include TRPC3 and 4 from the TRPC subfamily; TRPM2 and 7 from the TRPM subfamily; and TRPV1 from the TRPV subfamily (Bogeski et al. 2011; Takahashi et al. 2011; Miller & Zhang 2011; Miller 2006). One of these, the TRPM2, is often referred to as an ‘oxidative stress–activated cation channel’ (Takahashi et al. 2011).

TRPM2 is mainly expressed in the brain, bone marrow, pancreas, spleen, liver and heart (Fonfria et al. 2006). Similarly, to other TRP channels, it has six transmembrane (TM) domains with intracellular N- and C-termini. The C-terminus holds a domain with adenosine diphosphate (ADP) ribose (ADPR) pyrophosphatase activity, and a Nudix (nucleoside diphosphate linked moiety X) box, called ‘Nudix-type motif 9 homology’ (NUDT9-H) (Perraud et al. 2001). During oxidative stress, free ADPR is produced by hydrolysis of NAD$^+$ and cyclic-ADPR (cADPR) in various cellular compartments; this ADPR interacts with the NUDT9-H of the TRPM2 channel, gating the channel through an unknown mechanism. Open TRPM2 channels allow $\text{Ca}^{2+}$ to enter the cell, and increase free $[\text{Ca}^{2+}]_c$.

It has been proposed that ROS production and oxidative stress are the key underlying cause of many pathological processes, such as aging, cancer, neurodegenerative disorders (including Alzheimer’s disease), diabetes mellitus (DM), ischemia-reperfusion injury and many cardiovascular problems (such as atherosclerosis and myocardial infarction). It is also believed to be involved in the damaging effects of a range of toxicities, including drug toxicities. Overproduction of ROS is a cause of the toxicity of many drugs, including analgesic and anti-inflammatory drugs, such as acetaminophen and diclofenac; anti-neoplastic drugs, particularly doxorubicin and cisplatin; antipsychotic chlorpromazine; and anti-retroviral agents, such as azidothymidine (Deavall et al. 2012). Often, it is a metabolite of the drug—rather than the drug itself—that has a major role in the toxicity (Bissell et al. 2001). In this case, the liver is the main organ metabolising the drugs, and is thus the most susceptible to
damage organ by those metabolites (Bissell et al. 2001; Leung, Kalgutkar & Obach 2012).

The liver performs a multitude of functions, including synthesis of many proteins, as well as immunoglobulins, synthesis and homeostasis of lipids, storage and homeostasis of glucose, production of bile and detoxification of many endogenous and exogenous compounds. Due to this range of functions, the liver is a highly aerobic and oxygen-dependent tissue, and is very susceptible to hypoxia and toxic insults (Malhi, Guicciardi & Gores 2010).

Acetaminophen (paracetamol; N-acetyl-p-aminophenol) is one of the most widely used antipyretic and analgesic drugs. Due to its unrestricted availability, acetaminophen toxicity is one of the leading causes of emergency attendance for drug poisoning (Khan, Oniscu & Powell 2010). In fact, acetaminophen toxicity is the major cause of acute liver failure, requiring liver transplantation (Craig et al. 2010; Khan, Oniscu & Powell 2010). Despite extensive research, the exact mechanisms of acetaminophen toxicity remain unclear. Nevertheless, the central role of acetaminophen metabolites that cause oxidative damage in liver and kidneys is well recognised (Letelier et al. 2011). In an overdose, the regular pathways of acetaminophen conjugation and excretion are saturated, resulting in metabolism of acetaminophen by various cytochrome P450 enzymes (mainly CYP2Es and CYP2E1) in hepatocytes to several compounds, some of which are powerful oxidants (Burke, Macmillan-Crow & Hinson 2010; Letelier et al. 2011; Wolf et al. 2007). The major metabolite of acetaminophen with oxidative properties is N-acetyl-p-benzoquinone imine (NAPQI). The possible mechanism of acetaminophen toxicity is through accumulation of NAPQI and resulting oxidative stress. Increases in $[\text{Ca}^{2+}]_c$ observed in acetaminophen overdose may also play a significant role in development of liver damage, however, the molecular pathways leading to $\text{Ca}^{2+}$ rise remain unclear (Burcham & Harman 1991; Andringa et al. 2008).

This review analyses the current knowledge about (i) the role of TRPM2 channels in oxidative damage–induced cell death, and (ii) the mechanisms of acetaminophen toxicity in the liver. This analysis leads to the main hypothesis of this thesis—that $\text{Ca}^{2+}$ entry mediated by TRPM2 channels contributes to ROS-induced hepatocellular damage. Chapters 2 and 3 investigate the role of TRPM2 channels in the increase of intracellular $\text{Ca}^{2+}$ concentration mediated by acetaminophen overdose, and the protective effects of TRPM2 channel blockers and a reduction in TRPM2 expression in acetaminophen-mediated hepatocellular damage. The results presented in Chapter 4
demonstrate that TRPM2 channels are trafficked from intracellular compartments to the plasma membrane in response to oxidative damage, and Chapter 5 investigates the blocking effects of curcumin and chlorpromazine on TRPM2 channels.

1.2 Oxidative Stress

In general, the electrons of atoms are in pairs. Oxidation is the process of removing an electron, while reduction is the process of gaining an electron. The process of transferring an electron is called a ‘redox’ (reduction-oxidation) reaction. Radicals are molecules that are capable of oxidising other components and usually have a free electron, such as O$_2$· and hydroxyl radical (OH’). However, there are molecules, such as H$_2$O$_2$, that have electrons in pairs, but can easily take electrons from others and oxidise them. All the molecules with oxidising capability are called ‘reactive species’. Reactive compounds involved in oxidative stress are separated into two groups: ROS and RNS.

ROS include O$_2$·-, H$_2$O$_2$, OH’, singlet oxygen and peroxyl radical (LOO’). The major source of production of O$_2$·- is the mitochondrial membrane electron transport system. The highly reactive O$_2$·- radicals are converted to H$_2$O$_2$ by the enzyme superoxide dismutase (SOD). As well as being much less toxic than O$_2$·-, H$_2$O$_2$ is also easily diffusible through cell membranes. In turn, it is converted into several innocuous compounds by enzymes such as catalase and glutathione peroxidase. H$_2$O$_2$ can also be reduced to OH’ in the presence of transition elements, especially Fe$^{2+}$. OH’ is highly reactive and interacts with any molecule close to the site of its generation. Singlet oxygen—another ROS—is generated in phototoxic conditions with a photosensitiser pigment. The energy transferred to the oxygen produces a highly reactive compound that initiates lipid peroxidation (LPO) and damages the cell (Delaney & Glatstein 1988). LOO’ is generated during the breakdown of lipids in the cellular plasma membrane, and is a highly reactive compound.

RNS are diverse nitric oxide–derived molecules, such as nitric oxide (NO’), dinitrogen trioxide (N$_2$O$_3$), nitrogen dioxide (NO$_2$’), nitroxyl (HNO) and peroxynitrate (ONOO’). NO’ is the main RNS, and is produced by enzymes called ‘nitric oxide synthases’
(NOS). NO’ is also the main source of other RNS (Martinez & Andriantsitohaina 2009).

1.2.1 Systems Eliminating Oxidative Compounds

There are several enzymes and molecules that remove ROS and RNS; these enzymes are the SODs, catalases (CATs) and peroxidises. SODs are the first line for detoxification of ROS. In mitochondria, SOD is responsible for reducing the highly reactive \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) (which is much less toxic and can be tolerated by cells in much higher amounts than \( \text{O}_2^- \)). The SOD family consists of different enzymes, and is either divided according to the cellular regions of highest expression—into SOD1 (cytoplasm and nuclei), SOD2 (mitochondria) and SOD3 (extracellular)—or according to the metals found in the enzyme structure—into those containing copper and zinc (CuZnSOD) and those containing manganese (MnSOD). CuZnSODs are homodimetric enzymes found mainly in the cytoplasm and nuclei and, in much lower amounts, the mitochondria. The glycosylated form of the enzyme is also found in extracellular fluids. In contrast, MnSOD are exclusively located in the mitochondria of oxygen-metabolising cells (Miriyala, Holley & St Clair 2011; Zelko, Mariani & Folz 2002). MnSOD is the main SOD detoxifying the \( \text{O}_2^- \) produced in oxidative stress and in high concentrations of \( \text{H}_2\text{O}_2 \) (Buettner 2011).

Although \( \text{H}_2\text{O}_2 \) is less toxic to cells than \( \text{O}_2^- \), high concentrations cannot be tolerated. Reduction of \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \) and \( \text{O}_2 \) is catalysed by other enzymes, such as CATs. These enzymes are predominantly \( \text{Fe}^{3+} \)-containing enzymes consisting of four peptides that interact with two molecules of \( \text{H}_2\text{O}_2 \) to produce two molecules of \( \text{H}_2\text{O} \) and one molecule of \( \text{O}_2 \) (Nicholls 2012). Peroxidases, especially glutathione peroxidase (GPx), are other enzymes that neutralise \( \text{H}_2\text{O}_2 \). GPx is a family of four isoenzymes that catalyse the interaction of glutathione (GSH) with \( \text{H}_2\text{O}_2 \) or other hydroperoxides, such as lipid hydroperoxides, to produce water or an alcohol (Finkel & Holbrook 2000; Margis et al. 2008).

There are also some low molecular weight antioxidants present in the cells that neutralise ROS and RNS, including GSH (the most abundant), ascorbic acid (Vitamin C) and \( \alpha \)-tocopherol (Vitamin E) (Valko, Morris & Cronin 2005; Valko et al. 2006). The amounts of these antioxidants vary between cell types.
1.2.2 Cellular and Molecular Targets of Oxidative Stress

Oxidative stress can be divided into either exogenous or endogenous, depending on the source of the reactive compounds involved. In exogenous oxidative stress, the cell is exposed to a large amount of oxidative compounds generated outside the cell, such as pollutants, tobacco smoke, radiation and iron salts. In contrast, intrinsic oxidative stress is mediated by the overproduction of ROS and RNS in the cell. The mechanisms involved in oxidative stress–mediated cellular damage are the same in both intrinsic and extrinsic pathways: reactive species accumulate in cells, reacting with nearly all components of the cell, including lipids, proteins and nucleic acids, and subsequently disrupting their functions (Sohal, Mockett & Orr 2002; Hoye et al. 2008).

One of the main functions of cellular lipids is in maintaining the structural integrity of cellular membranes. These membranes are usually composed of phospholipids, cholesterol, cholesterol esters and fatty acids, along with a variety of proteins. ROS, especially OH·, can oxidise the fatty acids, producing lipid peroxyl radicals. In turn, these can generate a variety of aldehydes, such as 4-hydroxy-2-nonenal (HNE)—one of the most toxic aldehydes. These aldehydes can also be generated from other lipids in the cell membrane, such as arachidonic acid (ArAc) or linoleic acid, upon interaction with peroxides. Aldehydes, especially HNE, disrupt the function of the cell membrane, but are also able to diffuse from the plasma membrane and affect other cellular components, including the nuclear membrane and mitochondria (Radak et al. 2011; Sohal, Mockett & Orr 2002).

Proteins are also susceptible to oxidative damage by ROS. While all amino acids are susceptible to damage, the sulphur-containing amino acids, methionine and cysteine, are the most affected. Oxidation of amino acids, which happens post-translation, alters protein function. Accumulation of large quantities of these altered proteins impairs cellular functioning, leading to irreversible cell damage.

Both ribonucleic acid (RNA) and DNA are susceptible to being damaged during oxidative stress. With respect to RNA, both messenger RNA (mRNA) and ribosomal RNA (rRNA) are very vulnerable to oxidative stress; however, unlike DNA, oxidative damage to these RNAs does not result in cell death (Kong & Lin 2010). The susceptibility of other types of RNA, including transfer RNA (tRNA) and micro RNA (miRNA), to oxidative damage still requires further investigation. The guanine
nucleotide of RNA is the most vulnerable to ROS-mediated damage, especially by OH’, which modifies the guanine, resulting in production of 8-hydroxyguanosine (8-OHG)—a marker of oxidative stress (Kong & Lin 2010). mRNA modified by oxidative stress fails to be translated, and protein synthesis is diminished. Similar consequences occur following ROS modification of rRNA (Ding et al. 2005).

DNA is the most important cellular component that is vulnerable to ROS. While other cellular components—RNA, proteins and lipids—can be regenerated in the cell, DNA cannot be regenerated, and damaged DNA must be repaired (Chakravarti & Chakravarti 2007; Evans, Dizdaroglu & Cooke 2004; Lyras et al. 1998; Hegde et al. 2012). ROS production during mitochondrial respiration is the main cause of DNA damage—more so than ultra-violet (UV) light, ionising radiation (IR), toxins and chemicals (Furukawa et al. 2010; Cramers et al. 2012).

Several distinct DNA lesions are generated by ROS, with the most common ones being oxidised DNA bases, abasic sites, single-strand breaks (SSBs) and double-strand breaks (DSBs) (Hirota et al. 2010). DNA damage mediated by oxidative stress occurs more often in mitochondrial DNA than in nuclear DNA (Yakes & Van Houten 1997); it has been suggested that this is due to the localisation of mitochondrial DNA (mDNA) near the inner mitochondrial membrane (the place most ROS are generated) (Aamann et al. 2010). The sensitivity of different DNA bases to ROS is different, with guanine more sensitive than other bases due to its low redox potential, and guanine oxidation to 8-oxoG the most abundant kind of base damage (Hirano et al. 1996).

DNA damaged by oxidative stress needs to be repaired for regular cell functioning. There are several DNA-repairing mechanisms in the cell. The main repair mechanism for ROS-mediated DNA damage is base excision repair (BER) (Hegde et al. 2012). The initial step of BER is the cleavage of an N-glycosyl bond between the damaged base and the sugar by DNA glycosylases. As a result the abasic site is formed, called an ‘apurinic/apyrimidinic’ (AP) site.

The generated AP sites are predominantly removed by the enzyme AP Endonuclease1 (APE1), although other enzymes—such as polynucleotide kinase and 8-oxoguanine glycosylase 1 (OGG1)—also play a role. These enzymes incise the DNA strand 5’ to the AP site, producing a SSB at that site (Ruchko et al. 2011). This process is critical in the repair mechanism because abasic sites are highly prone to mutagenesis and cancerogenesis (King et al. 2009). After excision of the AP site, the removed nucleotide
is replaced by polymerases—usually DNA polymerase β (Polβ) (Le Page et al. 2003). This is known as the ‘short-patch pathway’ (SPP) for DNA repair. However, it is also common that the 5’ terminal is refractory to Polβ, and that repair of the damage requires integration of multiple nucleotides and several enzymes together to remove the polymerase refractory 5’ terminal. After removal of the blocking terminal, DNA ligases, such as LIG1 or the LIG3-XRCC1 complex, regenerate the damaged part of the DNA (Kanamitsu & Ikeda 2010). ROS can also generate SSBs directly by removing the oxidised deoxyribose and generating a 3’-phosphoglycolate terminus. This type of damage is also repaired by the aforementioned APE1 enzyme (Hegde et al. 2011), which removes the 3’-phosphoglycolate group. This repair pathway also requires the activity of some accessory proteins and enzymes. The most important of these are poly ADP-ribose polymerase 1 (PARP1) and XRCC1 (Peddi et al. 2006), which change the chromatin configuration in order to aid repair.

PARP1 is located in nuclei and plays two opposing roles in the presence of oxidative stress: a role in DNA repair, which is discussed here, and a role in worsening the damage mediated by ROS, which is discussed later (Kauppinen & Swanson 2007). PARP1 detects the DNA nicks generated by ROS and attaches to them. This attachment activates PARP1, which mediates poly-ADP ribosylation (attachment of poly-ADPR) of the nearest histones at the damaged DNA region through degradation of NAD⁺. Poly-ADP ribosylation of the DNA loosens the histones and changes the configuration of the DNA, allowing repair enzymes—such as DNA ligases and DNA polymerases—to attach. After completion of DNA repair, the poly-ADPR is degraded by several enzymes to ADPR, which can leave the nuclei easily and enter the cytoplasm (Welsby, Hutin & Leo 2012). The ADPR produced in this process can damage the cell through a variety of mechanisms, which will be addressed later.

1.2.3 Mechanisms of Oxidative Stress Mediated Cellular Damage

Oxidative stress can damage the cells through a variety of mechanisms, including activation of protein kinases, mitochondrial malfunctioning and a persistent rise in [Ca²⁺]c (Sohal, Mockett & Orr 2002; Hoye et al. 2008). H₂O₂ has been shown to activate all three major mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-
terminal kinases (JNK) and p38 MAPK (Ryter et al. 2007). These MAPK pathways activate death receptors or pro-apoptotic enzymes, such as the Bcl2 family, inducing irreversible cell damage and cellular death.

In oxidative stress, mitochondrial function disruption is the most general mechanism of cellular damage in mammalian cells (Ravagnan, Roumier & Kroemer 2002). Loss of mitochondrial membrane integrity is one of the initial steps in the cellular damage mediated by the oxidative stress—a process called ‘mitochondrial outer membrane permeabilisation’ (MOMP) (Green & Kroemer 2004). MOMP is induced by mitochondrial permeability transition pores (mPTP), which play a significant role in cell death mediated by both necrosis and apoptosis (Halestrap 2005; Crompton 2004). These mPTPs are opened by a protein called cyclophilin D (Cyp-D), which is usually located in the mitochondrial matrix, attached to the inner mitochondrial membrane (Baines et al. 2005). However, during overproduction of ROS in mitochondria, Cyp-D is translocated from the mitochondrial matrix to the inner mitochondrial membrane. This triggers the opening of mPTP and leads to the release of proapoptotic factors, such as SMAC/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI) and cytochrome C, from the mitochondrial intermembrane space to the cytosol (Mehmeti, Lenzen & Lortz 2011; Kroemer, Galluzzi & Brenner 2007). Increase in cytosolic concentration of cytochrome C can activate the apoptotic enzyme caspase 9 (cysteinyl aspartate-specific protease 9). The activated caspase 9 together with Apaf-1 stimulates downstream caspases and mediates irreversible damage to the cell (Mehmeti, Lenzen & Lortz 2011; Kroemer, Galluzzi & Brenner 2007). The rise in \([\text{Ca}^{2+}]_c\) and subsequent rise in mitochondrial \(\text{Ca}^{2+}\) concentration during oxidative stress has a significant role in the activation of MOMP. It has been identified that mitochondrial \(\text{Ca}^{2+}\) rise activates calcium-dependent phospholipases, which results in mitochondrial membrane damage and the release of the aforementioned proapoptotic factors (Rizzuto et al. 2012; Armstrong 2006).

Another mechanism resulting in cellular death during oxidative stress is the depletion of the cells’ stored energy reserve. The enzymes PARP1 and PARP2—previously discussed in relation to their role in DNA repair—have a significant role in this matter. PARP1 and PARP2 are predominately located in the nuclei, but can also be detected in other parts of the cell, including the mitochondria (Welsby, Hutin & Leo 2012). PARP1 and PARP2 are generally activated by binding to certain nuclear proteins and by a range of DNA or chromatin damage, but can also be activated by phosphorylation or...
acetylation (Bai & Canto 2012). Until recently, it was believed that the main function of PARP1 and PARP2 was a critical role in repairing ROS-mediated DNA damage. However, it has recently been proposed that this role is not vital for the cell. In PARP1 and PARP2 knock out (KO) mice, the rate of DNA damage is similar to that in wild-type (WT) mice (Bai et al. 2011a; Bai et al. 2011b). Given this evidence, it appears that the main function of these enzymes during oxidative stress may actually be detrimental to the cell, rather than beneficial. Their role in the oxidative-mediated damage occurs through a variety of mechanisms. PARP1 is the main cause of NAD$^+$ depletion during oxidative damage, with activated PARP1 rapidly reducing the amount of NAD$^+$ to 10-20% of its normal level (Houtkooper et al. 2010). This results in increased consumption of adenosine triphosphate (ATP) in order to replenish the NAD$^+$ level. Furthermore, NAD$^+$ depletion inhibits mitochondrial glycolysis activity, which reduces energy levels of the affected cell even more. Thereby, during oxidative stress, the cell consumes NAD$^+$ to produce poly-ADPR, and consumes ATP to replenish NAD$^+$. Thus, most of the energy the cell produces is used to generate ADPR, and the cell is confronted with severe starvation. As a result, the lesions remain unrepaired, pushing the cell to the irreversible stages that lead the cell to death (Kauppinen & Swanson 2007; Krishnakumar & Kraus 2010).

PARP1 has been implicated in several other detrimental processes during oxidative damage, including prevention of mitochondrial respiration. In this process, activated PARP1 produces a local mitochondrial signal that leads to accumulation of poly-ADPR in the mitochondria, disrupting mitochondrial respiration (Niere et al. 2008). It has also been demonstrated that activated PARP1 conjugates mitochondrial proteins with poly-ADPR, and that poly-ADP ribosylation of the mitochondrial enzymes further disrupts their function (Lai et al. 2008).
1.3 Oxidative Damage and Ca\textsuperscript{2+} Signalling

Ca\textsuperscript{2+} is a vital intracellular second messenger, however, it is well recognised that excessively high [Ca\textsuperscript{2+}]\textsubscript{c} can be deleterious to normal cell function, and it is well accepted that a rise in [Ca\textsuperscript{2+}]\textsubscript{c} has a pivotal role in the cellular damage mediated by ROS (Gorlach, Klappa & Kietzmann 2006).

Ca\textsuperscript{2+} has an important role in mitochondrial ATP generation; pulses of Ca\textsuperscript{2+} are one of the most powerful stimulants of the oxidative-phosphorylation pathway and, hence, ATP production. Ca\textsuperscript{2+} also promotes the generation of nicotinamide adenine dinucleotide (NADH) by activation of pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Gunter et al. 2004). This increase in energy production has been detected only with a short moderate rise in mitochondrial Ca\textsuperscript{2+} concentration. In oxidative stress, the [Ca\textsuperscript{2+}]\textsubscript{c} is increased, promoting a rise in mitochondrial Ca\textsuperscript{2+} concentration beyond the point where it has a positive effect on energy production. Such a rise promotes the production of ROS, reduces ATP production, and disrupts the mitochondrial membrane integrity. As has been previously discussed, this leads to irreversible cell damage (Gunter et al. 2004). In the nucleus, the rise in Ca\textsuperscript{2+} facilitates DNA fragmentation and changes in enzymatic activity, leading to cellular death (Meneghini 1997).

The rise in [Ca\textsuperscript{2+}]\textsubscript{c} during oxidative stress is mediated through release of stored Ca\textsuperscript{2+} or influx of Ca\textsuperscript{2+} from the extracellular fluid. H\textsubscript{2}O\textsubscript{2} promotes the release of stored Ca\textsuperscript{2+}; however, the role of this release in the cellular damage mediated by oxidative stress is debateable. The contribution of the release of stored Ca\textsuperscript{2+} in the persistent increase in [Ca\textsuperscript{2+}]\textsubscript{c} during oxidative stress is minimal, and the influx of Ca\textsuperscript{2+} from extracellular space is the main source of that increase. It has been found that removal of extracellular Ca\textsuperscript{2+} protects cells from H\textsubscript{2}O\textsubscript{2}-mediated damage (Sato et al. 2009). Many channels on the plasma membrane have been shown to be activated during oxidative stress, resulting in this rise in [Ca\textsuperscript{2+}]\textsubscript{c}. While voltage-dependent Ca\textsuperscript{2+} channels and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers are activated by H\textsubscript{2}O\textsubscript{2}, a more important role in Ca\textsuperscript{2+} signalling in oxidative stress is played by TRP channels (Montell 2005).
1.4 TRP Channels in Oxidative Stress

TRP channels are non-selective cation channels, each with different cation conduction properties. Several of these channels are known to be involved in the rise of $[Ca^{2+}]_c$ during the overproduction of ROS (Miller & Zhang 2011). There are currently 28 known members of the mammalian TRP channel superfamily. These are divided into two groups and seven subfamilies (Gees, Colsoul & Nilius 2010; Montell 2005). The first TRP channel discovered was detected in *Drosophila*, and shown to be involved in the increase in $[Ca^{2+}]_c$ during an anoxic spell (Montell & Rubin 1989; Agam et al. 2000; Phillips, Bull & Kelly 1992). TRP channels have been detected in organisms ranging from yeasts to mammals, and, with many similarities in these channels between organisms, it has been proposed that TRP channels are one of the most ancient known channel types (Montell 2005).

Group 1 of the TRP superfamily contains five subfamilies: TRPC, TRPV, TRPM, TRPA (Ankyrin) and TRPN (NOMPC). Group 2 contains the remaining subfamilies: transient receptor potential polycystin (TRPP) and transient receptor potential mucolipin (TRPML) (Venkatachalam & Montell 2007). All TRP channels are composed of six TM segments with varying degrees of homology in segmental sequences. These channels all differ in their ability to conduct Ca$^{2+}$ (Chang et al. 2010). Among the 28 known TRP channels, some are activated during oxidative stress. These include some members of TRPC, TRPV and TRPM subfamilies (Figure 1.1).

Of the TRPC channel subfamily, the TRPC3 and TRPC4 channels have been shown to have a role in oxidative stress (Miller & Zhang 2011; Gees, Colsoul & Nilius 2010; Miller 2006). It has been demonstrated that oxidative stress activates TRPC3 and TRPC4 channels endogenously expressed in endothelial cells and overexpressed in HEK293T cells, where they contribute to the rise of intracellular Na$^+$ and Ca$^{2+}$ concentration. However, the role of these channels in the sustained increase of $[Ca^{2+}]_c$—which mediates irreversible cell damage—is still poorly understood (Figure 1.1), (Miller 2006; Tappia, Asemu & Rodriguez-Leyva 2010).

TRPV1 is the only channel of the TRPV subfamily that is known to be activated during oxidative stress. However, the mechanism of activation and the role of TRPV1 in oxidative stress–mediated damage are not known (Figure 1.1), (Miller & Zhang 2011; Gees, Colsoul & Nilius 2010).
In the TRPM subfamily, the TRPM2 and TRPM7 channels are activated in oxidative stress, with TRPM2 channels believed to be the most important (Figure 1.1). The TRPM2 channel is activated by ROS and RNS through various mechanisms, contributing to a persistent rise in $[Ca^{2+}]_c$, which leads to irreversible damage to cellular components and to cellular death (Sumoza-Toledo & Penner 2011; Miller & Zhang 2011). Due to its importance and the focus of this thesis, the properties of TRPM2 channel and its role in oxidative stress-induced cell damage are discussed later under a separate heading.

TRPM7 is another member of the TRPM channels that is believed to be involved in the damage mediated by ROS. TRPM7 has a serine-threonine kinase enzymatic activity located at the cytoplasmic C-terminal of the channel. TRPM7 is expressed in most tissues and is non-selectively permeable to Na$^+$ and Ca$^{2+}$, but, importantly, is permeable to Mg$^{2+}$. There is controversy regarding the role of TRPM7 in the rise of $[Ca^{2+}]_c$ during oxidative stress. Overexpression of TRPM7 increases cellular damage and death during oxidative stress, while knock down of its expression using siRNA in cortical neurons prevents ROS-mediated damage. However, it has been proposed that TRPM7 has a regulatory effect on TRPM2 expression—suppressing TRPM7 expression using siRNA reduces the expression levels of TRPM2. Therefore, it is possible that the role of TRPM7 in oxidative stress may be mediated by its effects on TRPM2 channel (Miller & Zhang 2011; Miller 2006).
Figure 1.1: TRP channels superfamily

Group 1 contains five subfamilies: TRPC (Canonical), TRPM (Melastatin), TRPV (Vanilloid), TRPA (Ankyrin) and TRPN (NOMPC). Group 2 contains TRPP (Polycystin) and TRPML (Mucolipin). Channels activated by oxidative stress are marked in red.
1.5 TRPM2 Channels

The TRPM2 channel is a non-selective cation channel that is mainly activated during oxidative stress and apoptosis (Hecquet et al. 2014). The channel activation correlates with the persistent increase in \([\text{Ca}^{2+}]_c\), leading to cellular damage (Perraud et al. 2001, Zhivotovsky & Orrenius 2011).

1.5.1 History

TRPM2 was first discovered in 1998 while searching for the genes responsible for genetic malformations, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and holoprosencephaly, which mainly affect the central nervous system (CNS) (Nagamine et al. 1998). The protein of the discovered gene was found to be a cation channel permeable to \(\text{Ca}^{2+}\) and have homology with the TRP channel superfamily. It was named ‘transient receptor potential-related channel 7’ (TRPC7) (Nagamine et al. 1998). At the time of its discovery, it was believed that TRPC7 and other TRP channels were composed of seven TM domains, and belonged to the group of unidentified store operated channels (Putney, 1990; Nagamine et al. 1998).

In 2000, 13 TRP and related channels were categorised into three subfamilies according to the size of the channel protein. These categories were short TRP channels (STRPC), long TRP channels (LTRPC) and Osm TRP channels (OTRPC). TRPC7 was assigned to the LTRPC channel group and named ‘LTRPC2’ (Harteneck, Plant & Schultz 2000). At that time, it was established that TRP channels were composed of six TM domains with cytoplasmic N- and C-termini (Miller & Zhang 2011).

A special region composed of 350 amino acids (aa) was detected in the C-terminal of LTRPC2 (TRPM2) in 2001. This region has pyrophosphatase activity and, due to its homology to NUDT9, has been named ‘NUDT9-H’. This region has the ability to bind to ADPR, and the application of ADPR through a patch pipette in HEK293 cells expressing LTRPC2 results in the activation of a non-selective cation current with linear current-voltage relationship (Perraud et al. 2001).

In 2002, the TRP Nomenclature Committee proposed a unified nomenclature for the TRP superfamily. TRP channels were categorised into three subfamilies according to
the homology between the channels: TRPC channels (formerly STRPs), TRPV channels (formerly OTRPs) and TRPM channels (formerly LTRPs). The LTRPC2 channel was renamed ‘TRPM2’ (the numbering of these channels was due to the order of discovery). Since that time, the channel has remained named TRPM2 (Montell et al. 2002).

1.5.2 TRPM Subfamily

TRPM channels have a 20% sequence identity with TRPC channels. The ‘melastatin’ in the name of this group is based on the observation that TRPM1 protein is down regulated in melanoma (Duncan et al. 1998). Three channels of this subfamily have a unique characteristic of having enzymatic activity in addition to their function as a channel; these channels are called ‘chanzymes’ (channel-enzymes). The most important chanzymes are TRPM2, TRPM6 and TRPM7 (Scharenberg 2005). TRPM4 and TRPM5 have a unique activation mechanism by changes in cell membrane potential. They are also activated by PIP2, while TRPM5 is also activated by temperature (Nilius et al. 2006; Liu & Liman 2003). TRPM8 channels are activated by low temperatures (<28ºC), and also by icilin and menthol (Kuhn, Kuhn & Luckhoff 2009). TRPM2 has a specific pyrophosphatase activity of NUDT9-H located in the C-terminal of the channel, which activates TRPM2 by cleaving ADPR (Perraud et al. 2001).

1.5.3 TRPM2 Channel Isoforms and Variants

There are two known isoforms of the TRPM2 channel: long (TRPM2-L) and short (TRPM2-S) (Zhang et al. 2003). Additionally, TRPM2-L splice variants have been identified, including a shorter form of TRPM2 (SSF-TRPM2), TRPM2 with an N-terminal truncation (TRPM2-ΔN), TRPM2 with a C-terminal truncation (TRPM2-ΔC), and TRPM2 with both N- and C-termini truncations (TRPM2-ΔNΔC), (Figure 1.2), (Perraud, Schmitz & Scharenberg 2003; Du, Xie & Yue 2009).
Figure 1.2: TRPM2 isoforms

TRPM2 Channels

1. Long Isoform (TRPM2L)
   - a. TRPM2L ≈ 171 KDa
   - b. SSF-TRPM2
   - c. TRPM2-ΔN
   - d. TRPM2-ΔC
   - e. TRPM2-ΔNΔC

2. Short Isoform (TRPM2S)
   ≈ 90 KDa
The gene of the TRPM2-L channel is transcribed to a 6.5 kb mRNA, which, in humans, produces a 1,503 aa long protein (1,507 aa in rat and mouse), with a molecular weight of 171 kDa (Perraud et al. 2001; Hofmann et al. 2000; Uemura et al. 2005). The TRPM2-L channel consists of six TM domains with cytoplasmic N- and C-termini. The C-terminal of TRPM2-L contains a region of ADPR pyrophosphatase activity (NUDT9-H) that plays an important role in the activation of the channel (Kuhn & Luckhoff 2004). NUDT9-H cleaves the bound ADPR and, through an unknown mechanism, gates the channel. The pore of the TRPM2-L channel is located in the S5-Loop-S6 region of the channel, with channel activation allowing Na⁺ and Ca²⁺ to enter the cell. The rise in [Ca²⁺]c also contributes to the full activation of the TRPM2-L channel (Kuhn et al. 2010).

In this thesis, wherever the TRPM2 channel is mentioned, this refers to the TRPM2-L channel, unless otherwise stated.

1.5.3.1 TRMP2-S

The short isoform of the TRPM2 channel (TRPM2-S) has a molecular mass of 95 KDa (for comparison, TRPM2-L is 171 KDa). TRPM2-S was first isolated from human bone marrow, and contains just the N-terminal and first two TM domains of TRPM2-L (Zhang et al. 2003). This variant is generated by a stop codon located between exon 16 and 17 of the mRNA of the TRPM2 channel (Sumoza-Toledo & Penner 2011). This isoform is not activated by ADPR because there is no ADPR binding site on the TRPM2-S. In fact, TRPM2-S prevents activation of the TRPM2 long isoform. HEK293T cells transfected with both TRPM2-L and TRPM2-S have increased cell viability in the presence of H₂O₂, compared with those transfected with TRPM2-L alone (Zhang et al. 2003). Immunoprecipitation studies suggest that this may be the result of a direct interaction between TRPM2-L and TRPM2-S (Zhang et al. 2003).

1.5.3.2 SSF-TRPM2

The splice variant SSF-TRPM2 is similar to the full TRPM2-L protein, apart from missing 214 aa from the N-terminal end. This splice variant was first detected in the striatum of the human brain. The mRNA of SSF-TRPM2 is 5.5 Kb (compared to 6.5
Kb of TRPM2-L). SSF-TRPM2 maintains the characteristic of being activated by H$_2$O$_2$ (Uemura et al. 2005).

1.5.3.3 TRPM2-$\Delta$N

TRPM2-$\Delta$N lacks a 20 aa N-terminal $\Delta$N stretch (aa 538–557), a region that contains two binding sites for calmodulin (CaM; Ca$^{2+}$ binds to TRPM2 via CaM). HEK293 cells transfected with cDNA for TRPM2-$\Delta$N do not respond to either ADPR or H$_2$O$_2$ (Wehage et al. 2002). However, this appears to be independent of the CaM binding sites, as deletion of either or both CaM binding sites does not affect the activation of the channel by ADPR or H$_2$O$_2$ (Kuhn et al. 2009).

1.5.3.4 TRPM2-$\Delta$C

TRPM2-$\Delta$C was first detected in neutrophil granulocytes and HL-60 cells. This variant has aa residues of 1,292 to 1,325 deleted, and is resistant to ADPR, but can be activated by H$_2$O$_2$ (Wehage et al. 2002).

1.5.3.5 TRPM2-$\Delta$N$\Delta$C

The TRPM2-$\Delta$N$\Delta$C variant is also detected in HL-60 cells, and has missing aa in both N- and C-termini. This variant is resistant to both ADPR and H$_2$O$_2$ (Wehage et al. 2002).

1.5.4 TRPM2 Structure

1.5.4.1 TRPM2 Channel Topology

As mentioned above, the TRPM2 channel is composed of six TM domains, with N- and C-termini both cytoplasmic (Figure 1.3). The C-terminal of TRPM2 is responsible for the enzymatic activity of TRPM2 channel. This region has homology to NUDT9, and has been called the ‘NUDT9-H region’ (Figure 1.3), (Perraud et al. 2001; Kuhn & Luckhoff 2004). The importance of this region in TRPM2 activation will be discussed in detail later in this thesis. The N-terminal also plays a fundamental role in TRPM2
activation, and its deletion prevents TRPM2 from being activated by ADPR (Kuhn et al. 2009). There are several CaM binding sites in the N-terminus of TRPM2 protein; however, their role in the function of TRPM2 channel is still in doubt (Wehage et al. 2002; Kuhn et al. 2009).

Gating of TRPM2 is mediated by the pore, which is formed by S5, extracellular loop and S6 regions. The pore is responsible for allowing movement of cations, mainly Na\(^+\) and Ca\(^{2+}\) (Kuhn et al. 2010). It has been recognised that the two cysteine residues in the pore region of TRPM2 channel at positions 996 and 1008 are important in TRPM2 gating; mutation at those positions prevents TRPM2 activation by ADPR (Mei, Mao & Jiang 2006). It has been suggested that the pore functions solely as a gate, as exchanging the pore region of TRPM2 (S5 domain, pore loop and S6 domain) with that of TRMP8 (S5, S6 and the loop, which have 83, 70 and 30% homology with the respective regions of TRPM2) results in no change in function in response to ADPR, or in conductance of cations (Kuhn et al. 2010).

Study of the three-dimensional structure of the TRPM2 channel using electron microscopy has revealed that the TRPM2 is bell shaped at the extracellular region (Maruyama et al. 2007). The remaining part of the TRPM2 consists of TM domains, and part of the cytoplasmic region and the end cytoplasmic region is prism-like. This study also showed that 6% of the TRPM2 protein (11 kDa) is extracellular, 8% (14 kDa) of the channel is TM and the rest of the protein (86%, 148 kDa) is intracellular (Maruyama et al. 2007).
TRPM2 channel consists of six TM domains with cytoplasmic C- and N-termini. ADPR activates TRPM2 channel by interacting with NUDT9-H located in the C-terminus of the channel. During activation, the pore located between TM5 and TM6 opens and lets Na$^+$ and Ca$^{2+}$ into the cell. Ca$^{2+}$ interacts with the N-terminus of the channel and potentiates the ADPR effect.
1.5.4.2 Nudix Box and NUDT9-H

The Nudix box—a 23 aa sequence motif (GX\_5EX\_7REUXEEXGU, where X represents any aa and U is a large hydrophobic residue)—is a fundamental part of a superfamily of enzymes called ‘Nudix hydrolases’. These enzymes require Mg\textsuperscript{2+} for functioning and are involved in catalysing the hydrolysis of nucleoside diphosphates attached to other moieties (Mildvan et al. 2005). Nudix hydrolases have been detected in a wide range of organisms, including eukaryotes, bacteria, viruses and archaea (McLennan 2006). In humans, 24 Nudix hydrolase genes and five pseudogenes have been isolated and classified, from NUDT1 to NUDT22 (McLennan 2006; Takagi et al. 2012). Some of them have exhibited ADPR pyrophosphatase activity, including NUDT5, NUDT6, NUDT9, NUDT9-H and NUDT12 (McLennan 2006). NUDT9-H is the ADPR pyrophosphatase located on the C-terminal of the TRPM2 channel, which is directly involved in TRPM2 channel activation (McLennan 2006; Perraud et al. 2001). NUDT5 and NUDT9 are also important in the ADPR activation of the TRPM2 channel. NUDT5 has a negative effect on TRPM2 channel activation. It is widespread and freely located in the cytoplasm and is responsible for the hydrolysis and detoxification of ADPR (Ishibashi et al. 2005). NUDT5 hydrolyses ADPR to non-toxic compounds, adenosine monophosphate (AMP) and ribose 5'-phosphate, reducing the amount of ADPR to protect the cells from further ROS-mediated damage (Arimori & Yamagata 2011).

In comparison to NUDT9-H, NUDT5 has a high $K_m$ (Michaelis constant—indicates the affinity of an enzyme for its substrate, with a lower $K_m$ meaning higher affinity) for ADPR. In addition, it is not as specific as NUDT9-H for ADPR; NUDT5 hydrolyses many other toxic components, including 8-oxoGua (Yu et al. 2007; Arimori et al. 2011). NUDT5 has been shown to exhibit some protective effect against oxidative stress. Besides its effect on reducing the amount of ADPR, NUDT5 hydrolases 8-oxoGua, reducing its cellular concentration, so the cells can better tolerate the consequences of oxidative stress (Ishibashi et al. 2005). NUDT5 activity is increased in cells exposed to oxidative stress. By measuring the amount of ADP ribosylation of cysteine residue on NUDT5 protein, it has been identified that NUDT5 activity is increased by treating the cells with exogenous nitric oxide or TNF-α/INFγ (Yu et al. 2007). It has been concluded that under oxidative stress cells increase NUDT5 activity to recover from ROS-mediated damage (Arimori & Yamagata 2011).
NUDT9-H has a 39\% sequence homology with mitochondrial NUDT9 extracted from the spleen with similar affinity to ADPR ($K_m$ is $100\pm10$ $\mu$M) (Perraud et al. 2001; Shen et al. 2003). The NUDT9-H is responsible for hydrolysing ADPR and activating the TRPM2 channel (Perraud et al. 2001; Shen et al. 2003). There are specific sites identified on NUDT9-H that are critical for ADPR activation of the TRPM2 channel. It has been shown that mutations I1405E/L1406F in the NUDT9-H prevent the resultant TRPM2 channel being activated by ADPR, even at very high concentrations (Kuhn & Luckhoff 2004).

### 1.5.5 Cellular localizations of TRPM2 channel

Studies using heterologous expression in HEK293 cells suggest that TRPM2 channels are mainly located in the plasma membrane (Mei, Mao & Jiang 2006), and are involved in the persistent rise of $[Ca^{2+}]_c$ during oxidative stress (Perraud et al. 2001, Zhivotovsky & Orrenius 2011). However, in many primary cell types where TRPM2 is expressed endogenously, TRPM2 protein has been detected not only on the plasma membrane, but also in intracellular organelles, particularly, in lysosomes (Sumoza-Toledo et al. 2011). Lysosomes are small cytoplasmic organelles that are mainly responsible for digesting cellular waste; they can also act as storage organelles for some compounds, including Ca$^{2+}$ (Sumoza-Toledo et al. 2011). In pancreatic β-cells, apart from being expressed on the plasma membrane, TRPM2 is also expressed on the lysosomal membrane. The lysosomal TRPM2 channel is involved in the release of stored Ca$^{2+}$ from the lysosomes (Lange et al. 2009). This release has a significant contribution in ROS-mediated β-cells’ death (Lange et al. 2009). In dendritic cells of immune system, the TRPM2 channels expressed on the lysosomal membrane play a role in releasing stored Ca$^{2+}$. It has been shown that lysosomal TRPM2 is important for dendritic cell migration to the site of infection, which is impaired in TRPM2 KO mice. The expression of cytokine receptors in these cells is also impaired in TRPM2 KO mice (Knowles et al. 2011, Sumoza-Toledo et al. 2011).
1.5.6 TRPM2 Channel Activation

1.5.6.1 TRPM2 Gating

The exact mechanism of the TRPM2 channel gating is not fully understood. The enzymatic degradation of ADPR by NUDT9-H located in the C-terminal of the TRPM2 channel is the suggested mechanism. Binding of ADPR to NUDT9-H changes the TRPM2 channel conformation from closed to open, which provides a pathway for Na\(^+\) and Ca\(^{2+}\) into the cell (Kuhn et al. 2010; Magnone et al. 2012). The open probability of the TRPM2 channel was measured through determining the closure time in single channel recordings, and it has been identified that the open probability of the gated channel is close to one (Csanady & Torocsik 2009, Heiner, Eisfeld & Luckhoff 2003). This means that, when the channel is activated, it remains open for long periods of time, which are paused by very short closures. This unique characteristic of the TRPM2 channel kinetics allows a persistent increase in [Ca\(^{2+}\)]\(_c\) (Perraud et al. 2001; Heiner, Eisfeld & Luckhoff 2003).

Both intra- and extracellular Ca\(^{2+}\) play a role in TRPM2 channel gating (Csanady & Torocsik 2009). It is likely that Ca\(^{2+}\) promotes opening of the TRPM2 channel through a CaM-dependent mechanism; possibly, binding of Ca\(^{2+}\)-CaM complex to the N-terminus of TRPM2 protein allows the channel to be activated by much lower concentrations of ADPR (discussed later at section 1.5.7.1.1) (Csanady & Torocsik 2009).

1.5.6.2 TRPM2 Channel Activators

TRPM2 is activated by several compounds, including ADPR, cADPR, NAD\(^+\) and H\(_2\)O\(_2\). The mechanisms of TRPM2 activation by these substances are still unclear, and it has been suggested that different mechanisms may be involved in TRPM2 channel activation by different agonists (Kolisek et al. 2005). The activators of the TRPM2 channel are divided into two groups: direct and indirect. Direct activators are those that directly interact with TRPM2 and gate the channel, whilst indirect activators usually work through production of some intracellular mediators. There is doubt, however, about this categorisation in regards to some activators, including H\(_2\)O\(_2\). The indirect effect of H\(_2\)O\(_2\) on the TRPM2 channel through the overproduction of ADPR is well
established (Hara et al. 2002; Perraud et al. 2005); however, there is controversy regarding the direct activation of TRPM2 channel by H$_2$O$_2$ (Toth & Csanady 2010). The mechanisms of TRPM2 activation by different agonists have been primarily investigated using inside-out patch-clamp technique (Toth & Csanady 2010).

1.5.6.3 Direct TRPM2 Activators

Ca$^{2+}$, ADPR, NAD$^+$ and nicotinic acid adenine dinucleotide phosphate (NAADP) are the main direct activators of TRPM2 channel. NAD$^+$ and NAADP, however, are much less potent than Ca$^{2+}$ and ADPR.

1.5.6.3.1 Ca$^{2+}$

The notion that TRPM2 activation is regulated by Ca$^{2+}$ comes from several independent observations. In U937 cells (the U937 cell line was first isolated from histiocytic lymphoma to study monocytes) the endogenously expressed TRPM2 channels, show resistance to high concentrations of ADPR when [Ca$^{2+}$]$_c$ is buffered to low levels using 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (Perraud et al. 2001). In the experiments using the inside-out patches of TRPM2-transfected Xenopus oocytes, where the concentration of the TRPM2 ligands at the cytoplasmic face of the channel is controlled, ADPR fails to activate TRPM2 channels, even at millimolar range of concentrations, if Ca$^{2+}$ is buffered to submicromolar levels (Toth & Csanady 2010). Furthermore, 32 µM of ADPR, which fully activated the TRPM2 channel in the presence of 125 µM Ca$^{2+}$, had no effect at 4 µM Ca$^{2+}$ (Csanady & Torocsik 2009). At least 7.6 to 15 nM of intracellular Ca$^{2+}$ concentration is required for partial TRPM2 activation by ADPR. For a complete ADPR-mediated TRPM2 channel activation, the Ca$^{2+}$ concentration at the intracellular face of the channel has to be at least 125 µM. The data suggested that the rise in inward current amplitude measured in the whole cell patch clamp in response to a rise in intracellular Ca$^{2+}$, is mostly due to increase in the open probability of the activated channels, rather than activation of additional channels (Csanady & Torocsik 2009). To ascertain a possible mechanism of Ca$^{2+}$-mediated TRPM2 channel gating, single-channel gating kinetics was investigated at different Ca$^{2+}$ concentrations, to determine the opening and closing rates. Taking in an account
homotetrameric structure of TRPM2 channel (Maruyama et al. 2007), Monod-Wyman-Changeux model was used to describe the dependence of single channel gating parameters on \([Ca^{2+}]_c\) (Csanady & Torocsik 2009). The modelling suggested that binding of four \(Ca^{2+}\) ions are needed for maximum activation of each single TRPM2 channel, with each binding event shifting equilibrium constant towards opening \(~33\) fold (Csanady & Torocsik 2009).

Both intra- and extracellular \(Ca^{2+}\) has been investigated in relation to the mechanism of TRPM2 activation, and it has been revealed that cytoplasmic \(Ca^{2+}\) is more critical than extracellular \(Ca^{2+}\) (Starkus et al. 2007; Csanady & Torocsik 2009). In the absence of both intra- and extracellular \(Ca^{2+}\), ADPR fails to activate TRPM2 channels; however, at zero extracellular \(Ca^{2+}\), at least 30 nM \([Ca^{2+}]_c\) is needed for partial activation of the TRPM2 channel (Starkus et al. 2007). In another study, it has been shown that extracellular \(Ca^{2+}\) has minimal effect on initiation of TRPM2 channel activation even at very high concentrations; but, it was shown to play a role in keeping the activated channel open for long periods of time (Csanady & Torocsik 2009).

It is likely that \(Ca^{2+}\) activates the TRPM2 channel through its complex with CaM (Tong et al. 2006). CaM plays a role of \(Ca^{2+}\) sensor in \(Ca^{2+}\)-mediated TRPM2 channel activation. Co-transfection of HEK 293T cells with TRPM2 cDNA and mutant cDNA of CaM (which produces a non-functional CaM protein) prevents the TRPM2 channel being activated by \(H_2O_2\) or TNFα (Tong et al. 2006). Using western blot technique, it has been shown that CaM strongly interacts with the N-terminal of TRPM2, which contains two motifs similar to CaM-binding IQ motif (Tong et al. 2006, Rhoads & Friedberg 1997). Furthermore, adding 100 µM CaM to the internal solution containing 10 µM ADPR facilitates the activation of the TRPM2 channels by ADPR (Starkus et al. 2007). The importance of CaM in mediating \(Ca^{2+}\) dependence of TRPM2 activation is supported by the experiments using calmidazolium, a potent inhibitor of CaM. Application of calmidazolium to the internal solution prevents TRPM2 channel activation by ADPR. Therefore, it can be concluded that CaM is indispensable for TRPM2 channel gating by \(Ca^{2+}\) (Starkus et al. 2007).

However, the CaM binding site on TRPM2 channel, which mediates its \(Ca^{2+}\) dependence, remains unidentified. Further experiments into the roles of two IQ-like motifs located on the N-terminal of TRPM2 channel showed that the deletion of a stretch of 20 aa (aa 537–556) within these motifs produced no changes in the gating
properties of the channel. Thus, these IQ-like motifs are not necessary for Ca\textsuperscript{2+}-mediated TRPM2 activation (Kuhn et al. 2009).

1.5.6.3.2 ADPR

ADPR is the main activator of TRPM2. It interacts with the NUDT9-H motif and gates the channel (Perraud et al. 2001). Animal cells can generate ADPR through various distinct mechanisms. In mitochondria, ADPR is produced by direct glycohydrolisation of NAD\textsuperscript{+}, generating nicotinamide and poly-ADPR (Kim et al. 1993). In nuclei, poly-ADPR is produced by PARP enzymes, including PARP1, which is activated by oxidative stress-induced DNA damage (Wang et al. 2012). PARP1 is involved in the repair of damaged DNA. In oxidative stress, PARP1 detects the nicks in the affected DNA, attaches to them and becomes activated. Activated PARP1 catalyses poly-ADP ribosylation of the damaged DNA using NAD\textsuperscript{+} (Hassa & Hottiger 2008). This process increases the concentration of poly-ADPR several hundred fold. As mentioned above, poly-ADP ribosylation of the DNA facilitates the repair of the damaged area. After repair, the produced poly-ADPR is removed from the histones by enzymes called ‘poly-ADPR glycohydrolases’ and broken down into ADPR. ADPR freely passes through the nuclear membrane and accumulates in the cytoplasm (Hassa & Hottiger 2008). In the cytoplasm, enzyme called ‘ADPR pyrophosphatase’ (ADPRase) metabolise the accumulated ADPR, generating AMP and ribose-5-phosphate. Up to four different kinds of ADPRase have been identified: ADPRase-I (which is similar to NUDT9-H), ADPRase-II (similar to NUDT5), ADPRase-Mn (activated by Mn\textsuperscript{2+} and inhibited by Mg\textsuperscript{2+}) and ADPRase-m (located in mitochondria). Most of these enzymes require Mg\textsuperscript{2+} for activation (Arimori et al. 2011; Carloto et al. 2006; Kuhn & Luckhoff 2004; Shen et al. 2003).

NUDT9-H has a pyrophosphatase activity, catalysing ADPR to ribose 5-phosphate and AMP in the process of activation the TRPM2 channel (Shen et al. 2003; Kuhn & Luckhoff 2004). Ribose 5-phosphate is an innate substance and there is no evidence that it has any effect on TRPM2 channel activation. There is controversy, however, regarding the effect of AMP, another metabolite of ADPR, on TRPM2 channel activation. While some studies reported that AMP has no effect on ADPR-mediated TRPM2 channel activation (Toth & Csanady 2010), others have shown that AMP
prevents activation of TRPM2 channels by ADPR with IC$_{50}$ of 10 µM (Lange et al. 2008).

1.5.6.3.3 NAD$^+$

Several studies conducted on various cell types and TRPM2-transfected cell lines have shown that the addition of NAD$^+$ to intracellular solution leads to activation of TRPM2 current (Sano et al. 2001; Hara et al. 2002; Heiner et al. 2003). They identified that the efficacy of NAD$^+$ in TRPM2 channel activation was much lower, compared to ADPR (EC$_{50}$ >1 mM and 1 µM, respectively), (Hara et al. 2002; Kolisek et al. 2005; Toth & Csanady 2010). Later it was found that the commercially available NAD$^+$ was contaminated with ADPR, which was confirmed using TLC analysis. Therefore the direct effect of NAD$^+$ on TRPM2 channel activation remains unclear (Toth & Csanady 2010; Sumoza-Toledo & Penner 2011).

1.5.6.3.4 NAADP

NAADP was shown to be another activator of TRPM2, albeit much less potent than ADPR. NAADP probably activates the TRPM2 channel via both direct and indirect mechanisms (Beck et al. 2006; Kolisek et al. 2005). Using the inside-out patch-clamp technique, it has been identified that, compared to ADPR, NAADP activates the TRPM2 channel only partially and with much lower apparent affinity; EC$_{50}$ of NAADP and ADPR for TRPM2 channel was measured at 35 and 1 µM, respectively (Toth & Csanady 2010).

1.5.6.4 Indirect TRPM2 Channel Activators

H$_2$O$_2$, cADPR and heat are likely to activate TRPM2 channels by triggering the generation of direct activators.

1.5.6.4.1 H$_2$O$_2$

H$_2$O$_2$ has been shown to activate a large non-selective cation current in different cell types and HEK293 cells expressing TRPM2 channels. In TRPM2-expressing HEK293
cells, application of 100 µM H₂O₂ to the bath causes development of an inward current with a mean amplitude of 117 pA/pF, which reaches its’ maximum in about 300 s (Hara et al. 2002). The properties of the current activated by H₂O₂ are similar to those of the TRPM2 current activated by ADPR. Silencing of TRPM2 protein expression in neurons prevents development of a current in response to H₂O₂ (Kaneko et al. 2006). This suggests that H₂O₂ is a TRPM2 activator. There is controversy, however, regarding the mechanisms of H₂O₂-mediated TRPM2 channel activation.

The indirect mechanism of TRPM2 activation involves rise of cytoplasmic ADPR concentration due to H₂O₂-mediated damage to mitochondria and DNA (Hara et al. 2002; Perraud et al. 2005). The indirect mechanism of TRPM2 channel activation by H₂O₂ is supported by several observations. PARP1 inhibitors, which prevent generation of poly-ADPR in response to DNA damage, also prevent TRPM2 channel activation in response to H₂O₂ in HEK293 heterologous expression system (Perraud et al. 2005). In primary rat megakaryocytes, H₂O₂ fails to activate the TRPM2 channel at all (Naziroglu 2011). Direct application of H₂O₂ to the cytoplasmic face of TRPM2 in inside-out patches also failed to activate TRPM2 current, which suggested that possible direct oxidation of some intracellular residues in TRPM2 protein, is unlikely to have any functional role in activation of the channel (Toth & Csanady 2010). It has also been suggested that H₂O₂ may potentiate ADPR-mediated TRPM2 channel activation (Kolisek et al. 2005; Lange et al. 2008).

In contrast, the evidence in favour of direct TRPM2 activation by H₂O₂ is limited. The main observation that supports direct action of H₂O₂ is that TRPM2-ΔC lacking the C-terminal, and therefore the NUDIX box, is activated by H₂O₂, although, the channel is resistant to ADPR, even at high concentrations (Wehage et al. 2002).

1.5.6.4.2 cADPR

cADPR, as a possible activator of TRPM2 channels has been investigated in several studies (Togashi et al. 2006; Kolisek et al. 2005; Toth & Csanady 2010). It has been shown that cADPR activates the TRPM2 channels expressed in HEK293T cells, but much with much lower efficacy, compared to ADPR (Kᵯ/ᵦ = 700 µM) (Kolisek et al. 2005). In contrast, in single-channel recording using inside-out patch clamping, there is no TRPM2 channel activation in response to application of cADPR. Thus, it has been proposed that in whole-cell recordings cADPR is metabolised to ADPR, thereby
activating the TRPM2 channel (Kolisek et al. 2005). On the other hand, it has been shown that commercial cADPR is contaminated with ADPR, and purified cADPR has no effect on activation of TRPM2 channels expressed in HEK293T cells (Toth & Csanady 2010).

It has been reported that cADPR-mediated TRPM2 channel activation is significantly potentiated by heat (Togashi et al. 2006). In TRPM2-expressing HEK293T cells, cADPR has a negligible effect on TRPM2 channel activation in temperatures of 25°C or below; however, increasing the temperature to 36°C significantly potentiated cADPR-mediated TRPM2 channel activation. In pancreas islets, cADPR activates TRPM2 current at normal body temperature, increasing insulin secretion (Togashi et al. 2006).

1.5.6.4.3 Heat

High temperatures (> 36°C) can activate some TRP channels, including TRPV1 (Walder et al. 2012), TRPV3 and TRPV4 (Saito & Shingai 2006) from the TRPV subfamily, and TRPM4 and TRPM5 (Talavera et al. 2005) from the TRPM subfamily. There is currently no clear explanation regarding the role of temperature in TRPM2 channel activation; however, it has been reported that temperatures above 36°C could potentiate TRPM2 channel activation mediated by 100 μM ADPR or 1 mM NAD⁺ in HEK 293 cells (Togashi et al. 2006). Thus, it has been proposed that normal body temperature might be an ‘endogenous co-activator’ of the TRPM2 channel (Togashi et al. 2006). The possibility of a direct activation of the TRPM2 channel by heat cannot be dismissed yet; however, there is no evidence to support this so far (Togashi et al. 2006).

1.5.7 TRPM2 Channel Blockers

The capability to inhibit TRPM2 current using a specific blocking agent is essential for understanding the function and gating of the channel. Potentially, TRPM2 specific inhibitors can be considered as therapeutic agents for treating oxidative stress-mediated pathologies. Unfortunately, no such specific TRPM2 channel inhibitor has been identified yet. Currently, known inhibitors of TRPM2 channel are categorised
according to their structure and the mechanism of action into fenamates, azoles, N-(p-amylcinnamoyl) anthranilic acid (ACA) and 2-aminoethoxydiphenyl borate (2-APB).

1.5.7.1 ACA

ACA is a potent inhibitor of phospholipase A$_2$ (PLA$_2$) (Konrad et al. 1992). PLA$_2$ is a family of enzymes that catalyses hydrolysis of phospholipids into arachidonic acid and lysophospholipids (Blackwell et al. 1977). Different types of PLA2 are activated during inflammation and cellular damage (Kitsiouli, Nakos & Lekka 2009). Its main product, arachidonic acid, is the precursor of many inflammatory mediators, including prostaglandins (produced through cyclooxygenase (COX) pathway) and leukotrienes (produced through lipoxygenase pathway) (Murakami & Kudo 2002). ACA completely blocks ADPR-activated TRPM2 channel in HEK cells (IC$_{50}$ = 4.9 µM), and this inhibition is reversible (Harteneck, Frenzel & Kraft 2007). However, it is likely that this blockade is independent of PLA$_2$ inhibition. Two other PLA$_2$ inhibitors—para-bromophenacyl bromide (PBP) and arachidonyl trifluoromethyl ketone—have no obvious effect on TRPM2 current, even at very high concentrations (Kraft et al. 2006). ACA blocks not only TRPM2, but also other TRP channels, including TRPM8 and TRPC6 (Harteneck, Frenzel & Kraft 2007). ACA inhibits the menthol-mediated TRPM8 channel activation in HEK cells (IC$_{50}$ = 3.9 µM). It also blocks aluminium fluoride (AlF)–mediated TRPC6 channel activation with IC$_{50}$ of 2.3 µM in HEK293T cells (Kraft et al. 2006). There is no blocking effect of PBP on TRPM8 and TRPC6 channels. Thus, it is concluded that ACA blocks TRP channels through a mechanism unrelated to its inhibition of PLA$_2$ (Kraft et al. 2006).

1.5.7.2 Fenamates

Fenamates are a family of chemicals that are derivatives of N-phenyl-substituted anthranilic acid. These chemicals are categorised as non-steroidal anti-inflammatory drugs (NSAIDs), and the most commercially used drug of this group is mefenamic acid. These drugs are broad-spectrum COX inhibitors. COX enzymes convert the arachidonic acid resulting from PLA2 action into prostaglandins. Prostaglandins are mediators of fever, inflammation, pain sensation and clotting (Choi, Aid & Bosetti
Many fenamates, including flufenamic acid (FFA), have been shown to inhibit the TRPM2 current (Hill et al. 2004).

FFA blocks the TRPM2 current in concentrations ranging from 50 to 1,000 µM, with the rate of blockade directly correlating to the concentration of FFA (Hill et al. 2004). FFA is not a specific TRPM2 channel inhibitor—it interacts with other channels, including Cl− conducting γ-Aminobutyric acid A (GABA_A) receptor and ClC-Ka channel (Sinkkonen et al. 2003; Liantonio et al. 2006). Similar to other fenamates, FFA inhibits some other TRP channels, such as TRPM3, TRPM4 and TRPM5 (Klose et al. 2011; Ullrich et al. 2005). In contrast, FFA activates TRPC6 (c.f. to ACA) (Foster et al. 2009) and TRPA1 (Hu et al. 2010).

Fenamates exhibit different potency in blocking the TRPM2 channel. Among mefenamic acid, FFA, niflumic acid (NIF), tolfenamic acid, meclofenamic acid, 3′-5′-dichlorodiphenylamine-2-carboxylic acid (DCDPC) and S645648, DCDPC is the most potent (IC_50=31.1), whereas NIF, mefenamic acid and S645648 are the least potent (DCDPC > meclofenamic acid > tolfenamic acid > FFA > NIF, mefenamic acid, S645648) (Klose et al. 2011).

A newly synthesised fenamate, 2-(3-methyfenyl) aminobenzoic acid (3-MFA), has been found to be more selective in inhibiting TRPM2 than FFA. In 3-MFA, the trifluoromethyl group of FFA has been substituted with meta–CH_3. 3-MFA is much less potent in releasing Ca^{2+} from the intracellular stores than FFA, but has similar potency in TRPM2 channel inhibition (Chen et al. 2012). It has also been identified that 3-MFA inhibits TRPM2 current with similar potency to 2-APB and econazole, with minimal effects on the other TRP channels and stored Ca^{2+} (Chen et al. 2012).

The mechanism of fenamates-mediated TRPM2 channel inhibition is still unclear. FFA inhibits the channel in both reversible and irreversible ways. Following removal of FFA, 10-15% of the current recovered in a short time (~ 3 s) but the rest remained unrecovered (Hill et al. 2004). FFA probably inhibits the channel from the outside, as intracellular application of FFA has shown no inhibitory effect on the TRPM2 current (Hill et al. 2004).

1.5.7.3 Clotrimazole and other Azoles

Azoles are antifungal drugs that inhibit the synthesis of ergosterol, leading to the accumulation of 14-α-methylsterols, disruption of fungal respiration and fungal death.
Many of these agents interact with mammalian ion channels (Gupta et al. 2003). It has been shown that econazole, miconazole and clotrimazole completely block the ADPR-activated TRPM2 current at concentrations ranging between 3-30 µM (Hill, Mcnulty & Randall 2004; Togashi, Inada & Tominaga 2008). The mechanism of this blockade is not fully understood. However, it has been proposed that econazole and clotrimazole act from extracellular side and that their inhibition of the TRPM2 channel is irreversible (Hill, Mcnulty & Randall 2004).

1.5.7.4 2-APB

2-APB inhibits the TRPM2 channel in a manner that is complete and reversible. The 2-APB blockade of TRPM2 current is more prominent and potent than of other TRP channels, including TRPC1, TRPC3, TRPC5, TRPC6, TRPM3, TRPM7 and TRPM8 (Togashi, Inada & Tominaga 2008). In DRG neurons, 100 µM 2-APB completely blocks the ADPR-activated TRPM2 current (Naziroglu et al. 2011). As 2-APB inhibits the TRPM2 channel rapidly and reversibly, it has been suggested that 2-APB interacts with either extracellular domains of the TRPM2 channel or its pore (Togashi, Inada & Tominaga 2008).

1.5.8 The Role of TRPM2 Channels in oxidative stress-related pathologies

As described before, activation of TRPM2 channels in cells damaged by oxidative stress leads to a persistent rise in [Ca²⁺]c and cellular death. Because the aetiology of a large number of diseases lies in the increased production of ROS and oxidative stress, TRPM2 channels have been investigated in relation to a number of pathologies of immune, nervous, vascular and endocrine systems. Aberrant activation of TRPM2 channels may have a deleterious effect in a number of human diseases, including Alzheimer’s disease, diabetes mellitus, Behcet’s disease, brain trauma and some cancers (Fonfría et al. 2005; Lange et al. 2009; Naziroglu et al. 2014; Cook et al. 2010). TRPM2 channels play a significant role in normal functioning of the immune system. Compared to wild-type mice, the function of immune cells in TRPM2 deficient mice is severely impaired, with a strongly diminished response to infections (Yamamoto et al. 2008; Knowles et al. 2011). Using transgenic animals it has been found that, the cytokine production in monocytes of TRPM2 KO mice is notably decreased making the
mice highly susceptible to listeriosis. This is an infectious disease caused by the invasion of Listeria monocytogenes, and is manifested by septicaemia, meningitis, encephalitis, corneal ulcers and pneumonia, leading to death (Wehrhahn et al. 2010; Knowles et al. 2011). Another study, using dextran sulphate sodium-induced colitis, has shown a significant reduction in the amount of inflammation and the size of ulceration in TRPM2 KO mice, compared to WT mice, due to impairment of monocyte activation and migration (Yamamoto et al. 2008). Furthermore, it has been discovered that maturation and chemotaxis of dendritic cells is highly impaired in TRPM2 KO mice, resulting in lack of lymphatic trafficking of these cells in response to bacterial infection (Sumoza-Toledo et al. 2011). Findings show that TRPM2 channel is an essential factor for immune cells activation to combat infections. On the other hand, preventing TRPM2 channel activation in pathologies caused by over-activation of immune system, including Behcet’s disease, significantly reduces the severity of the disease (Naziroglu et al. 2014). Behcet’s disease, an autoimmune disorder of small vasculature, is usually manifested by ulceration of mucosal membranes and ocular problems, but also likely to affect internal organs, including GI tract, lungs, and cardiovascular and nervous systems. The aetiology of this disease is not fully understood, but the role of the immune system and the oxidative stress is well recognized (Hatemi, Yazici, & Yazici 2013). Abnormal homeostasis of T cells (Th1, Th17 and regulatory T cells, in particular) triggers neutrophil hyperactivation, causing oxidative stress and small blood vessels damage, which may lead to blood flow occlusion and tissue necrosis (Pineton de Chambrun et al. 2012). Using Ca^{2+} imaging it has been demonstrated that [Ca^{2+}₈] in neutrophils collected from patients with Behcet’s disease is increased significantly, compared to [Ca^{2+}₈] in neutrophils from normal individuals. This increase in [Ca^{2+}₈] could be inhibited by TRPM2 channel blocker, 2-APB (Naziroglu et al. 2014), which suggests that in Behcet’s disease, an abnormal activation of TRPM2 channels in neutrophils leads to vascular damage (Naziroglu et al. 2014).

In nervous system, overproduction of ROS and oxidative stress contribute to development of several pathologies, including Alzheimer’s disease, Parkinson’s disease and brain trauma, possibly, through activation of TRPM2 channels (Fonfria et al. 2005; Lee et al. 2013). The role of TRPM2 mediated Ca^{2+} entry in oxidative stress-induced neuronal damage has been investigated in several studies. It has been shown that reducing the expression of the TRPM2 protein prevents H₂O₂-mediated neuronal
cells death (Kaneko et al. 2006). Furthermore, preventing TRPM2 channel activation by overexpression of TRPM2S reduces the H$_2$O$_2$- and β-amyloid-mediated neuronal cells damage (Fonfria et al. 2005). As treating the cultured rat striatal cells with H$_2$O$_2$ significantly increases the production of β-amyloid in those cells, it has been suggested that in Alzheimer’s disease, generation of β-amyloid and ensuing neuronal damage is mediated by TRPM2 channels activation in response to over production of ROS (Fonfria et al. 2005).

Using impact-acceleration model of brain trauma, it has been shown that the levels of TRPM2 mRNA and consequently TRPM2 protein are significantly increased in the cortex and hippocampus of injured rats, compared to sham-treated animals (Cook et al. 2010). These changes occur 3 days after inducing the injury, but not in the acute phase of the trauma. It is likely that an increase in TRPM2 channel expression in brain trauma is one of the main factors causing the enlargement of the necrotic area surrounding the original brain injury (Cook et al. 2010). Similarly, increase in TRPM2 expression is detected in glial cells one week after inducing ischemia-reperfusion injury in rat brain by transient occlusion of middle cerebral artery (Fonfria et al. 2006). Glial cells are known to play a fundamental role in the progression of brain damage due to ischemia-reperfusion (Zhang et al. 2013). There are several lines of evidence suggesting that TRPM2 channels may contribute to the development of Parkinsonism. The symptoms of Parkinson’s disease are caused by the deterioration of dopaminergic neurons in the substantia nigra pars compacta (SNC), and the deficit of dopamine in the striatum (Wichmann & Dostrovsky 2011). TRPM2 channels are expressed at high levels in basal ganglia of the brain, including SNC dopaminergic neurons (Fonfria et al. 2005). Experiments using rat brain slices have shown that intracellular application of ADPR through a patch pipette, or bath application of H$_2$O$_2$ (1-5 mM) results in activation of TRPM2 current in these neurons (Chung et al. 2011). Oxidative stress has been suggested as a main pathway of neuronal death in Parkinson’s (Surendran & Rajasankar 2012), and activation of TRPM2 channels by ROS may play a significant role in this process (Chung et al. 2011).

TRPM2 are also expressed in GABAergic neurons of the substantia nigra pars reticulata (SNr) (Lee et al. 2010). These SNr neurons normally exhibit regular pattern of action potential firing, but in Parkinsonism, this regular firing is replaced with bursts of action potentials interrupted by hyperpolarisations; these change in firing pattern may contribute to the pathophysiology of the disease (Ibáñez-Sandoval et al. 2007).
Experiments using SNr neurons from guinea pigs showed that H$_2$O$_2$, by activating TRPM2 channels, works synergistically with NMDA in converting these neurons firing pattern into bursting activity (Lee et al. 2013). Thus, activation of TRPM2 channels in GABAergic neurons by ROS may play a significant role in the mechanism of Parkinson’s disease (Lee et al. 2013). TRPM2 channels may be involved in the induction and progression of different types of epilepsy. Juvenile Myoclonic epilepsy (JEM), the most common form of idiopathic generalized epilepsy, is caused by the mutations in the EF-hand motif-containing protein, EFHC1 (de Nijs et al. 2012). Both EFHC1 and TRPM2 are expressed in hippocampal neurons, and immunoprecipitation analysis shows that EFHC1 protein interacts with N- and C-terminal cytoplasmic regions of TRPM2, enhancing its activity (Katano et al. 2012). It has been proposed that the mutation in EFHC1 changes its interaction with TRPM2 channel playing a role in JEM (Katano et al. 2012).

At least one genetic variant of TRPM2 channel (SNP rs1556314 in exon 11) has been found to correlate significantly with type 1 bipolar disorder; the exact mechanism of this association, however, has not been fully investigated (Xu et al. 2006).

In the cardiovascular system, TRPM2 channels have been shown to regulate endothelial cells permeability. Treatment of endothelial cell monolayers with H$_2$O$_2$ (300 μM) caused a decrease in trans-endothelial electrical resistance, which suggested opening of inter-endothelial junctions (Hecquet et al. 2008). At the same time, endothelial cells exhibited H$_2$O$_2$- dependent Ca$^{2+}$ entry and a non-selective cation current with properties similar to those of TRPM2 current. Disruption of TRPM2 activity by siRNA-mediated knock down, TRPM2 antibodies, or over-expression of a dominant negative splice variant of TRPM2, restored trans-endothelial resistance and significantly reduced Ca$^{2+}$ entry in H$_2$O$_2$-treated endothelial monolayers (Hecquet et al. 2008). These data suggest that TRPM2 channels mediate oxidative stress-induced endothelial dysfunction. Function of TRPM2 channels has different impact on different types of cancers. In glioblastoma cells, increasing TRPM2 channel expression boosts the cytotoxic effects of H$_2$O$_2$ without any effect on migration, proliferation or invasion of cancerous cells (Ishii et al. 2006); the authors suggest that activators of TRPM2 channels can be considered as possible anti-cancer drugs.

Studies exploring a link between non-coding RNAs (ncRNAs) and different types of cancer have recently discovered that some types of melanomas, lung cancers and
prostate cancers express aberrant antisense TRPM2 transcripts (TRPM2-AS and TRPM2-TE) which prevent expression of functional TRPM2 channels (Orfanelli et al. 2008, Orfanelli et al 2014). Knock-out of TRPM2-TE transcripts or over-expression of wild-type TRPM2 in melanoma cell line, increased its susceptibility to apoptosis and necrosis, suggesting that the expression of functional TPM2 channels is important in prevention of neoplastic transformation in melanocytes (Orfanelli et al. 2008). On the other hand, some other studies found that in PC-3 and DU-145 prostatic cancerous cells, inhibiting TRPM2 channel expression using siRNA prevents their growth without any obvious effect on normal cells (Zeng et al. 2010).

In the endocrine system, activation of TRPM2 channels by ROS has been implicated in the development of diabetes. In rats, pancreatic β-cells, which are responsible for producing insulin, express TRPM2 channels in both, plasma membrane and lysosomes (Uchida et al. 2011; Lange et al. 2009). Activation of TRPM2 channels in β-cells by H$_2$O$_2$ application, results in cellular damage, which can be prevented by TRPM2 channel inhibitor (ACA), (Bari et al. 2009). Recently it has been demonstrated that TRPM2 plays a role in the radiation-induced salivary gland damage (Liu et al. 2013). Irradiation is the therapy of choice in treating head and neck cancers, however, it has major side effects, inducing persistent salivary gland damage and generating dry mouth (xerostomia). It has been shown that in TRPM2 deficient mice, irradiation-induced xerostomia is significantly lower compared to WT mice. Data show that the effect of irradiation on [Ca$^{2+}$]$_c$ is significantly reduced in acinar cells of TRPM2 KO mice, compared to its effects in acinar cells of WT mice (Liu et al. 2013).

1.6 Oxidative Stress and Liver Diseases

The generation of ROS and RNS plays a crucial role in the pathogenesis of a large number of diseases, ranging from early life pathologies, such as premature labour and still birth, to age-related diseases, such as Alzheimer’s (Chen & Zhong 2014; Markesbery 1997; Migliore et al. 2005). Oxidative stress also plays a role in aetiology on a number of liver pathologies (Ishii, Kurose & Kato 1997; Roberts et al. 2009; Zhu et al. 2012). The liver is the largest internal organ responsible for a wide range of functions. Hepatocytes, which account for 80% of liver cells, synthesise most plasma proteins,
including albumin (major plasma protein), globulins (α and β globulins), most coagulating (fibrinogen, prothrombin and factors V, VII, IX, X and XI) and anti-coagulating factors (heparin, proteins C and S and plasminogen), acute phase reactant proteins, and many other proteins (Knell 1980). The liver is responsible for the synthesis of cholesterol, triglycerides and lipoproteins, and regulates the homeostasis of lipids in the blood (Knell 1980). It is also one of the major organs controlling blood glucose levels through insulin and glucagon. Gluconeogenesis (synthesis of glucose), glycogenolysis (generation of glucose from glycogen) and glycogenesis (formation of glycogen from glucose) are the related functions performed by the liver. The liver also produces some hormones, including thrombopoietin and insulin-like growth factor 1 (IGF-1), and is responsible for the production of bile. Furthermore, the liver is the main organ, which breaks down and detoxifies many endogenous and exogenous compounds (Herbarth et al. 2004).

Due to a large number of energy-demanding functions performed, hepatocytes have a high metabolic rate. Such high metabolism, accompanied by exposure to toxic compounds, exposes liver to oxidative stress due to overproduction of ROS and RNS. In normal conditions, hepatocytes have a high ROS scavenging capacity, and contain large amounts of antioxidants, including SODs and GSH (Mari et al. 2010). In pathology, the liver's free radical scavenging capacity is significantly reduced, and overproduction of ROS can damage the hepatocytes. Oxidative stress is a hallmark of many liver diseases, including alcoholic liver disease (ALD); liver fibrosis and cirrhosis; hepatocellular carcinoma; ischemia-reperfusion injury; drug-induced liver damage; viral hepatitis, particularly hepatitis C; and non-alcoholic fatty liver disease (Muriel 2009).

ALD is probably the major cause of oxidative stress–mediated liver damage. ALD is a term for a broad spectrum of disorders ranging from reversible fatty liver to severe irreversible liver injury, including alcoholic hepatitis, cirrhosis, liver fibrosis and hepatocellular carcinoma (Gao & Bataller 2011). Hepatocytes metabolise ethanol into acetaldehyde using alcohol dehydrogenase, cytochrome P450 and catalase. Similar to other ethanol metabolites, acetaldehyde generates ROS and RNS, inducing oxidative stress. Hepatocytes are very sensitive to the produced acetaldehyde as it can bind to proteins and DNA, making acetaldehyde adducts. These adducts accumulate in the hepatocytes, thereby preventing regular cellular functions, reducing the amount of
GSH, increasing LPO activity and inducing mitochondrial dysfunction (Gao & Bataller 2011).

The liver is the main organ responsible for drug metabolism, which makes it susceptible to drug-related toxicities. In the United States, drugs are responsible for more than 50% of acute liver failures (Lee 2003). Drug toxic reactions are categorised in two types. Type A drug reactions are predictable, dose dependent and usually occur due to overdose or drug interactions. Type B drug reactions—called ‘idiosyncratic drug reactions’—are usually rare, unpredictable and unrelated to the dose or duration of drug administration. Drugs can damage the liver through both types of reactions. Some of the liver toxic drugs cause damage through oxidative stress—these drugs are listed in Table 1. Most of these drugs affect the liver through Type B reaction.

### Table 1.1: Drugs that induce liver damage through oxidative stress and the corresponding pathologies

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Main pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen, isoniazid (INH), troglitazone, valproate, propiconazole</td>
<td>Acute hepatitis and acute liver failure</td>
</tr>
<tr>
<td>Diclofenac sodium, nitrofurantoin</td>
<td>Chronic hepatitis</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate, sulindac</td>
<td>Acute cholestasis</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Non-alcoholic liver fatty disease</td>
</tr>
<tr>
<td>Phenytoin, sulfonamides</td>
<td>Atypical hepatitis</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Fibrosis and cirrhosis</td>
</tr>
</tbody>
</table>

Antibiotics, antipsychotics and herbal supplements are the most common drugs that induce idiosyncratic liver damage. In the US, amoxicillin-clavulanate is responsible for more than 12% of all idiosyncratic drug-induced liver injuries (Amacher 2012). The mechanism of liver damage in Type B drug reaction is not completely understood; however, it is likely that the liver damage is mostly caused by the reactive metabolites of the drugs. Other mechanisms have also been proposed, including drugs interactions and immunological activation processes (Amacher 2012; Zhang et al. 2011).

Fewer drugs damage the liver through Type A drug reactions; however, these liver toxicities are more prevalent. The most common drug that affects the liver through Type A reaction is Acetaminophen.
1.7 Acetaminophen

Acetaminophen or paracetamol—the chemical name of N-acetyl-p-aminophenol (APAP)—is one of the most widely used antipyretic and analgesic drugs around the world. In Australia in 1999-2003, acetaminophen was the leading drug of poisoning in children below 2 years old leading to hospitalization (Cripps & Steel 2006). According to the reports of the US Food and Drug Administration, acetaminophen was the main drug causing poisoning in the US from 1995 to 1999. In severe cases acetaminophen hepatotoxicity is irreversible, leading to acute liver failure, for which the only available treatment is liver transplantation. In the US, acetaminophen hepatotoxicity is the main cause of acute liver failure leading to liver transplantation (Larson et al. 2005). In a six-year cohort study of 662 patients undergone liver transplantation after being diagnosed with acute liver failure, acetaminophen toxicity was the leading cause (42%) (Larson et al. 2005). The mean digested dose of acetaminophen in these patients was about 24 g (48 tablets of 500 mg of acetaminophen) (Larson et al. 2005).

1.7.1 History of discovery

Acetaminophen was discovered accidentally by a chemist David W Young while working at the Standard Oil Company of Ohio. However, officially acetaminophen was first synthesised by Harmon Northrop Morse in 1877 at Johns Hopkins University, by reducing p-nitrophenol with tin. Joseph von Mering published a paper on 1893 and for the first time described the effect of acetaminophen on humans (Prescott 2000). Later it was found that acetaminophen is a metabolite of acetanilide that was known before by the trade name of Antifebrin, which was removed from the market due to its life-threatening side effects, including cyanosis and methemoglobinemia. In the US, the marketing of acetaminophen began in 1953; however, due to the popularity of aspirin and incorrect beliefs about its safety, acetaminophen did not become widely used until the adverse effects of NSAIDs, especially aspirin, were publicized in the 1950s. When the side effects of NSAIDs became apparent—including gastric bleeding, renal failure and especially Ray’s Syndrome in children—their use reduced significantly, while the popularity of acetaminophen increased (Lamphier & Young 1958). Acetaminophen has similar therapeutic effects to NSAIDs—except for the anti-inflammatory effects—but
without the aforementioned side effects. Initially it was believed that acetaminophen was safe even in high doses; however, the hepatotoxicity and other toxic effects of acetaminophen overdose, including renal interstitial damage and ototoxicity became apparent (Boyd & Bereczky 1966; Peters et al. 1972).

1.7.2 Acetaminophen Pharmacokinetics

Acetaminophen is a weak acid that is almost completely absorbed from the gastrointestinal (GI) tract. It is easily distributed through the body and passes the blood brain barrier into the cerebrospinal fluid. Acetaminophen is mainly eliminated from the body via its metabolism in the liver, and less than 5% of digested acetaminophen is excreted unchanged by the kidneys (Prescott 1980).

In the liver, acetaminophen is metabolized via two pathways to become more water soluble (Figure 1.1). One of these pathways is conjugation, called Phase II Metabolism. During this process, the drug is conjugated with glucuronic acid, sulfates or GSH, and these conjugates are excreted from the kidney or GI tract. Glucuronidation (conjugation with glucuronic acid) is the main pathway of detoxification of acetaminophen, when it is taken at prescribed doses. The metabolism of acetaminophen is related to age—in young children, the main mechanism involved in acetaminophen metabolism is sulfation, while among older children (more than five years old) and adults, the main pathway is through glucuronidation (Miller, Roberts & Fischer 1976; van der Marel et al. 2003).

The other means of metabolizing acetaminophen by hepatocytes—Phase I Metabolism—is conducted using CYPs generating reactive compounds which covalently bind to the proteins (Jollow et al. 1974). In regular non-toxic doses, the amount of acetaminophen metabolized through this pathway is minimal which is easily detoxified by GSH (Jollow et al. 1974). The main CYP isoforms involved in acetaminophen metabolisms are CYP2E1, CYP1A2, CYP3A4, and CYP2D6 (Dong et al. 2000; Raucy et al. 1989). The most important of acetaminophen metabolites is NAPQI. Detoxification of NAPQI by GSH is very rapid and is catalyzed by glutathione transferase (Coles et al. 1988). In acetaminophen toxicity, however, overproduction of NAPQI causes depletion of GSH and NAPQI covalently binds to cellular proteins producing acetaminophen-protein adducts (Jollow et al. 1973). Acetaminophen-protein adducts disrupt mitochondrial functioning, causing overproduction of ROS and RNS
and leading to cell damage and liver necrosis (will be discussed in more detail in section 1.7.5), (Hinson et al. 1998; Knight et al. 200; Knight et al. 2003).

1.7.3 Acetaminophen Pharmacodynamics

The exact mechanism of acetaminophen’s therapeutic action is still not completely understood. It was believed that acetaminophen had a similar mechanism of action to NSAIDs; however, early studies based on in vitro experiments on visceral tissues indicated that the effect of acetaminophen in the prevention of prostaglandins (PGs) synthesis is minimal (Flower et al. 1972; Skjelbred, Album & Lokken 1977; Skjelbred & Lokken 1979). Later experiments, however, revealed that the effect of acetaminophen on PG synthesis is different in periphery and CNS (Jóźwiak-Bebenista & Nowak JZ 2014). Furthermore, acetaminophen is likely to have a dual action on the synthesis of PGs. At low concentrations (67–667 µM), acetaminophen increases the synthesis of PG, while, at higher concentrations, it prevents it (Robak, Wieckowski & Gryglewski 1978). In contrast to NSAIDs, acetaminophen has shown little (if any) effect on inflammation. It lacks the antiplatelet activity and the GI side effects; therefore it has been categorised as a separate drug (O’Brien, Finch & Clark 1970; Radack, Deck & Bloomfield 1987; Loebl et al. 1977).

Similar to NSAIDs, acetaminophen prevents the synthesis of PGs by blocking the COX; however, acetaminophen-induced COX inhibition varies between different types of COX in different tissues (Jóźwiak-Bebenista & Nowak JZ 2014). In 1991, the polymorphisms in the gene of COX were detected and classified as COX-1 and COX-2 (Xie et al. 1991; Kujubu et al. 1991), which were further divided into COX-1a and COX-1b, and COX-2a and COX-2b (Olsen et al. 2012; Morales-Sainz et al. 2008). Acetaminophen has lower potency in blocking COX-2 compared to COX-1; (Kis, Snipes & Busija 2005; Graham & Scott 2005), and COX enzyme derived from brain tissue is more susceptible to be blocked by acetaminophen than the spleen COX (Flower & Vane 1972). Some researcher have suggested that an unknown isoform of the COX enzyme that is blocked by acetaminophen—called ‘COX-3’—may be responsible for the acetaminophen effects; however, there is no evidence of the existence of such an enzyme yet (Botting 2000).
Acetaminophen is mainly excreted from the body through its metabolism in the liver. At regular doses, most of the absorbed acetaminophen is metabolized by glucoronidation and sulfation; the remaining small amount is metabolized by CYPs, mainly into NAPQI, which is easily detoxified by conjugation with GSH. In acetaminophen overdose, NAPQI overproduction depletes the cell from GSH. NAPQI accumulates and covalently binds to proteins, producing acetaminophen protein adducts, disrupting mitochondrial function, inducing cellular damage through oxidative stress and causing cell death.
1.7.4 Acetaminophen Overdose and Liver Damage

Since being reported for the first time in 1966 (Davidson & Eastham 1966), the acetaminophen-induced hepatocellular damage has been extensively investigated. The liver damage in acetaminophen toxicity is mainly initiated from centrilobular (Zone 3, perivenous) regions of the liver, and the extension of the damage to other zones (Zones 2 and 1, respectively) depends on the amount of consumed acetaminophen (Hinson, Roberts & James 2010). There is general agreement that acetaminophen mediates hepatocellular death through oncotic (haemorrhagic) necrosis (Jaeschke, Knight & Bajt, 2003). Apoptosis has also been proposed as a mechanism of hepatocellular death, (Ray et al. 1996), however, the role of apoptosis in acetaminophen toxicity has still not been clearly established.

The first pathology that appears as early as 2 h after induction of acetaminophen toxicity, and that can be detected by the histology of liver samples, is the loss of glycogen and the induction of vacuolization in centrilobular hepatocytes. This is followed by nuclear abnormalities in these hepatocytes, at around 3 h, and an extensive necrosis of centrilobular region is detectable after 6 h of induction (Mitchell et al. 1973; Walker, Racz & McElligott 1980).

Early investigations have shown that the liver damage is mediated by metabolism of acetaminophen to a reactive metabolite (NAPQI), which covalently binds to cellular proteins (Mitchell et al. 1973; Bessems & Vermeulen 2001). In regular doses, the NAPQI generated through cytochrome P450-mediated metabolism of acetaminophen is detoxified by conjugation with GSH (Jollow et al. 1974). NAPQI-GSH conjugation is mainly catalysed by glutathione transferase, and NAPQI is easily and rapidly removed from the body until the cellular levels of GSH are severely depleted (Coles et al. 1988).

In acetaminophen overdose, accumulation of large amounts of NAPQI in hepatocytes exhausts the GSH storage, however, GSH depletion by itself is not the sole mechanism of acetaminophen toxicity. It has been shown that in hepatocytes, GSH depletion mediated by diethylmaleate generates no cellular damage (Mitchel et al. 1973). Therefore, it is likely that other mechanisms, besides GSH depletion, are involved in hepatocellular damage induced by acetaminophen overdose.

As GSH is progressively depleted from hepatocytes, NAPQI covalently reacts with cellular proteins, forming acetaminophen-protein adducts (Green, Dabbs & Tyson...
Inhibition of the production of acetaminophen-protein adducts prevents cellular damage (Green, Dabbs & Tyson 1984; Tarloff et al. 1996). However, it is not just the level of covalent binding of acetaminophen metabolites to proteins is important, but more so the location of the protein adducts. In mice, N-acetyl-m-aminophenol (AMAP)—a regioisomer of acetaminophen—produces much higher levels of protein adducts than acetaminophen, when applied at similar doses, but without any signs of liver toxicity (Holme et al. 1991). Most of the produced AMAP-protein adducts are located in cytosol and ER. In contrast, the acetaminophen-protein adducts are mainly localised in mitochondria. Thus, it has been proposed that the location of the generation and accumulation of the adduct is more important than the amount of the protein adducts (Nelson et al. 1991).

The severity of liver damage by acetaminophen also depends on the type of proteins involved in covalent binding. Thiol-containing proteins are important for cellular structure and function. In acetaminophen toxicity, thiol-containing proteins are one of the main targets for acetaminophen metabolites and oxidation. The ratio of thiol-containing to total acetaminophen-protein adducts in acetaminophen-treated hepatocytes is much higher, compared to such ratio in hepatocytes treated with AMAP. Thus, the nature of the proteins affected by covalent binding is as important as the location of adducts (Pumford et al. 1989; Tirmenstein & Nelson 1989).

One of the key proteins affected in acetaminophen toxicity is glucose-6-phosphate dehydrogenase (G6PD). G6PD is an enzyme that catalyses the conversion of glucose-6-phosphate into 6-phosphoglucono-δ-lactone. G6PD activity is critical in the pentose phosphate pathway producing a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) from NADP⁺ (Pandolfi et al. 1995; Wang et al. 2014). In acetaminophen toxicity, G6PD becomes non-functional leading to a significant reduction in the amounts of NADPH (O'Brien et al. 2000). As NADPH is involved in several critical cellular metabolic functions, acetaminophen-treated hepatocytes lose one of the main sources of energy production, and lose one of the main reducing agents in the cell (Dietze et al. 1997).

Despite the important role of acetaminophen-protein adducts formation in the process of acetaminophen-induced liver damage, and similarly to GSH depletion, their formation alone is not enough to induce hepatocellular death. In glutathione transferase KO mice, applying high doses of acetaminophen resulted in 70% reduction in GSH level compared to more than 90% GSH reduction in WT mice (Henderson et al. 2000).
The KO mice was significantly less susceptible to acetaminophen-induced liver damage, compared to WT mice, despite similar levels of acetaminophen-protein adducts. This suggests that in acetaminophen toxicity, at least 90% reduction in GSH is needed to induce liver necrosis (Henderson et al. 2000). The rise in the amounts of NAPQI, significant GSH depletion and acetaminophen-protein adducts generation lead to overproduction of ROS and RNS, damaging the hepatocytes through LPO, DNA fragmentation and mitochondrial dysfunction, which lead to irreversible cell damage and death (Hinson, Roberts & James 2010). The role of reactive compounds in acetaminophen toxicity is discussed in the next section in detail.

1.7.5 Oxidative Damage in Acetaminophen Toxicity

There is large body of evidence that suggests that acetaminophen-induced liver damage is mediated through oxidative stress (Lores et al. 1995; Gibson et al. 1996; Chan et al. 2001). Early investigations showed a significant increase in lipid peroxidation in hepatocytes of acetaminophen-treated rats (Muller 1983), which was later confirmed in mice (Knight et al. 2003). Oxidative degradation of lipids is caused by ROS interacting with polyunsaturated fatty acids of the plasma membrane and formation of lipoperoxyl radical (LOO•), which subsequently generates lipid radicals and lipid hydroperoxide. The end products of lipid peroxidation, mostly aldehydes, are used as markers of ROS overproduction (Barrera 2012; Knight et al. 2003). In acetaminophen-induced liver toxicity the levels of LPO directly correlate with the amount of acetaminophen-protein adducts (Mitchell et al. 1981).

Initially, it was believed that the main cause of oxidative stress was acetaminophen metabolism by CYP450 and the oxidative properties of NAPQI itself (Wendel & Feuerstein 1981). However, soon after, it was recognised that NAPQI is not the immediate cause of oxidative stress-mediated cellular damage (Smith & Jaeschke, 1989). Therefore, other mechanisms of acetaminophen-induced oxidative stress have been proposed and investigated (Bajt et al. 2003).

Direct measurements suggested that in acetaminophen toxicity, the production of both ROS and RNS in hepatocytes is elevated (Lores et al. 1995; Bajt et al. 2003). Using in situ liver chemiluminescence, Lores Arneiz et al have shown a 72% increase in H₂O₂
intracellular concentration 15 min after the injection of 375 mg.kg\(^{-1}\) of acetaminophen to mice (Lores et al. 1995). These authors also found that 8 mM acetaminophen caused 2.4-fold increase in the production of superoxide by the liver microsomes (Lores et al. 1995). The role for ROS is also supported by the data showing that an iron chelator, deferoxamine, significantly attenuates acetaminophen-induced liver toxicity (Adamson & Harman 1993; Ito et al. 1994; Kyle et al. 1987). This is consistent with the elevation of \(H_2O_2\) concentration in hepatocytes and the production of highly reactive hydroxyl radical through Fenton reaction (\(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\)).

In addition to overproduction of ROS, acetaminophen toxicity causes generation of RNS. High levels of nitrotyrosine protein adducts, found in the livers damaged by acetaminophen overdose suggest high levels of peroxynitrite in the affected hepatocytes (Hinson et al. 1998; Knight et al. 2001). Peroxynitrite is generated in the reaction between nitric oxide and super oxide, and interacts with tyrosine to form 3-nitrotyrosine (Reiter, Teng & Beckman 2000). The levels of 3-nitrotyrosine in acetaminophen-treated hepatocytes strongly correlate with the levels acetaminophen-protein adducts and the levels of necrosis, suggesting a detrimental role for RNS in acetaminophen toxicity (Hinson et al. 1998). Early investigations suggested that inducible nitric oxide synthase (iNOS), which is responsible for the synthesis of nitric oxide from L-arginine in the cytoplasm, was activated in the hepatocytes of acetaminophen-treated rats (Gardner et al. 1998). This conclusion was based on the finding that pre-treatment of rats with aminoguanidine (relatively specific iNOS inhibitor) significantly reduces the acetaminophen-induced hepatocellular damage (Gardner et al. 1998). Further studies, however, showed that in iNOS KO mice, despite 50% reduction in blood ALT levels, compared to WT mice, acetaminophen-induced liver necrosis was similar. Therefore, it was concluded that the role of iNOS in acetaminophen-induced liver damage was minimal (Michael et al. 2001). Burke, Macmillan-Crow & Hinson also found no obvious role of iNOS in acetaminophen toxicity; but confirmed that the peroxynitrite production in acetaminophen-treated hepatocytes is significantly increased, suggesting that NO is generated in the mitochondria (Burke, Macmillan-Crow & Hinson 2010; Ghafourifar & Cadenas 2005). Other studies supported this suggestion, showing peroxynitrite interactions with mitochondrial protein (adduct production) and DNA (DNA fragmentation) in mice treated with toxic doses of acetaminophen (Agarwal et al. 2011; Cover et al. 2005b). Disruption of the mitochondrial membrane integrity and
increased permeability is likely to be responsible for peroxynitrite production during acetaminophen toxicity (Cover et al. 2005b).

As oxidative stress is the likely cause of acetaminophen toxicity, the pathways leading to generation of ROS and RNS in hepatocytes have been investigated in some detail, including uncoupling of CYPs, particularly CYP2E1 (Koop 1992) and mitochondrial dysfunction (Knight et al. 2001; Brand et al. 2004; Casteilla, Rigoulet & Penicaud 2001).

CYP2E1 is one of the main enzymes metabolising acetaminophen in the liver, and CYP2E1 KO mice are less susceptible to acetaminophen toxicity than WT mice (Chen et al. 2008). Initially, it was concluded that the reduction in acetaminophen–induced liver damage was due to a decrease in acetaminophen metabolism to NAPQI. However, further experiments showed no difference between CYP2E1 KO and WT mice in the ability to produce NAPQI, as CYP2E1 KO mice treated with toxic doses of acetaminophen had similar NAPQI concentration in the urine (Harman et al. 1991; Tong et al. 1998). The explanation was is that CYP2E1 is likely to act as Fe$^{2+}$ donor for Fenton-mediated ROS production (Al-Ghamdi et al. 2004). In this pathway, Fe$^{2+}$ directly interacts with H$_2$O$_2$ producing OH' and hydroxide ion (Wink et al. 1994). It has been shown that, CYP2E1 inhibitors prevent the production of OH' from H$_2$O$_2$ in H$_2$O$_2$-treated LLC-PK1 cells (Al-Ghamdi et al. 2004), which suggests that CYP2E1 mainly contributes to acetaminophen toxicity through generation of OH'.

Another major contributor to acetaminophen toxicity is mitochondria, and mitochondrial dysfunction has been shown to play a significant role in mediating liver necrosis (Jaeschke et al. 2012a). Using electron microscopy, Walker, Racz & McElligott have shown a significant change of the mitochondrial morphology in the livers damaged by acetaminophen overdose (Walker, Racz & McElligott 1980). Further studies revealed that NAPQI interrupts the mitochondrial respiration at complexes I and II, reducing ATP generation and leading to overproduction of reactive compounds (Burcham & Harman 1991; Donnelly, Walker & Racz 1994). Increased concentration of glutathione disulfide (a marker of the intracellular reactive compounds overproduction) in acetaminophen toxicity suggests mitochondrial dysfunction as the main source of oxidative stress (Tirmenstein & Nelson, 1990).

Mitochondrial dysfunction is initiated by the production of high amounts of acetaminophen-protein adducts in the mitochondria. Consistent with that is a higher
susceptibility of mice to acetaminophen toxicity than rats, as mice exhibit much higher levels of the mitochondrial acetaminophen-protein adducts than rats (Mcgill et al. 2012). In addition to interrupting ATP synthesis, formation of acetaminophen-protein adducts causes opening of mitochondrial permeability transition (MPT) pore, resulting in mitochondrial inner membrane depolarization and mitochondrial Ca\(^{2+}\) release (Weis, Kass & Orrenius 1994; Kon et al. 2004; Reid et al. 2005). Cyclosporine A, a MPT inhibitor, significantly protects the cultured mouse hepatocytes from acetaminophen-induced damage without producing a noticeable change in NAPQI production (Kon et al. 2007). It has been demonstrated that cyclosporine A largely prevents the acetaminophen-induced mitochondrial swelling and mitochondrial membrane depolarisation (Masubuchi, Suda & Horie 2005). In vivo study using cyclosporine A in acetaminophen-treated rats showed a significant reduction in liver toxicity and damage (Nieminien et al. 1997). Thus, it can be concluded that mitochondria plays a pivotal role in the development of acetaminophen toxicity in the liver.

Acetaminophen overdose, in addition to initiating MPT, also causes mitochondrial outer membrane permeabilisation (MOMP), which leads to a release of pro-death factors, including endonucleases that facilitate DNA fragmentation, from the intermembrane space to the cytosol (Jaeschke & Bajt 2006). In acetaminophen toxicity, endonuclease G, released from mitochondria into the cytoplasm due to MOMP, is translocated into the nuclei, cleaving DNA and causing DNA fragmentation (McGill et al. 2012). This DNA fragmentation is one of the early indicators of acetaminophen-mediated hepatocellular damage (Ray et al. 1993; Shen et al. 1992).

Besides the role of hepatocellular reactive compounds production, immune cell activation has been shown to play a role in acetaminophen-mediated oxidative liver damage. There is strong evidence of inflammation and activation of pro-inflammatory cells in the liver affected by high doses of acetaminophen (Lawson et al. 2000). The exact mechanism of inflammation, however, is still not well understood. It has been proposed that the inflammatory processes are mainly initiated by the release of cytokines and debris from dead and dying cells, as a consequence of acetaminophen-induced hepatocellular damage (indirect activation). However, there is some evidence to suggest that the inflammatory pathways can be activated directly in the liver by high doses of acetaminophen. Mitochondrial dysfunction and permeabilization leads to release of mitochondrial compounds including high-mobility group box 1 protein, heat shock proteins and DNA fragments into the liver parenchyma, which activate
parenchymal and non-parenchymal inflammatory cells, including Kupffer cells (Jaeschke et al. 2012b; Lawson et al. 2000; Liu et al. 2006). Granulocyte receptor-1 (Gr-1) is a receptor located on many immune cells including neutrophils, monocytes and macrophages. Anti-Gr-1 antibody is usually used to deplete an animal from neutrophils (Matsuzaki et al. 2003). It has been identified that neutrophil depletion using anti-Gr-1 antibody before the application of acetaminophen significantly diminishes hepatocellular damage (Liu et al. 2006).

Finding that Kupffer cells and non-parenchymal macrophages release inflammatory mediators, including interleukins, cytokines and chemokines, in acetaminophen-induced liver damage, supports the role of pro-inflammatory cells (Gardner et al. 2003; Dambach et al. 2002). Reducing the number of natural killer (NK) cells using anti-NK monoclonal antibody reduces the production Interferon-γ (INF-γ) and the hepatocellular damage in acetaminophen overdose, compared to control mice. Therefore, it is likely that NK cells, by producing INF-γ, have a significant role in acetaminophen hepatotoxicity (Liu, Govindarajan & Kaplowitz 2004).

1.7.6 Current Clinical Treatment of Acetaminophen Toxicity, Advantages and Disadvantages

Currently, NAC is the main recommended treatment of acetaminophen toxicity. GSH is the main natural thiol-containing intracellular antioxidant (Manov, Hirsh & Iancu 2002). Depletion of GSH leads to increased levels of ROS and RNS, which can disrupt various cellular functions, leading to irreversible cellular damage and cell death (Chia et al. 2010; Casanova & Heck Hd 1987). It has been shown that, in acetaminophen overdose, the GSH levels in hepatocytes drop rapidly, and the extent of liver damage is related to the extent of GSH depletion. Replenishment of GSH can ameliorate the damage mediated by acetaminophen (Mitchell, Acosta & Bruckner 1985; Cigremis et al. 2009).

GSH is a three-peptide composed of glutamate, glycine and cysteine. Of these amino acids, cysteine has the lowest intracellular concentration, and plays a role of the limiting factor in the rate of de novo synthesis of GSH (Dickinson et al. 2003). Increase in intracellular concentration of cysteine can accelerate the synthesis of GSH (Kozer & Koren 2001). For this reason, the early treatment suggested for acetaminophen overdose was administration of cysteamine (cysteamine is a sulphydryl containing
compound derived from degradation of cysteine). Early studies found that in hepatocytes cysteamine inhibited the CYP450 from metabolizing acetaminophen into NAPQI, and therefore they concluded that this inhibition was the main mechanism the protective effect of cysteamine in acetaminophen toxicity (Miller & Jollow 1986). Although administration of cysteamine in acetaminophen overdose efficiently protects the liver from damage, due to significant side effects, including GI complaints (the most common side effect), hyperthermia, lethargy and rash, this treatment is no longer used. The other reason for removing cysteamine from clinical use is the availability of tolerable and safer cysteine-containing compounds (Miller & Jollow 1986; Besouw et al. 2011).

N-Acetyl Cysteine (NAC) is a cysteine containing pro-drug. NAC is easily metabolised in the cell to cysteine, which can be used to replenish the intracellular levels of GSH (Lauterburg, Corcoran & Mitchell 1983). Despite having a similar chemical structure, NAC has a much lower thiol reactivity, greater water solubility, greater resistance to oxidation and, importantly, lower toxicity than cysteamine (Prescott, Stewart & Proudfoot 1978). Currently, NAC is the main recommended treatment of acetaminophen toxicity. It is quite safe; however, it should be used with caution for pregnant women and patients with a history of asthma (Manov, Hirsh & Iancu 2002).

NAC is currently the only approved treatment for acetaminophen toxicity and is widely used; but as discussed earlier there are a number of disadvantages. Therefore, researchers and clinicians have searched for better treatments.

### 1.7.7 Antioxidants in Treatment of Acetaminophen Toxicity

Evidence suggests that oxidative stress is the main pathway of acetaminophen toxicity; hence, several studies have looked at the protective effects of different types of antioxidants in acetaminophen poisoning. Some of those antioxidants have shown some protection against acetaminophen toxicity in the liver. Most of them are of plant origin, including curcumin, green tea and silymarin.

Curcumin is a phenol-containing chemical obtained from the rhizome of the plant *Curcuma longa*. It has powerful antioxidant properties and has been shown to reduce liver damage in acetaminophen overdose. Liver protection against acetaminophen toxicity by curcumin is similar to the protective effect of NAC. It was shown that
curcumin, at 100 mg/kg, significantly reduced acetaminophen-induced liver and kidneys damage in rats (Kheradpezhouh et al. 2010; Yousef et al. 2010). There is controversy regarding the effect of green tea on acetaminophen toxicity. Green tea extracted from minimally processed leaves of *Camellia sinensis* contains high levels of flavonoids, powerful antioxidants. It has been shown that green tea extract administered before applying toxic dose of acetaminophen afford a significant protection against acetaminophen-induced hepatocellular damage in mice (Salminen et al. 2012; Roomi et al. 2008). It was also concluded that the timing of green tea administration is important for its protective effect, as applying green tea extract after acetaminophen injection increased the acetaminophen-induced hepatocellular damage in mice (Salminen et al. 2012). This happens probably due to facilitation of GSH depletion mediated by green tea extracts, as administration of green tea extract at 500 or 1000 mg/kg induces a significant reduction in GSH levels in mouse hepatocytes (Salminen et al. 2012). However, pre-treatment with green tea extract can protect the liver from acetaminophen toxicity through an unknown mechanism probably preventing NAPQI production (Salminen et al. 2012).

Silymarin is the extract of milk thistle (*Silybum marianum*) seeds, containing silibinin as an active component. Silibinin is a flavonolignan that exhibits an antioxidant activity. Its hepatoprotective effect has been extensively investigated and proven (Abenavoli et al. 2010). It has been demonstrated that applying 25 mg/kg sylimarin, post acetaminophen administration reversed the acetaminophen-induced liver and kidneys damage in rats (Gopi et al. 2010; Das et al. 2011).

Vitamin E is a natural antioxidant that has been shown to be effective in the treatment of acetaminophen toxicity. Administering Vitamin E before and after inducting acetaminophen toxicity reduces the extent of hepatocellular damage (Sener, Sehirli & Ayanoglu-Dulger 2003).

The observations that different types of antioxidants can reduce or prevent liver damage induced by acetaminophen overdose strongly support the idea that oxidative stress plays a significant role the mechanism of acetaminophen toxicity.

### 1.7.8 Role of Ca^{2+} in Acetaminophen-induced Hepatocellular Damage

Several early studies showed a significant rise in free cytoplasmic and mitochondrial Ca^{2+} concentration in hepatocytes subjected to high concentrations of acetaminophen.
(Corcoran, Bauer & Lau 1988; Ray et al. 1991). Measurements of the mitochondrial Ca$^{2+}$ concentration in the livers of mice affected by toxic doses of acetaminophen, showed a four-fold increase in mitochondrial Ca$^{2+}$ concentration six hours after the administration, compared to the control hepatocytes. This rise in Ca$^{2+}$ leads to mitochondrial Ca$^{2+}$-ATPase complex dysfunction and cellular damage (Tirmenstein & Nelson 1989). Using glycogen phosphorylase $a$ activity as an index of [Ca$^{2+}$]$_c$, it was suggested that acetaminophen overdose causes a significant rise in [Ca$^{2+}$]$_c$ in rat liver, as the activity of the enzyme increased significantly after administration of acetaminophen, and remained at high levels for several hours (Tsokos-Kuhn 1989). Several independent investigations have demonstrated this rise in [Ca$^{2+}$]$_c$ during acetaminophen toxicity (Tsokos-Kuhn 1989; Harman et al. 1992; Grewal & Racz 1993); however, it has been proposed that this rise in [Ca$^{2+}$]$_c$ is a secondary event in hepatocellular damage (Harman et al. 1992; Grewal & Racz 1993). In one study, it was elicited that the hepatocytes’ [Ca$^{2+}$]$_c$ was increased after 2 h of exposure to 5 mM acetaminophen despite observing some cellular damage after 1 h; then it was concluded that the notion that acetaminophen-induced [Ca$^{2+}$]$_c$ rise in hepatocytes was not an initial event (Harman et al. 1992). Nevertheless, these data did not exclude a possibility that rise in [Ca$^{2+}$]$_c$ contributes to hepatocellular death in acetaminophen overdose.

1.7.9 Evidence for Possible Involvement of TRPM2 Channels in Acetaminophen Toxicity

Sustained increases in free intracellular Ca$^{2+}$ concentration in hepatocytes treated with toxic doses of acetaminophen suggests activation of a Ca$^{2+}$ entry pathway across the plasma membrane, most likely mediated by Ca$^{2+}$ permeable channels. One of the candidates that can provide such a pathway for Ca$^{2+}$ entry in hepatocytes is TRPM2 non-selective cation channel. Several lines of evidence indirectly support this hypothesis. In patch-clamping experiments, TRPM2 is activated in response to intracellular ADPR and extracellular application of H$_2$O$_2$ (Toth & Csanady 2010). Concentrations of both H$_2$O$_2$ and ADPR increase in acetaminophen toxicity (Cover et al. 2006; Lores et al. 1995). It has been shown previously that acetaminophen overdose results in activation of PARP1 (Cover et al. 2005a), the enzyme catalysing generation of poly-ADPR, which probably causes significant increase in intracellular concentration of ADPR, the main ligand of TRPM2. Intracellular GSH, which normally
detoxifies highly reactive oxygen species, is depleted in hepatocytes in acetaminophen overdose (Chia et al. 2010; Casanova & Heck Hid 1987). On the other hand, depletion of GSH in neuronal cells has been shown to facilitate activation of TRPM2 channels in response to H$_2$O$_2$. Incubation of DRG neurons with buthionine sulfoximine to prevent synthesis of GSH increases the activation of TRPM2 channels by H$_2$O$_2$. In contrast, application of NAC prevents the TRPM2 channel activation by H$_2$O$_2$ (Ozgul & Naziroglu 2012).

There are experiments that showed the protective effects of Chlorpromazine (calmodulin inhibitor) and 4-aminobenzamide (PARP inhibitor) on acetaminophen-induced cellular damage in mice (Ray et al. 2001). As mentioned before, both calmodulin and PARPs have significant roles in TRPM2 channel activation, which indirectly supports the notion that activation of TRPM2 channels contributes to acetaminophen-induced hepatocellular damage.

1.8 Conclusions and Project Aims

During toxicity, hepatocytes metabolize the accumulated acetaminophen into NAPQI; the NAPQI depletes the hepatocytes from GSH and covalently interacts with proteins producing acetaminophen protein adducts. These GSH depletion and acetaminophen protein adducts disrupt mitochondrial function resulting in ROS and RNS overproduction. Therefore, oxidative stress is the mainstay of acetaminophen-induced hepatocellular damage. As in oxidative stress, TRPM2 channel activation via ADPR overproduction has a major role in persistent rise in [Ca$^{2+}$]$_c$, and in acetaminophen toxicity significant rise in [Ca$^{2+}$]$_c$ has been detected; then in conclusion, there is a possibility that in hepatocytes facing acetaminophen overdose, oxidative stress increases ADPR production resulting in TRPM2 channel activation and consequently continuous rise in [Ca$^{2+}$]$_c$ and cell death.

The research presented in this thesis aims to investigate the role of TRPM2 channels in oxidative stress-induced hepatocellular damage, particularly in acetaminophen toxicity. The specific aims of the first experimental chapter are:

- to determine TRPM2 channel expression and the properties of TRPM2 current in rat hepatocytes
• to investigate the role of TRPM2 channels in H$_2$O$_2$- and acetaminophen-induced Ca$^{2+}$ entry in rat hepatocytes.

To provide a better understanding of TRPM2 channel involvement in acetaminophen toxicity, the aims of the second experimental chapter are:

• to compare the effects of acetaminophen overdose on TRPM2 WT and KO mice.
• to investigate H$_2$O$_2$- and acetaminophen-induced Ca$^{2+}$ entry and membrane currents in TRPM2 WT and KO mice.
• to investigate the role of TRPM2 channels in acetaminophen-induced liver toxicity

The findings of the first two experimental chapters indicate that TRPM2 channels expressed in hepatocytes are activated by H$_2$O$_2$ and acetaminophen and contribute to liver damage in acetaminophen overdose. The results of some experiments in these chapters indicated that the localisation of TRPM2 channels is changing in response to oxidative stress.

Thus, the aim of the third experimental chapter is:

• to investigate the trafficking of the TRPM2 channels in hepatocytes in response to treatment by H$_2$O$_2$ and acetaminophen.

The findings of the first two experimental chapters reveal the role of TRPM2 channels in acetaminophen-induced hepatocellular damage. Currently, there is no TRPM2 channel inhibitor suitable for human administration. However, there is a possibility that some substances with known hepatoprotective properties are, in fact, TRPM2 inhibitors.

Thus, the aim of the fourth experimental chapter is:

• to investigate curcumin and chlorpromazine, which are known to protect liver against acetaminophen-induced hepatocellular damage, as inhibitors of TRPM2 channel.
Chapter 2: The Role of the TRPM2 Channel in Acetaminophen-mediated $[\text{Ca}^{2+}]_c$ rise in rat hepatocytes

2.1 Introduction

Acetaminophen is a common over-the-counter drug used to treat pain and fever. Acetaminophen is safe when used at prescribed doses, its’ overdose, however, can be life threatening, causing severe liver and kidney damage (Davidson & Eastham 1966; Mitchell et al. 1973; Edwards et al. 1971). In western countries, acetaminophen-induced hepatotoxicity is a leading cause of acute liver failure requiring liver transplantation (Larson et al. 2005). Acetaminophen is a lipophilic drug, and more than 98% of digested acetaminophen is metabolised in the liver, while less than 2% of unchanged acetaminophen is excreted in urine (Miller, Roberts & Fischer 1976; van der Marel et al. 2003). In the liver, therapeutic doses of acetaminophen are metabolised by glucuronidation and sulphation into non-toxic compounds (Thomas 1993). Only a small amount of acetaminophen is converted by hepatic cytochrome P450 (CYP)-dependent mixed function oxidases to the reactive intermediate metabolite N-acetyl-para-benzoquinoneimine (NAPQI). The NAPQI generated by a therapeutic dose of acetaminophen is rapidly metabolised to non-toxic products by conjugation with glutathione (GSH) (Thomas 1993; Bessems & Vermeulen 2001). With large doses of acetaminophen, however, hepatic GSH becomes depleted resulting in the accumulation of toxic amounts of NAPQI. Covalent binding of NAPQI to cellular proteins has previously been considered the main cause of liver cell death under these circumstances. Indeed, it has been shown that covalent binding precedes hepatocellular death and treatments that prevent covalent binding, also prevent liver necrosis (Cohen & Khairallah 1997). More recently, however, it has been suggested that, by itself, the covalent binding of NAPQI is not sufficient to induce apoptosis or necrosis. The toxic signal produced by covalent binding undergoes further amplification through the formation of reactive oxygen and nitrogen species (ROS and RNS), deregulation of $\text{Ca}^{2+}$ homeostasis and increased intracellular $\text{Ca}^{2+}$, causing oxidant stress in mitochondria and inducing the mitochondrial membrane permeability transition (Jaeschke & Bajt 2006; Muriel 2009). Although widely acknowledged, the role of $\text{Ca}^{2+}$ in acetaminophen toxicity is poorly
understood and has not been thoroughly investigated. Non-selective Ca\textsuperscript{2+} channels blockers chlorpromazine and verapamil have been shown to attenuate liver injury in mice (Ray et al. 2001; Tsutsui et al. 2003), however, the mechanism of their protective properties in acetaminophen overdose is not clear, and it is not known whether it involves any Ca\textsuperscript{2+} permeable channels on the plasma membrane of hepatocytes.

The only Ca\textsuperscript{2+} selective channel that has been clearly identified in hepatocytes so far is Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} (CRAC) channel activated by the depletion of intracellular Ca\textsuperscript{2+} stores downstream of PLC\textbeta{} and PLC\textgamm{} signalling (Litjens et al. 2004; Barritt et al. 2009). In addition, a number of Ca\textsuperscript{2+} permeable non-selective cation channels with no clearly defined functions, mostly from TRP family of channels, have been shown to be present in hepatocytes and liver cells (Rychkov & Barritt 2011; Fonfria et al. 2006). One of these channels, TRPM2, which presence in the liver has only been demonstrated at the mRNA level (Fonfria et al. 2006), is activated in response to oxidative stress and, potentially, can be involved in acetaminophen-induced Ca\textsuperscript{2+} rise in hepatocytes.

The TRPM2 channel is a non-selective cation channel formed by four identical subunits each having six transmembrane domains and cytoplasmic N- and C-termini (Perraud et al. 2001; Kuhn & Luckhoff 2004). Two isoforms of TRPM2 channel have been identified: short and long. Only one of them – the long isoform acts as a channel (Zhang et al. 2003). The C-terminal of the long isoform has an ADPR binding motif (NUDT9-H) ‘homologous to NUDT9 ADPR pyrophosphatase’. ADPR binding to NUDT9-H is responsible for gating the channel (Perraud et al. 2001; Kuhn & Luckhoff 2004). During oxidative stress, the overproduction of ROS and RNS results in DNA damage in the nuclei and mitochondria, and activation of PARP1 and PARP2 (Kim, Jacobson & Jacobson 1993; Wang et al. 2012). PARP1 and PARP2 use NAD\textsuperscript{+} as a substrate to produce poly-ADPR (in the nucleus) or ADPR (in mitochondria). Poly-ADPR is then hydrolysed by poly-ADPR glycohydrolases to ADPR, which results in a rapid rise in ADPR concentration in cytosol and activation of TRPM2 channels (Kim, Jacobson & Jacobson 1993; Wang et al. 2012; Perraud et al. 2001; Kuhn & Luckhoff 2004).

The production of poly-ADPR in hepatocytes, resulting in ADPR generation, is one of the hallmarks of acetaminophen liver toxicity (Cover et al. 2005a). It is quite likely that ADPR produced as a result of acetaminophen-induced oxidative stress interacts with NUDT9-H motif in TRPM2 and gates the channel. In this study, we investigated the role of TRPM2 channels in acetaminophen-induced [Ca\textsuperscript{2+}]\textsubscript{c} rise in hepatocytes.
2.2 Methods and Materials

2.2.1 Chemicals

Acetaminophen, NAPQI, 3,4-Dihydro-5-[4-(1-piperidinyl) butoxyl]-1(2H)-isoquinolinone (DPQ), pleuronic acid, bovine serum albumin (BSA), ionomycin, dimethyl sulfoxide (DMSO), chlorotrimazole, ACA and chlorpromazine were purchased from Sigma-Aldrich (Rockville, Maryland, US). Dulbecco’s Modified Eagle Medium (DMEM), penicillin/streptomycin, and trypsin-ethylene glycol tetraacetic acid (EGTA) were purchased from GIBCO (Grand Island, New York, US). Fura2-AM and Fast SYBR® Green Master Mix were purchased from Invitrogen (Carlsbad, California, US). Foetal bovine serum (FBS) was purchased from Bovogen (Melbourne, Australia), and collagenase was purchased from Worthington (Lakewood, New Jersey, US).

2.2.2 Animals

Hooded Wistar rats were housed and bred in the controlled environment with a 12-hour light–dark cycle at least three weeks before beginning the experiments. Male rats aged eight to 12 weeks were used for the experiments. All experiments were approved by the Animal Ethics Committees of the University of Adelaide and Flinders University of South Australia.

2.2.3 Solutions

Wash media for hepatocyte preparation (mM): 136 NaCl, 4.7 KCl, 0.85 Na₂HPO₄, 0.45 KH₂PO₄, 24 NaHCO₃, 20 glucose, 1.3 CaCl₂, 0.8 MgSO₄ + 7 H₂O, BSA (10% W/V), penicillin (100 U/ml), streptomycin (100 µg/ml) and phenol red (0.001% W/V). Krebs-Ringer-Hepes (KRH) solution (mM): 136 NaCl, 4.7 KCl, 1.3 CaCl₂, 1.25 MgCl₂, 10 glucose and 10 Na-HEPES with pH adjusted to 7.4 by NaOH. Control bath solution for patch clamping (mM): 140 NaCl, 4 CsCl, 2 CaCl₂, 2 MgCl₂ and 10 Na-HEPES, adjusted to pH 7.4 with NaOH. Pipette solution for patch clamping (mM): 130-caesium glutamate, 5 MgCl₂, 5 CaCl₂ and 10 EGTA, adjusted to pH 7.3 with NaOH. Tris-buffered saline (TBS solution) (mM): 150 NaCl and 25 Tris with pH adjusted to 7.4 by...
NaOH. TBST solution: TBS solution plus 0.1% Tween-20. Phosphate buffered saline (PBS) solution (mM): 137 NaCl, 2.7 KCl, 10 Na$_2$HPO$_4$ and 1.8 KH$_2$PO$_4$.

2.2.4 Hepatocyte Isolation and Culture

The rat was briefly anesthetised with inhaled isoflurane, and then, was immediately injected with ketamin/xylazine (50 and 8 mg/kg, respectively) into the intra-peritoneal (IP) space. After the rat was fully anesthetised, it was moved to the 37°C incubator and fixed on a plate. The abdomen was opened medially and, by two lateral incisions near the ribs (in an inverted T-shape), was fully exposed. The portal vein was cannulised and the liver perfused with the perfusion media warmed to 37°C and bubbled with 2 L/min carbogen [95% O$_2$ and 5% CO$_2$]. The rat was exsanguinated by cutting the inferior vena cava (IVC) below the kidneys. The anterior part of the chest was then removed completely and the upper part of the IVC was cannulised. The abdominal part of the IVC was clamped and the liver was first perfused with media containing EGTA 0.5 mM and then (for around 15 minutes) with the perfusion media containing CaCl$_2$ 1.3 mM and collagenase (25 mg/100 ml). After being fully digested, the liver was harvested, placed in the sterile container on ice and chopped using scissors. The hepatocytes were isolated from the mixture by several washings with the pre-chilled (4°C) wash media, followed by 80 s centrifugation at 500 rpm. The isolated hepatocytes were cultured on glass coverslips at 37°C in 5% CO$_2$ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v) for 16 to 96 hours before the experiments.

2.2.5 Calcium Imaging

Fura2-AM was dissolved in 5 µl of 20% pluronic acid in DMSO (w/v) and diluted in KRH buffer to the final concentration of 5 µM. Sixteen hours after plating on glass coverslips, the hepatocytes were loaded with Fura2-AM for 30 minutes, washed and incubated in KRH solution for 10 minutes in the CO$_2$ incubator at 37°C. The fluorescence of Fura-2 was measured using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, Omega XF04 filter set for Fura-2, Photonic Science ISIS-3 ICCD camera and UIC Metafluor software. Fluorescence images were obtained every 10 seconds using a 20× objective. Fluorescence ratio
values (340/380 nm) were transformed to $[\text{Ca}^{2+}]_c$ using the equation derived by Grynkiewicz, Poenie & Tsien (1985); a $K_d$ of 224 nM for binding of Fura-2 to $\text{Ca}^{2+}$; and ionomycin and EGTA to determine $R_{\text{max}}$ and $R_{\text{min}}$, respectively (Figure 2.1).

### 2.2.6 Immunofluorescence Imaging

Isolated hepatocytes plated on glass coverslips were transferred to a 24-well tray (one coverslip/well). The coverslips were then washed 3 times with PBS, and fixed with -20°C methanol for five minutes. Afterwards, the cells were again washed 3 times with PBS, and blocked with 20% FCS in PBS for 15 minutes. After washing with PBS, the coverslips were incubated with primary antibody (Ab) for 3 hours (diluent: 0.1% Tween 20 in PBS, antibody concentration: 1/100–250). After incubation with primary antibody the coverslips were washed 3 times with diluent and incubated for 1 hour with secondary antibody (fluorescein isothiocyanate [FITC] type, 1/2,000–5,000 in diluent). After washing 3 times with PBS, the coverslips were mounted on microscope slides using Gold Antifade, and kept in darkness overnight. The following day, after sealing the edges with clear nail polish, the slides were used for imaging or stored at 4°C for later use. The immunofluorescence was imaged using an Olympus BX-52 microscope equipped with appropriate filter sets and XM-10 Olympus camera. The images were captured using Cell^B Olympus software.
Figure 2.1: Calibration of Ca\(^{2+}\) imaging system using ionomycin and EGTA

**a.** The fluorescence intensity of hepatocytes loaded with Fura2-AM at 340 and 380 nM excitation wavelengths. **b.** The 340/380 ratio of fluorescence intensity of hepatocytes loaded with Fura2-AM. Data from this graph was used to calculate \([\text{Ca}^{2+}]_c\) by using equation \([\text{Ca}^{2+}]_c = K_d \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times S_f^2 / S_b^2;\) \(S_f^2\) and \(S_b^2\) are respectively the values of proportional fluorescent activity measured at 380 nM for Ca\(^{2+}\) free (f represents free) and Ca\(^{2+}\) saturation (b represents bond) states.
2.2.7 Western Blotting

After preparation, 2-5×10^6 hepatocytes were washed with cold PBS 3 times, and centrifuged at 1,000 rpm for 10 minutes. The hepatocytes were then incubated with 100 µL lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X, 3 mM EDTA and 10 µl Protease Inhibitor) for 30 minutes on ice, followed by 15 seconds of vortexing. After centrifuging at 8,000 rpm for 15 minutes at 4°C, the supernatant was collected. The amount of protein was measured using the BCA Protein Determination Kit (Sigma). Next, 20 µl supernatant was mixed with 20 µl sample buffer (final concentration: 125 mM Tris, 10% glycerol, 4% sodium dodecyl sulfate (SDS), 4% 2-mercapto-ethanol and 0.2% bromophenol-blue) and incubated at 75°C for 15 minutes. The sample was then put on ice and centrifuged at 8,000 rpm for five minutes in a cold room (4°C). Equal amounts of proteins (around 5 µg) and ladders (visible and biotinylated ladders) were then separated on the SDS-PAGE gel using Pharmacia Electrophoresis EPS 3500XL, adjusted at 60 V and 90 to 110 mA, until the protein of interest reached the middle part of the lower gel.

The prepared gel consisted of two parts. The lower part was more condensed and contained 4.6 ml double distilled water (DDW), 2.5 ml of 30% acrylamide, 2.5 ml Tris (1.5M, pH 8.8), 100 µl of 10% SDS (w/v), 60 µl of 10% APS (w/v) and 25 µl tetramethylethylene diamine (TEMED). The small proportion on top (from 1 cm below the comb) consisted of 3.4 ml DDW, 830 µl of 30% acrylamide, 630 µl Tris (1M, pH 6.6), 50 µl of 10% SDS, 50 µl of 10% APS and 5 µl TEMED. After separation, the protein and the ladders were transferred to a pure nitrocellulose blotting membrane by electrophoresis using Pharmacia Electrophoresis EPS 3500XL at 60 V and 150 mA at 4°C in transfer buffer (SDS, Tris, glycine and methanol). After the transfer, the paper was blocked by incubation with TBST solution (150 mM NaCl, 25 mM Tris, pH 7.4, 0.1% Tween-20) containing 5% skim milk (w/v) for one hour at room temperature on a shaker.

The paper was then washed four times with TBST for 15 minutes each, and kept with anti-TRPM2 polyclonal antibody (diluted 1:1,000, Abcam) and anti-GAPDH monoclonal antibody (1:1,000, Abcam) overnight on a shaker at 4°C. The following day, the paper was washed with TBST 4 times, and incubated with horseradish peroxidase (HRP) secondary antibodies against primary antibodies and biotin (to visualise the biotinylated ladder) at room temperature on a shaker for 1 hour. The paper
was then washed 4 times with TBST. The protein bands were visualised using H$_2$O$_2$ and enhanced chemiluminescence (ECL). After developing, the film was scanned using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, California, US) and the band intensities quantified using Quantity One software, version 4.3.1 (Bio-Rad Laboratories).

### 2.2.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative RT-PCR

After preparation, $2 \times 10^6$ hepatocytes were washed 3 times with cold PBS, and centrifuged. The pellet was gently mixed with 1 ml Trizole. The cells were lysed using 300 µl chloroform vortexing and incubating the tube at room temperature for three minutes. RNA was extracted by centrifugation at 9,000 rpm for 15 minutes at 4°C. The clear supernatant was taken out to a new Eppendorf tube (without touching the white DNA layer) and 375 µl isopropanol was added to the supernatant, vortexed for 10 seconds and kept at room temperature for 10 minutes. The tube was then centrifuged at 7,500 rpm for 10 minutes at 4°C. The supernatant was removed carefully and the pellet was washed twice with pre-chilled 75% ethanol to remove the isopropanol. The pellet was dried in the air for 10 minutes (at room temperature) and then dissolved in 50 µl nuclease free water (NFW) for 10 minutes at 56°C. Afterwards, the prepared RNA was kept on ice. The amount of RNA was measured using Nanodrop ND-8000. Contaminated DNA was removed using a DNase enzyme, and the activity of DNase was stopped using a DNase stop solution. cDNA from RNA was prepared using a reverse transcriptase (RT) enzyme (50 minutes at 37°C). The produced cDNA was used for conventional or quantitative PCR. The primers used for detecting TRPM2 isoforms and quantitative PCR are listed in Table 2.1.

Conventional PCR was performed using an Eppendorf Mastercycler nexus machine according to published methods (Wehage et al. 2002). The products were then run on the gel containing 1 µl Gel Red per gel, and illuminated and captured using Imagemaster (VCD). To perform quantitative PCR, 2 µl cDNA was mixed with 10 µl Express SYBR Green ER qPCR Supermix Universal and 200 nM of each sense and antisense primers. The volume size was then increased to 20 µl using NFW. The mixture was run on a Rotor Gene 3000 (Qiagen) real-time PCR machine. The qPCR condition was one cycle at 50°C for two minutes, then one cycle at 95°C for five
minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for one minute. The condition for the melting curve was set on ramping from 60 to 94°C, with 1°C rise in each step and 3 seconds waiting. The raw data were processed by Rotor Gene 3000 software using the comparative quantitation method, and exported into a Microsoft Excel file for further analysis with GraphPad Prism 5. TRPM2 mRNA was compared with the expression of β-actin as a housekeeping gene to determine the efficiency of the expression and to validate the $2^{-\Delta\Delta C_T}$ (Figure 2.2). The slope of the difference between both genes was near zero (0.0439; Figure 2.2B); then evaluating TRPM2 gene expression using $2^{-\Delta\Delta C_T}$ is valid (Livak & Schmittgen 2001).

<table>
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<th>Table 2.1: The primers used in RT-PCR and quantitative RT-PCR experiments</th>
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<td>∆C-Stretch</td>
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TRPM2 primers detect all isoforms and variants of the TRPM2 channel, except TRPM2S (a short isoform of the TRPM2 channel that contains the cytoplasmic N-terminal and first two TM domains). ∆C-Stretch primers detect the NUDT9-H of the TRPM2 channel, and their product is 311 bp for TRPM2L and 255 for TRPM2-∆C variant, which is missing in TRPM2S. ∆N-Stretch 1 primers detect the N-terminus of TRPM2; their product is missing in TRPM2-∆N, but it is 268 bp for TRPM2L. ∆N-Stretch 2 primers also detect the N-terminus of TRPM2; their product is 464 bp for TRPM2L and 406 bp for TRPM2-∆N. The products of ∆N-Stretch 1 and ∆N-Stretch 2 sets of primers for TRPM2S are similar to the products of TRPM2L. The primers were designed for rat according to previous data on human samples (Wehage et al. 2002).
The efficacy of the amplification of the TRPM2 gene and the internal control (β-actin) was determined using quantitative RT-PCR and SYBR Green detection.
2.2.9 Patch-clamp Recording

Membrane currents were measured at room temperature (23°C) using standard patch clamping in a whole-cell mode, and a computer-based EPC-9 patch-clamp amplifier run by PULSE software. In order to monitor the development of membrane currents, voltage ramps between -120 to +120 mV were applied every two seconds following the achievement of whole-cell configuration. The holding potential was -40 mV. The data were analysed using PULSEFIT software. The TRPM2 current of hepatocytes was activated by adding 1 mM ADPR to the pipette solution, respectively. Patch pipettes were pulled from borosilicate glass and fire-polished to a resistance between 1.5 and 2.5 MΩ. The series resistance did not exceed 7.5 MΩ and was 50 to 70% compensated.

2.2.10 TRPM2 Knocked Down (KD) Hepatocytes

Four hours after plating, the hepatocytes were transfected with siRNA against TRPM2 or control siRNA using HiPerfect transfection reagent (Qiagen, Germany). After 36 to 48 hours, the hepatocytes were used for Ca²⁺ imaging and patch-clamp experiments. The level of TRPM2 mRNA and protein knockdown was assessed using qRT-PCR and western blotting.

2.2.11 HEK 293T Cells Culture and Transfection

HEK 293T cells were cultured in 75 cm² flasks at 37°C in 5% CO₂ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v). Cells were harvested using PBS containing EGTA. The harvested cells were plated onto glass coverslips and 8 h later transfected with pCIneo expression vector containing human TRPM2 cDNA (generously provided by Professor Yasuo Mori, Japan) using TrueFect transfection reagent (United BioSystems Inc., US). Sixteen hours later, the transfected HEK 293T cells were used for the patch-clamp experiments.
2.2.12 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA), followed by the Bonferroni post hoc test and Student’s t-test (two tailed).

2.3 Results

2.3.1 Expression of functional TRPM2 channel in rat hepatocytes

Previous investigation demonstrated the presence of TRPM2 mRNA in the liver; however, it did not specify the cell type (Fonfria et al. 2006). To determine whether TRPM2 channels are expressed in hepatocytes, we extracted the RNA from isolated rat hepatocytes, and conducted conventional RT-PCR using different sets of primers designed for detect different isoforms of TPM2 (Table 2.1). We have found that rat hepatocytes express the TRPM2L variant, which contains NUDT9-H motif in its’ C-terminus (Figure 2.3). To ascertain whether hepatocytes express TRPM2 on a protein level, we conducted western blotting and immunofluorescence imaging. Western blot analysis demonstrated that the long isoform of TRPM2 with a protein size of 171 KDa is expressed in hepatocytes at high levels (Figure 2.3b). Immunofluorescence imaging using TRPM2 specific antibodies confirmed the presence of TRPM2 protein and suggested that most of TRPM2 protein is localised intracellularly (Figure 2.3c.2).

To investigate whether any TRPM2 channels are expressed on the plasma membrane and are functional we used whole-cell patch clamping. ADPR was added to the pipette solution at 1 mM concentration to activate the channels. Data presented in Figure 2.4a, shows a robust activation of TRPM2 current by ADPR, which reached steady state within 2-5 min after establishing whole-cell configuration. As expected, the current exhibited a linear I-V plot (Figure 2.4b) with zero reversal potential, confirming poor selectivity of TRPM2 channel between cations (Xia et al. 2008). Replacement of 140 mM Na\(^+\) with N-Methyl-D-glucamine (NMDG) in the bath solution resulted in a drastic reduction of the inward current (Figure 2.4a). We used this test with NMDG in all further patch clamping experiments to discriminate between TRPM2 current and a non-
specific leakage. Previous studies using heterologous expression of TRPM2 in HEK293 cells showed that intracellular Ca\(^{2+}\) plays a role of an important co-factor in activation of TRPM2 channels by ADPR and the efficacy of ADPR strongly depends on intracellular Ca\(^{2+}\) concentration. However, in hepatocytes it was difficult to use pipette solutions with elevated Ca\(^{2+}\) concentrations, as the seal between the pipette and the membrane was quickly getting unstable. Therefore, all patch clamping was conducted using pipette solution with 120 nM free Ca\(^{2+}\). Under these conditions 10 µM ADPR had no effect on the membrane current, however, 100 µM consistently elicited TRPM2 current of a considerable amplitude (20-40 pA/pF). The dose-response curve for ADPR yielded EC\(_{50}\) of 484±6.55 µM (n=5) (Figure 2.4c).
Heterologously expressed TRPM2 channels are inhibited by ACA, clotrimazole, FFA and 2-APB (Harteneck, Frenzel & Kraft 2007; Togashi, Inada & Tominaga 2008; Hill et al. 2004; Naziroglu et al. 2011). In the next series of experiments, we investigated whether these blockers inhibit TRPM2 channels expressed in hepatocytes. The addition of either 1 or 10 µM in the bath solution resulted in a complete inhibition of ADPR-activated TRPM2 current (Fig 2.5a). However, there was a significant difference in the time course of the block: the higher concentration resulted in a much faster block (fig 2.5b). The time constant of the block was reduced from 871±17 at 1 µM ACA (n=3) to 149±4 s at 10 µM ACA (n=5) (Figures 2.5b). Similar to ACA, 25 µM clotrimazole completely inhibited ADPR-activated TRPM2 current (fig 2.5d & e).
Previously it has been shown that application of H\(_2\)O\(_2\) to the bath causes activation of heterologously expressed and endogenous TRPM2 channels (Perraud et al. 2005; Hara et al. 2002; Kaneko et al. 2006). The concentrations of H\(_2\)O\(_2\) used and the time course of activation varied between publications (Perraud et al. 2005; Hara et al. 2002). In hepatocytes, application of 1 mM H\(_2\)O\(_2\) to the bath did not activate any current within 10 min or recording. At 10 mM, however, H\(_2\)O\(_2\) was very effective in activation of a large current similar to the current activated by ADPR (Figure 2.6). It is likely that the failure of relatively low concentrations of H\(_2\)O\(_2\) to activate TRPM2 current in hepatocytes was due to the fact that in our experiments the intracellular free Ca\(^{2+}\) concentration was strongly buffered at 120 nM, whereas other studies used higher levels of Ca\(^{2+}\), which would facilitate TRPM2 activation (Perraud et al. 2005; Csanady & Torocsik 2009).
Figure 2.3: TRPM2 expression in hepatocytes

a. Detection of TRPM2 expression in hepatocytes by RT-PCR. The product of ΔC-Stretch primer set demonstrated the presence of NUDT9-H of TRPM2 channel. The products of ΔN-Stretch 1 & 2 primer sets provided evidence that the full length TRPM2 channel is expressed in hepatocytes. b. Western blot analysis of TRPM2 channel expression in hepatocytes. The detected TRPM2 band corresponds to the long isoform of TRPM2. c. Immunofluorescence imaging using antibody against TRPM2 channel: (1) the background (only secondary antibody was applied) and (2) TRPM2-specific immunofluorescence (both primary and secondary antibodies were applied).
**Figure 2.4: TRPM2 current in rat hepatocytes**

**a.** Activation of TRPM2 current in response to intracellular application of ADPR (1 µM) through the patch pipette. Each data point corresponds to an inward membrane current measured at -100 mV. The amplitude of the current at -100 mV was taken from the current traces recorded in response to voltage ramps between -120 and 120 mV, applied every 2 seconds. **b.** I-V plots of hepatocyte membrane currents obtained in response to voltage ramps between -120 and 120 mV immediately after achieving whole cells configuration (trace 1) (before the development of TPM2 current); after full development of the TRPM2 current (trace 2); and after the replacement of 140 mM NaCl in the bath with 140 mM NMDG Cl (trace 3). **c.** The dose-dependence of TRPM2 current amplitude on ADPR-concentration. The EC$_{50}$ was 484±6.55 µM (n=5).
Figure 2.5: Inhibition of TRPM2 current by ACA and clotrimazole

a. The time course of TRPM2 current inhibition by ACA. The amplitude of the current was measured from current traces obtained in response to voltage ramps between -120 and 120 mV, applied every 2 seconds. b. The average time constants of TRPM2 inhibition by 1 and 10 µM ACA. c. The I-V plots of TRPM2 currents activated by 1 mM ADPR. Trace 1 – control, trace 2 - NMDG, trace 3 - 10 µM ACA. d. The time course of TRPM2 current inhibition by 25 µM clotrimazole. The amplitude of the current was measured from current traces obtained in response to voltage ramps between -120 and 120 mV, applied every 2 seconds. e. The I-V plots of TRPM2 currents activated by 1 mM ADPR. Trace 1 – control, trace 2 - NMDG, trace 25 µM Clotrimazole.
Figure 2.6: Activation of TRPM2 current in response to H$_2$O$_2$ in hepatocytes

**a.** The time course of non-selective cation current activation by 10 mM H$_2$O$_2$ in hepatocytes. The amplitude of the current was measured from current traces obtained in response to voltage ramps between -120 and 120 mV, applied every 2 seconds. Activated current was reduced by replacing Na$^+$ with NMDG.

**b.** The I-V plots of TRPM2 currents activated by 10 mM H$_2$O$_2$. Trace 1 – baseline (before applying H$_2$O$_2$), trace 2 - H$_2$O$_2$-activated current, trace NMDG.

**c.** The average amplitude of the current measured at -100 mV: (1) before application of H$_2$O$_2$ in the bath; (2) after full development of the TPM2 current in response to 10 mM H$_2$O$_2$; and (3) after the replacement of 140 mM NaCl in the bath with 140 mM NMDG-Cl.
2.3.2 Activation of $Ca^{2+}$ entry and non-selective cation current in rat hepatocytes in response to treatment by $H_2O_2$ and acetaminophen

In order to investigate the effect of acetaminophen on the free $[Ca^{2+}]_c$ in hepatocytes, the plated cells were incubated with 10 mM acetaminophen for one hour in the presence of 1.3 mM $Ca^{2+}$, and then loaded with Fura2-AM in $Ca^{2+}$ free media. To measure the change in free $[Ca^{2+}]_c$ in response to $H_2O_2$, the hepatocytes were loaded with Fura2-AM, and then 0.5 mM $H_2O_2$ was applied in $Ca^{2+}$ free media. The addition of 1.3 mM g $Ca^{2+}$ to the bath solution resulted in a significant rise in the $[Ca^{2+}]_c$ in both the acetaminophen- and $H_2O_2$-treated hepatocytes (Figure 2.7). However, in the hepatocytes treated with concomitant 10 mM acetaminophen and The addition of 50 µM clotrimazole or 10 µM ACA into the incubation medium during the treatment of hepatocytes with acetaminophen or $H_2O_2$ resulted in almost complete inhibition of $[Ca^{2+}]_c$ rise (Figure 2.7a & c). Application of 10 µM ACA after the incubation of the hepatocytes with 0.5 mM $H_2O_2$ or 10 mM acetaminophen was prevented the $[Ca^{2+}]_c$ rise (Figures 2.7b and 2.7d, respectively). Inhibition of acetaminophen- and $H_2O_2$-induced $Ca^{2+}$ entry by TRPM2 channel blockers is consistent with the notion that oxidative stress activates TRPM2 channels in hepatocytes.

The liver toxicity of acetaminophen is believed to be largely mediated by its’ reactive metabolite, NAPQI (Jollow et al. 1973; Hinson et al. 1998; Knight et al. 200; Knight et al. 2003). To ascertain whether NAPQI activates $Ca^{2+}$ entry , 100 µM NAPQI was applied to Fura-2 loaded hepatocytes in $Ca^{2+}$ free media for 15 minutes Re-introduction of 1.3 mM $Ca^{2+}$ to the bath solution produced a significant rise in $[Ca^{2+}]_c$ (Fig 2.8). NAPQI-induced $Ca^{2+}$ entry could be completely prevented by concomitant treatment with 50 µM clotrimazole or 10 µM ACA (Figure 2.8).
Figure 2.7: Acetaminophen and H$_2$O$_2$ activate Ca$^{2+}$ entry in rat hepatocytes

a. Ca$^{2+}$ entry in hepatocytes treated with 0.5 mM H$_2$O$_2$ for 30 min under the mentioned conditions (n=3). Both clotrimazole (50 μM) and ACA (10 μM) were applied to the bath 5 min before the addition of Ca$^{2+}$. b. Ca$^{2+}$ entry in hepatocytes treated with 0.5 mM H$_2$O$_2$. The 10 μM ACA applied for 15-minute after incubation of the hepatocytes with H$_2$O$_2$. c. Ca$^{2+}$ entry in hepatocytes treated with 10 mM acetaminophen for 60 min under the mentioned conditions (average data from three separate cell preparations). Both clotrimazole (50 μM) and ACA (10 μM) were applied to the bath 5 min before the addition of Ca$^{2+}$. d. Ca$^{2+}$ entry in hepatocytes treated with 10 mM acetaminophen. The 10 μM ACA applied for 15-minute after incubation of the hepatocytes with acetaminophen.
Figure 2.8: The effect of NAPQI on $[\text{Ca}^{2+}]_c$ in hepatocytes

The dependence of $[\text{Ca}^{2+}]_c$ on time of Fura2-AM-loaded hepatocytes. $[\text{Ca}^{2+}]_c$ was measured in the presence of (1) 100 µM NAPQI; (2) 100 µM NAPQI and 50 µM clotrimazole; and (3) 100 µM NAPQI and 10 µM ACA.
Increase in $[\text{Ca}^{2+}]_c$ in response to $\text{Ca}^{2+}$ add back in $\text{H}_2\text{O}_2$ and acetaminophen treated hepatocytes suggests activation of $\text{Ca}^{2+}$ permeable channels on the plasma membrane. Although inhibition of $\text{Ca}^{2+}$ entry by clotrimazole and ACA suggests that the channel responsible may be TRPM2, $\text{Ca}^{2+}$ imaging alone cannot provide a definitive answer. To ascertain what type of channels is activated in hepatocytes in response to the treatments by $\text{H}_2\text{O}_2$ and acetaminophen, we used whole cell patch clamping. Isolated hepatocytes plated on glass coverslips for 24h were first incubated in a bath solution containing 2 mM $\text{Ca}^{2+}$ with either 1 mM $\text{H}_2\text{O}_2$ for 30 min or 10 mM acetaminophen for 45 minutes and then transferred to the recording chamber. In control hepatocytes, the current recorded in response to voltage ramp between -102 and 120 mV showed some outward rectification and amplitude between 2 and 5 pA/pF at -100 mV. That current was reduced by only 1.2±0.5 pA/pF (n=5) when NaCl in the bath solution was replaced with NMDG. In hepatocytes treated with either $\text{H}_2\text{O}_2$ or acetaminophen, the membrane current was almost linear and its’ amplitude was reduced by 16.33±5.0 (n=4) and 13.7±2.4 pA/pF (n=5), respectively, when NaCl in the bath solution was replaced with NMDG Cl (Figure 2.9).

2.3.3 siRNA-mediated knock down of TRPM2 protein attenuates $\text{H}_2\text{O}_2$- and acetaminophen-induced $\text{Ca}^{2+}$ entry and the cation current

In order to confirm that $\text{H}_2\text{O}_2$- and acetaminophen-induced non-selective cation current and $\text{Ca}^{2+}$ entry are mediated by TRPM2 channels, we used siRNA to reduce TRPM2 expression in rat hepatocytes. The level of the siRNA-mediated knockdown was ascertained using western blot analysis and quantitative RT-PCR. SiRNA against TRPM2 caused approximately 60% reduction in TRPM2 expression on both, mRNA level and protein level (Figure 2.10), (P < 0.0001).
Figure 2.9: H₂O₂ and acetaminophen activate a non-selective cation current in rat hepatocytes

**a.** The amplitude of the inward current, measured at -100 mV in control, 1 mM H₂O₂- and 10 mM acetaminophen-treated hepatocyte. **b.** The amplitude of the Na-mediated current, determined by replacing NaCl in the bath solution with NMDG Cl. There was a significant difference between the amplitudes of the currents measured in control, H₂O₂- and acetaminophen-treated hepatocytes (P = 0.0046 and 0.0084, respectively). **c.** I-V plots of membrane currents measured in control hepatocytes, and hepatocytes treated with 0.5 mM H₂O₂ in control bath solution, after replacement of 140 mM NaCl in the bath solution with 140 mM NMDG Cl and after the addition of 50 μM clotrimazole to the bath (n=7 for each trace). **d.** I-V plots of membrane currents measured in control hepatocytes, and hepatocytes treated with 10 mM acetaminophen for 60 min in control bath solution, after replacement of 140 mM NaCl in the bath solution with 140 mM NMDG Cl and after the addition of 50 μM clotrimazole to the bath (n=22 for each trace). Error bars are omitted for clarity here and all other I-V plots.
Figure 2.10: siRNA-mediated knockdown of TRPM2 expression

a. The level of TRPM2 channel expression in the control (Control-siRNA) and TRPM2 KD (TRPM2-siRNA) hepatocytes determined by using western blot. There was a 60% reduction in TRPM2 channel expression in TRPM2 KD hepatocytes compared to that in the control hepatocytes. 

b. The relative expression of TRPM2 mRNA in control and TRPM2 KD hepatocytes, determined by calculating $2^{-ΔΔC_T}$.
Ca$^{2+}$ imaging using Fura2-AM and Ca$^{2+}$ add-back protocol showed a significant reduction in the H$_2$O$_2$- and acetaminophen-induced Ca$^{2+}$ entry in TRPM2 KD hepatocytes, compared to control hepatocytes (treated with control siRNA) (Figure 2.11a). The peak Ca$^{2+}$ entry measured in H$_2$O$_2$-treated hepatocytes 15 minutes after introducing 1.3 mM Ca$^{2+}$ to the bath was reduced from 867±47 nM (n=3) in control hepatocytes, to 163±11 nM (n=3) in TRPM2 KD hepatocytes, (P = 0.0002) (Figure 2.11b). Similarly, peak Ca$^{2+}$ entry in TRPM KD hepatocytes treated with 10 mM acetaminophen for 1 h, was reduced to 111±5 nM (n=3), compared to 557±16 nM (n=3) measured in control hepatocytes under the same conditions, (P < 0.0001) (Figure 2.11d).

To confirm the results of the Ca$^{2+}$ imaging, whole-cell patch-clamp recording was performed on the control (Control-siRNA) and TRPM2 KD (TRPM2-siRNA) hepatocytes. The amplitude of TRPM2 current activated by 1 mM ADPR in the pipette solution at -100 mV was reduced from -149.7±19 pA/pF (n=5) in control hepatocytes to 44.46±8.9 pA/pF (n=5) in TRPM2 KD hepatocytes (P < 0.001) (Figure 2.12). The amplitude of the inward current induced by a 15-minute incubation of the control hepatocytes with 1 mM H$_2$O$_2$, or by the 45-minute incubation with 10 mM acetaminophen was -14.67±2.56 pA/pF (n=7) and -8.4±1.4 pA/pF (n=22) correspondingly. This amplitude was reduced to -5.24±1.2 pA/pF (n=7) and -2.56±0.5 pA/pF (n=17) in TRPM2 KD hepatocytes correspondingly, (P < 0.001) (Figure 2.12).

### 2.3.4 H$_2$O$_2$ and acetaminophen-induced Ca$^{2+}$ entry requires ADPR

Acetaminophen overdose has been shown to cause DNA fragmentation in hepatocytes and, as a consequence, activation of polyADP-ribose polymerase (PARP), which generates polyADPR, the main precursor of cytoplasmic ADPR (Cover et al. 2005a). Using antibodies against polyADPR and immunofluorescence we were able to demonstrate an increase in polyADPR production in hepatocytes treated for 45 min by either 10 mM acetaminophen or 1 mM H$_2$O$_2$ (Fig 2.13). Incubation with 10 mM acetaminophen overnight induced much stronger polyADPR production.

To investigate whether generation of ADPR in hepatocytes in response to H$_2$O$_2$ and acetaminophen contributes to activation of Ca$^{2+}$ entry we used PARP inhibitor DPQ (3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinoline) (Takahashi et al. 1999). Measurements of [Ca$^{2+}$]$_c$ showed that DPQ strongly inhibited Ca$^{2+}$ entry in rat
hepatocytes, supporting the notion that Ca\textsuperscript{2+} rise induced by H\textsubscript{2}O\textsubscript{2} and acetaminophen is mediated by ADPR-activated TRPM2 channels (Fig 2.14).

2.4 Discussion

In this study we show that hepatocytes express long isoform of TRPM2 channels. These channels function as a cation entry pathway across the hepatocyte plasma membrane and similarly to TRPM2 channels heterologously expressed in HEK293 cells are activated by ADPR included in the patch pipette and H\textsubscript{2}O\textsubscript{2} added to the bath solution (Perraud et al. 2001; Kuhn & Luckhoff 2004; Hara et al. 2002). Novel and important finding of this study is that TRPM2 channels mediate a substantial increase in [Ca\textsuperscript{2+}]\textsubscript{c} in hepatocytes treated with toxic concentrations of acetaminophen.
Figure 2.11: The effect of TRPM2 knockdown on hepatocyte Ca\(^{2+}\) entry

a. The effect of TRPM2 knockdown on Ca\(^{2+}\) entry in rat hepatocytes preincubated with 0.5 mM H\(_2\)O\(_2\) for 30 min. b. [Ca\(^{2+}\)]\(_c\) of 0.5 mM H\(_2\)O\(_2\)-treated control and TRPM2 KD hepatocytes 15 minutes after introducing 1.3 mM Ca\(^{2+}\). There was a significant difference in [Ca\(^{2+}\)]\(_c\) detected between the control and TRPM2 KD hepatocytes (P = 0.0002). c. The effect of TRPM2 knockdown on Ca\(^{2+}\) entry in rat hepatocytes preincubated with 10 mM acetaminophen for 60 min. d. [Ca\(^{2+}\)]\(_c\) of 10 mM acetaminophen-treated control and TRPM2 KD hepatocytes 15 minutes after introducing 1.3 mM Ca\(^{2+}\). There was a significant difference in [Ca\(^{2+}\)]\(_c\) detected between the control and TRPM2 KD hepatocytes (P value<0.0001).
The absolute amplitude of the current at -100 mV was measured from current traces obtained in response to voltage ramps from -120 to 120 mV. The TRPM2-mediated current was activated by either 1 mM ADPR in the pipette solution, pre-incubation of hepatocytes for 15 minutes with 1 mM H₂O₂, or pre-incubation with 10 mM acetaminophen for 45 minutes.
Figure 2.13: The role of ADPR in activation of $\text{Ca}^{2+}$ entry in hepatocytes

Immunofluorescence detection of poly-ADPR in: a. control hepatocytes; b. 10 mM acetaminophen-treated hepatocytes for 45 minutes; c. 1 mM H$_2$O$_2$-treated hepatocytes for 45 minutes; and d. 10 mM acetaminophen-treated hepatocytes for 16 hours.
Figure 2.14: The effect of PARP inhibitor on Ca$^{2+}$ entry in hepatocytes

Inhibition of H$_2$O$_2$- (a) and acetaminophen (b)-induced Ca$^{2+}$ entry in hepatocytes by PARP inhibitor DPQ (10 μM). DPQ was added to the incubation medium 2 min before the addition of H$_2$O$_2$ or acetaminophen (n=3).
The mechanism of acetaminophen hepatotoxicity has been under intensive investigation for several decades (Hinson et al. 2010). It has been established that acetaminophen overdose causes a multitude of interrelated cellular events (Hinson et al. 2010), but the relative importance of each of these events in hepatocellular death is not well understood. Briefly, the main steps that lead to acetaminophen hepatotoxicity can be summarised as follows. Saturation of glucuronidation and sulphation pathways by excessive levels of acetaminophen leads to the increased acetaminophen metabolism by several isoforms of cytochrome P450 (CYP2E1, CYP1A2, CYP3A4, and CYP2D6) into the reactive metabolite NAPQI (Qiu et al. 1998; 39). NAPQI saturate and deplete intracellular GSH and covalently binds to proteins (Qiu et al. 1998; Srivastava et al. 2010). Lack of GSH causes accumulation of reactive oxygen and nitrogen species and oxidative stress. Increased oxidative stress, together with covalent binding, causes mitochondrial dysfunction, DNA fragmentation and deregulation of Ca\(^{2+}\) homeostasis ((Hinson, Roberts & James 2010; Bessem & Vermeulen 2001). Oxidants and increased [Ca\(^{2+}\)]\(_{c}\) promote mitochondrial permeability transition, which, in turn, initiates further oxidative stress, loss of mitochondrial potential and cessation of ATP synthesis (Ishii et al. 2006). Finally, loss of ATP triggers necrosis of hepatocytes. Two cellular processes in this sequence are likely to result in an increase in cytoplasmic concentration of ADPR, the main ligand of the Ca\(^{2+}\) permeable TRPM2 channels (Perraud et al. 2001; Fonfria et al. 2004). Opening of the mitochondrial permeability transition pore in the inner membrane releases ADPR from mitochondria, while activation of PARP by DNA damage results in generation of ADPR precursor, polyADPR (Fonfria et al. 2004; Buelow, Song & Scharenberg 2008). The importance of the events that produce cytoplasmic ADPR is emphasised by the findings that the inhibitors of mitochondrial permeability transition and the PARP inhibitors protect liver against acetaminophen overdose (Kröger et al. 1997). However, not all studies agree on the role of PARP in the development of liver damage in acetaminophen toxicity (Cover et al. 2005a). A sustained rise of [Ca\(^{2+}\)]\(_{c}\) was one of the first findings of early work investigating acetaminophen toxicity (Tirmenstein & Nelson 1989; Tsokos-Kuhn 1989). Involvement of [Ca\(^{2+}\)]\(_{c}\) in the mechanism of liver damage was supported by observations that some Ca\(^{2+}\) channel blockers and calmodulin inhibitors prevented acetaminophen-induced hepatocellular damage (Saville et al. 1988; Satorres et al. 1995).
In this chapter we show that hepatocytes express functional TRPM2 channels that can be activated in the patch clamp experiments by the addition of ADPR into the pipette solution. The time course of TPM2 current activation, the linear I-V plot and its inhibition by ACA and clotrimazole in hepatocytes were similar to those observed in other cell types (Harteneck, Frenzel & Kraft 2007; Togashi, Inada & Tominaga 2008). The concentration of ADPR required to fully activate TRPM2 channels in hepatocytes was relatively higher than in other cells types, most likely due to the strong buffering of intracellular Ca²⁺ to 120 nM in this study (Perraud et al. 2001; Csanady & Torocsik 2009). Strong Ca²⁺ buffering was also probably the reason that the TRPM2 current in hepatocytes could only be activated by very high concentrations of H₂O₂ in the bath. Our attempts to use weak Ca²⁺ buffering in patch clamping resulted in a quick loss of the seal between glass pipettes and plasma membrane.

In Ca²⁺ imaging experiments, however, relatively low concentrations of H₂O₂ were required to activate Ca²⁺ entry, as intracellular Ca²⁺ under these conditions was unbuffered. After the addition of Ca²⁺ to the bath solution, [Ca²⁺]ₐ continued to rise for 10-15 minutes in H₂O₂ pre-treated hepatocytes, which is consistent with the positive role of Ca²⁺ in activation of TRPM2 channels. Ca²⁺ entering the cell through TRPM2 channels provides positive feedback mechanism, increasing the number of active TRPM2 channels thus further promoting the increase in [Ca²⁺]ₐ.

Similar Ca²⁺ entry was activated by pre-incubation of hepatocytes with acetaminophen. The notion that Ca²⁺ entry activated by H₂O₂ and acetaminophen is mediated by TRPM2 is supported by following observations: (i) this Ca²⁺ entry was inhibited by TRPM2 blockers - clotrimazole and ACA, and (ii) this Ca²⁺ entry was attenuated by siRNA-mediated knockdown of TRPM2 protein in hepatocytes.

In patch clamping experiments hepatocytes pre-treated with H₂O₂ or acetaminophen exhibited a linear non-selective current which was also inhibited by clotrimazole, ACA and siRNA-mediated knockdown of TRPM2. It should be noted here that achieving a gigaseal with the membrane of pre-treated hepatocytes presented a considerable challenge due to membrane blebbing. Only cells that showed little or no visible damage were amenable to patch clamping. Therefore, it is likely that the amplitude of TRPM2 current developed in response to pre-treatment with H₂O₂ or acetaminophen was underestimated. These cells also showed some non-specific leakage, which was not blocked by ACA, clotrimazole or NMDG.
One of the splice variants of TRPM2, TRPM2ΔC, lacks the ADPR binding motif and has been shown to be activated by H₂O₂, but not ADPR (Wehage et al. 2002). Our RT-PCR data showed that TRPM2ΔC is not expressed in hepatocytes. Pre-treatment of hepatocytes with H₂O₂ and acetaminophen resulted in production of poly-ADPR, as determined by immunofluorescence using poly-ADPR-specific antibodies, and therefore ADPR. Theoretically, in our experiments using H₂O₂ and acetaminophen, TRPM2 could be activated by both, a direct action of H₂O₂ on the channel and through generation of ADPR (Hara et al. 2002; Perraud et al. 2005). Inhibition of H₂O₂- and acetaminophen-induced Ca²⁺ entry by PARP inhibitor, DPQ, suggested that in hepatocytes activation of TRPM2 channels mainly occurs through generation of ADPR, rather than a direct action of H₂O₂.

In conclusion, findings of this study add considerably to our current understanding of the mechanism of acetaminophen liver toxicity. Currently, the only clinically available treatment for acetaminophen overdose is N-acetylcysteine, a GHS precursor, which to be effective has to be administered within 15-16 h after acetaminophen ingestion (Chun et al. 2009). If this time window is lost, the efficacy of N-acetyl-cysteine in preventing liver damage is significantly reduced, and liver failure is the likely outcome (Chun et al. 2009). The TRPM2 channel offers an alternative therapeutic target, which may allow treatment over a wider window of time. Moreover, inhibitors of TRPM2 offer the potential to treat other ROS-mediated liver diseases such as non-alcoholic liver disease, hepatitis and hepatocellular carcinoma.
Chapter 3: The role of TRPM2 Channel in acetaminophen-induced Hepatocellular Damage

3.1 Introduction

Acetaminophen (paracetamol) is a widely used medicine to treat pain and fever. At prescribed doses, acetaminophen is safe, however, its overdose can be life threatening, causing severe liver and kidney damage (Davidson & Eastham 1966; Mitchel et al. 1973). Acetaminophen overdose is the most common cause of acute liver failure and the leading cause of chronic liver damage requiring liver transplantation in developed countries (Larson et al. 2005; Craig et al. 2010; Khan, Oniscu & Powell 2010). The only currently available treatment for acetaminophen overdose is the injection of N-acetyl-cysteine, a GSH precursor, which has to be administered within 8 hours after the ingestion to be fully effective. Its’ usefulness drastically drops after 8 h, and it is usually not recommended after 24 h of overdose. Acetaminophen liver toxicity arises from its metabolism by hepatic cytochromes P450, including CYP2E1, into a highly reactive compound, NAPQI. Normally, generated NAPQI is detoxified by GSH conjugation and excretion of the conjugate into the bile (Bessem & Vermeulen 2001). In the process of detoxification, the GSH stores in hepatocytes are significantly depleted, which leads to the accumulation of NAPQI. NAPQI interacts with cellular proteins, generating acetaminophen-protein adducts (Chia et al. 2010; Casanova & Heck Hd 1987; Henderson et al. 2000; (Hinson, Roberts & James 2010), leading to disruption of mitochondrial functions, increase in mitochondrial membranes permeability, decrease in ATP production, and overproduction of ROS and RNS resulting in oxidative stress-mediated hepatocellular damage (Kon et al. 2004; Reid et al. 2005; Weis, Kass & Orrenius 1994; Jaeschke & Bajt 2006).

One of the well-known, but poorly understood consequences of acetaminophen overdose is disruption of Ca$^{2+}$ homeostasis and increase in intracellular Ca$^{2+}$ concentration in hepatocytes (Corcoran, Bauer & Lau 1988; Ray et al. 1991). In the previous chapter, we have shown that acetaminophen-induced [Ca$^{2+}$]$_e$ rise in hepatocytes is mediated by the Transient Receptor Potential Melastatin 2 (TRPM2) cation channels. Hepatocytes express long isoform of TRPM2 channel, gated by ADP-
ribose binding to NUDT9-H motif in its C-terminus (Perraud et al. 2001; Kuhn & Luckhoff 2004). Treatment of hepatocytes with high concentrations of acetaminophen or \( \text{H}_2\text{O}_2 \) results in activation of TRPM2-mediated non-selective cation current and \( \text{Ca}^{2+} \) entry, most likely due to ADPR production in response to the oxidative stress-induced damage to mitochondria and DNA (Kim, Jacobson & Jacobson 1993; Wang et al. 2012). Previous research into the mechanisms of acetaminophen toxicity showed that some \( \text{Ca}^{2+} \) channel blockers and calmodulin antagonists, including verapamil and chlorpromazine, effectively attenuate acetaminophen-induced liver damage (Saville et al. 1988; Satorres et al. 1995). This suggests that \( \text{Ca}^{2+} \) entry and \( \text{Ca}^{2+} \) signalling play a significant part in development of acetaminophen toxicity. Our data presented in the previous chapter show that TRPM2 channels are the principle \( \text{Ca}^{2+} \) entry pathway responsible for acetaminophen-induced \( \text{Ca}^{2+} \) entry, however, it is not yet known whether TRPM2 plays any part in liver damage.

In this study, we used isolated hepatocytes and TRPM2 KO mouse model to investigate the role of TRPM2 channels in development of acetaminophen induced hepatocellular death. Using patch clamping and \( \text{Ca}^{2+} \) imaging, we show that TRPM2 KO mouse hepatocytes lack acetaminophen and \( \text{H}_2\text{O}_2 \)-induced cation current and \( \text{Ca}^{2+} \) entry. Furthermore, pharmacological inhibition of TRPM2 channels protects hepatocytes from acetaminophen-induced damage in culture, and animal experiments demonstrate that TRPM2 KO mice are largely protected from acetaminophen toxicity.

3.2 Methods and Materials

3.2.1 Chemicals

Acetaminophen, pleuronic acid, propylene glycol, BSA, ionomycin, DMSO, clotrimazole and ACA were purchased from Sigma-Aldrich (Rockville, Maryland, US). DMEM, penicillin/streptomycin, and trypsin-EGTA were purchased from GIBCO (Grand Island, New York, US). Fura2-AM and Fast SYBR® Green Master Mix was purchased from Invitrogen (Carlsbad, California, US). FBS was purchased from Bovogen (Melbourne, Australia), and collagenase was purchased from Worthington (Lakewood, New Jersey, US).
3.2.2 Animals

TRPM2 KO mouse model was generously provided by Professor Yasuo Mori, Japan. Hooded Wistar rats, and TRPM2 KO, Heterozygote and WT mice were housed and bred in the controlled environment with a 12-hour light–dark cycle in the animal house of Flinders University at least three weeks before beginning the experiments. Male rats aged eight to twelve weeks were used for the experiments. Both male and female mice aged eight to ten weeks were used for the experiments.

3.2.3 Solutions

Wash media for hepatocyte preparation (mM): 136 NaCl, 4.7 KCl, 0.85 Na$_2$HPO$_4$, 0.45 KH$_2$PO$_4$, 24 NaHCO$_3$, 20 glucose, 1.3 CaCl$_2$, 0.8 MgSO$_4$, BSA (10% W/V), penicillin (100 U/ml), streptomycin (100 µg/ml) and phenol red (0.001% W/V). KRH solution (mM): 136 NaCl, 4.7 KCl, 1.3 CaCl$_2$, 1.25 MgCl$_2$, 10 glucose and 10 Na-HEPES with pH adjusted to 7.4 by NaOH. Control bath solution for patch clamping (mM): 140 NaCl, 4 CsCl, 2 CaCl$_2$, 2 MgCl$_2$ and 10 Na-HEPES, adjusted to pH 7.4 with NaOH. Pipette solution for patch clamping (mM): 130 caesium glutamate, 5 MgCl$_2$, 5 CaCl$_2$ and 10 EGTA, adjusted to pH 7.3 with NaOH. TBS solution (mM): 150 NaCl and 25 Tris with pH adjusted to 7.4 by NaOH. TBST solution: TBS solution plus 0.1% Tween-20. PBS solution (mM): 137 NaCl, 2.7 KCl, 10 Na$_2$HPO$_4$ and 1.8 KH$_2$PO$_4$.

3.2.4 Estimation of the number of dead cells using Trypan blue.

Isolated rat hepatocytes were plated on glass coverslips and incubated for 4-6h at 37°C and 5% CO$_2$. When cells were well attached to the coverslips 10 mM acetaminophen, or 10 µM ACA, or 10 mM acetaminophen with 10 µM ACA were added to the medium and hepatocytes were incubated for 16 hours. At the end of the treatment the number of dead or damaged cells was counted using Trypan Blue.
3.2.5 Induction of In Vivo Acetaminophen Toxicity in Mice

TRPM2 KO, WT and heterozygote mice were divided into the following groups (number of mice in brackets):

1. WT-control: WT mice, untreated (n=7)
2. WT-vehicle: WT mice received IP injection of 7 ml/Kg vehicle solution (propylene glycol/water, 50–50%, v/v) (n=7)
3. KO-control: TRPM2 KO mice, untreated (n=6)
4. WT-acetaminophen: WT mice received IP injection of 500mg/kg acetaminophen (n=10)
5. Heterozygote-acetaminophen: TRPM2 heterozygote mice received IP injection of 500mg/kg acetaminophen (n=7)
6. KO-acetaminophen: TRPM2 KO mice acetaminophen received 500 mg/kg IP injection (n=9)

The mice were restricted from access to food for 12 hours before and three hours after the beginning of the experiment. IP injections were performed after the induction of a brief anaesthesia using isoflurane. Twenty-four hours post injection, each mouse in each group was anesthetised with ketamine-xylazine (50 and 8 mg/kg, respectively, IP injection) immediately after short-course sedation with inhaled isoflurane. After the induction of surgical anaesthesia, the abdomen was opened vertically, the anterior part of the chest was removed, and 400 µL of blood was taken from the left ventricle and transferred to a heparinised tube for further analysis. The liver was immediately perfused with KRH, harvested and fixed in buffered formaldehyde (10% in PBS) for histopathology. Fixed liver tissue was blocked in paraffin, sliced with microtome (5 µm thickness), stained with Haematoxylin and Eosin (H&E) and mounted on glass slides using mounting reagent.

3.2.6 Blood Liver Enzymes Assay

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured at the Biochemistry Facility of Flinders Medical Centre and reported in U/l.
3.2.7 Histopathology

The images of H&E-stained liver sections were captured at magnification 4x using a Bright-Field BX53 Olympus microscope equipped with Olympus DP72 camera. Total, normal and necrotic areas were measured and processed by CellSense software in µm² using ‘magic wand’ or ‘free hand polygon’ patterns. The examples of selected areas are shown in Figure 3.1.

3.2.8 Hepatocyte Isolation and Culture

First, the WT or TRPM2 KO mouse was briefly anesthetised with inhaled isoflurane, and then, was immediately injected with ketamine/xylazine (50 and 8 mg/kg, respectively) into the intra-peritoneal (IP) space. After the rat was fully anesthetised, it was moved to the 37°C incubator and fixed on a plate. The abdomen was opened medially and, by two lateral incisions near the ribs (in an inverted T-shape), was fully exposed. The anterior part of the chest was then removed completely, followed by complete rupturing of the portal vein using a scissor. Immediately, the intra-thoracic part of IVC was cannulised towards the liver, the liver was first perfused with media containing EGTA 0.5 mM and then (for around 15 minutes) with the perfusion media containing CaCl₂ 1.3 mM and collagenase (25 mg/100 ml). After being fully digested, the liver was harvested, placed in the sterile container on ice and chopped using scissors. The hepatocytes were isolated from the mixture by several washings with the pre-chilled (4°C) wash media, followed by 80 s centrifugation at 500 rpm.

The isolated hepatocytes were cultured on glass coverslips at 37°C in 5% CO₂ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v) for 16 to 96 hours before the experiments.
Figure 3.1: Area selection in mouse liver sections using CellSense software

The areas with blue borders are the lumens of blood vessels and non-tissue areas. The areas with red borders are the necrotic liver tissues. The remaining is the normal liver tissue.
3.2.9 Calcium Imaging

Fura2-AM was dissolved in 5 µl of 20% pluronic acid in DMSO (w/v) and diluted in KRH buffer to the final concentration of 5 µM. Sixteen hours after plating on glass coverslips, the hepatocytes were loaded with Fura2-AM for 30 minutes, washed and incubated in KRH solution for 10 minutes in the CO₂ incubator at 37°C. The fluorescence of Fura-2 was measured using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, Omega XF04 filter set for Fura-2, Photonic Science ISIS-3 ICCD camera and UIC Metafluor software. Fluorescence images were obtained every 10 seconds using a 20× objective. Fluorescence ratio values (340/380 nm) were transformed to [Ca²⁺]c using the equation derived by Grynkiewicz, Poenie & Tsien (1985); a K_d of 224 nM for binding of Fura-2 to Ca²⁺; and ionomycin and EGTA to determine R_max and R_min, respectively.

3.2.10 Western Blotting

After preparation, 2-5×10⁶ hepatocytes were washed with cold PBS 3 times, and centrifuged at 1,000 rpm for 10 minutes. The hepatocytes were then incubated with 100 µL lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X, 3 mM EDTA and 10 µl Protease Inhibitor) for 30 minutes on ice, followed by 15 seconds of vortexing. After centrifuging at 8,000 rpm for 15 minutes at 4°C, the supernatant was collected. The amount of protein was measured using the BCA Protein Determination Kit (Sigma).

Next, 20 µl supernatant was mixed with 20 µl sample buffer (final concentration: 125 mM Tris, 10% glycerol, 4% sodium dodecyl sulfate (SDS), 4% 2-mercapto-ethanol and 0.2% bromophenol-blue) and incubated at 75°C for 15 minutes. The sample was then put on ice and centrifuged at 8,000 rpm for five minutes in a cold room (4°C). Equal amounts of proteins (around 5 µg) and ladders (visible and biotinylated ladders) were then separated on the SDS-PAGE gel using Pharmacia Electrophoresis EPS 3500XL, adjusted at 60 V and 90 to 110 mA, until the protein of interest reached the middle part of the lower gel.

The prepared gel consisted of two parts. The lower part was more condensed and contained 4.6 ml double distilled water (DDW), 2.5 ml of 30% acrylamide, 2.5 ml Tris (1.5M, pH 8.8), 100 µl of 10% SDS (w/v), 60 µl of 10% APS (w/v) and 25 µl tetramethylethylenediamine (TEMED). The small proportion on top (from 1 cm below
the comb) consisted of 3.4 ml DDW, 830 µl of 30% acrylamide, 630 µl Tris (1M, pH 6.6), 50 µl of 10% SDS, 50 µl of 10% APS and 5 µl TEMED. After separation, the protein and the ladders were transferred to a pure nitrocellulose blotting membrane by electrophoresis using Pharmacia Electrophoresis EPS 3500XL at 60 V and 150 mA at 4°C in transfer buffer (SDS, Tris, glycine and methanol). After the transfer, the paper was blocked by incubation with TBST solution (150 mM NaCl, 25 mM Tris, pH 7.4, 0.1% Tween-20) containing 5% skim milk (w/v) for one hour at room temperature on a shaker.

The paper was then washed four times with TBST for 15 minutes each, and kept with anti-TRPM2 polyclonal antibody (diluted 1:1,000, Abcam) and anti-GAPDH monoclonal antibody (1:1,000, Abcam) overnight on a shaker at 4°C. The following day, the paper was washed with TBST 4 times, and incubated with horseradish peroxidase (HRP) secondary antibodies against primary antibodies and biotin (to visualise the biotinylated ladder) at room temperature on a shaker for 1 hour. The paper was then washed 4 times with TBST. The protein bands were visualised using H₂O₂ and enhanced chemiluminescence (ECL). After developing, the film was scanned using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, California, US) and the band intensities quantified using Quantity One software, version 4.3.1 (Bio-Rad Laboratories).

3.2.11 RT-PCR

After preparation, 2×10⁶ hepatocytes were washed 3 times with cold PBS, and centrifuged. The pellet was gently mixed with 1 ml Trizole. The cells were lysed using 300 µl chloroform vortexing and incubating the tube at room temperature for three minutes. RNA was extracted by centrifugation at 9,000 rpm for 15 minutes at 4°C. The clear supernatant was taken out to a new Eppendorf tube (without touching the white DNA layer) and 375 µl isopropanol was added to the supernatant, vortexed for 10 seconds and kept at room temperature for 10 minutes. The tube was then centrifuged at 7,500 rpm for 10 minutes at 4°C. The supernatant was removed carefully and the pellet was washed twice with pre-chilled 75% ethanol to remove the isopropanol. The pellet was dried in the air for 10 minutes (at room temperature) and then dissolved in 50 µl nuclease free water (NFW) for 10 minutes at 56°C. Afterwards, the prepared RNA was kept on ice. The amount of RNA was measured using Nanodrop ND-8000.
Contaminated DNA was removed using a DNase enzyme, and the activity of DNase was stopped using a DNase stop solution. cDNA from RNA was prepared using a reverse transcriptase (RT) enzyme (50 minutes at 37°C). The produced cDNA was used for conventional PCR. The primers used for detecting TRPM2 isoforms and quantitative PCR are listed in Table 3.1.

Conventional PCR was performed using an Eppendorf Mastercycler nexus machine according to published methods (Wehage et al. 2002). The products were then run on the gel containing 1 µl Gel Red per gel, and illuminated and captured using Imagemaster (VCD).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Nucleotide position</th>
<th>Predicted size, bp</th>
<th>Isoform or genotype detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM2-NUDT9-H</td>
<td>Sense (P6)</td>
<td>5’-TGCCAACATCCTGCTGCTTA-3’</td>
<td>3,413–3,432</td>
<td>651</td>
<td>TRPM2L</td>
</tr>
<tr>
<td></td>
<td>Antisense (P7)</td>
<td>5’-ATGCGGGCATTTGGGATAG-3’</td>
<td>4,063–4,044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPM2-Pore</td>
<td>Sense (P8)</td>
<td>5’-CATCCCGAGCGACGCTTAC-3’</td>
<td>2,969–2,987</td>
<td>368</td>
<td>TRPM2 WT and heterozygote mice</td>
</tr>
<tr>
<td></td>
<td>Antisense (P9)</td>
<td>5’-CAGGACACCTTAGCTGTAG-3’</td>
<td>3,336–3,317</td>
<td>missing</td>
<td>TRPM2 KO mice</td>
</tr>
</tbody>
</table>

Primers used to detect Nudix box (NUDT9-H) in TRPM2 WT and the pore region in TRPM2 WT, heterozygote and KO mouse hepatocytes.

### 3.2.12 Patch-clamp Recording

Membrane currents were measured at room temperature (23°C) using standard patch clamping in a whole-cell mode, and a computer-based EPC-9 patch-clamp amplifier run by PULSE software. In order to monitor the development of membrane currents, voltage ramps between -120 to +120 mV were applied every two seconds following the achievement of whole-cell configuration. The holding potential was -40 mV. The data were analysed using PULSEFIT software. The TRPM2 current of hepatocytes was activated by adding 1 mM ADPR to the pipette solution, respectively. Patch pipettes
were pulled from borosilicate glass and fire-polished to a resistance between 1.5 and 2.5 MΩ. The series resistance did not exceed 7.5 MΩ and was 50 to 70% compensated.

3.3 Results

3.3.1 TRPM2 channel inhibitor, ACA, attenuates acetaminophen-induced hepatocellular death in culture

The main hypothesis of this chapter is that Ca^{2+} entry through TRPM2 channels activated due to oxidative stress contributes to acetaminophen-induced hepatocellular death. To demonstrate this experimentally, we first used cultured rat hepatocytes treated for 16 h with either acetaminophen alone or with acetaminophen with ACA, to block activation of TRPM2 channels. After the treatment the damaged cells were counted using Trypan blue. The results show that hepatocytes incubated with acetaminophen and ACA were significantly less damaged, compared to hepatocytes threatened with acetaminophen alone (Table 3.2).

Table 3.2: The protective effect of ACA against acetaminophen-induced hepatocellular damage

<table>
<thead>
<tr>
<th></th>
<th>Total counted cells</th>
<th>Intact cells (undamaged)</th>
<th>Damaged cells (stained)</th>
<th>Damaged percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,626</td>
<td>1,449</td>
<td>180</td>
<td>11.1±1.31</td>
</tr>
<tr>
<td>ACA</td>
<td>1,615</td>
<td>1,464</td>
<td>151</td>
<td>9.27±0.72</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>1,287</td>
<td>694</td>
<td>593</td>
<td>54.26±4.33*</td>
</tr>
<tr>
<td>Acetaminophen+ACA</td>
<td>1,702</td>
<td>1,344</td>
<td>358</td>
<td>22.15±2.55**</td>
</tr>
</tbody>
</table>

The conditions of the experiment are described in the Methods section.
* - P<0.001, compared to the control cells
** - P<0.01, compared to the cells treated with acetaminophen alone

3.3.2 TRPM2 expression in mouse hepatocytes

To establish whether activation of TRPM2 channels plays a role in acetaminophen-induced liver damage in vivo we used TRPM2 KO mice. First, we investigated TRPM2 channel expression in mouse hepatocytes, using western blot analysis of the protein
extracts from the livers of TRPM2 WT, heterozygote and KO mice, and anti-TRPM2 Ab. The western blot showed that, similarly to rat hepatocytes, TRPM2 WT and heterozygote mouse hepatocytes express high levels of TRPM2 protein (band detected at 171 kDa, Figure 3.2). In the TRPM2 KO mice, however, no band corresponding to TRPM2 at 171 kDa was detected. This confirms that, in the TRPM2 KO mice, there are no functional TRPM2 channels expressed (Figure 3.2). RT-PCR showed that TRPM2 was also absent in TRPM2 KO mice on the mRNA level, whereas TRPM2 WT and heterozygote mouse hepatocytes expressed TRPML isoform with the NUDT9-H motif in the C-terminus (Figure 3.2).

3.3.3 The effect of knocking-out TRPM2 channel on Ca\textsuperscript{2+} entry and non-selective cation current in H\textsubscript{2}O\textsubscript{2}- or acetaminophen-treated mouse hepatocytes

Before conducting the in vivo experiments using TRPM2 KO mouse model, we investigated whether ablation of TRPM2 expression in mice results in the ablation of oxidative stress-induced Ca\textsuperscript{2+} entry. To measure the rise in [Ca\textsuperscript{2+}]\textsubscript{c} in response to H\textsubscript{2}O\textsubscript{2} hepatocytes isolated from TRPM2 KO and WT mice were loaded with Fura-2AM and transferred to the microscope stage. Hepatocytes were then incubated with 0.2 mM H\textsubscript{2}O\textsubscript{2} in Ca\textsuperscript{2+} free solution for approximately 15 minutes. Introduction of 1.3 mM Ca\textsuperscript{2+} to the bath solution resulted in a rapid increase of [Ca\textsuperscript{2+}]\textsubscript{c} in WT hepatocytes to the levels above 1,500 nM. As at such high Ca\textsuperscript{2+} concentrations Fura-2 is close to saturation, the real [Ca\textsuperscript{2+}]\textsubscript{c} is likely to be even higher. This rise was prevented by the addition of 50 µM clotrimazole or 10 µM ACA the bath together with 0.2 mM H\textsubscript{2}O\textsubscript{2} (Figure 3.3a). In H\textsubscript{2}O\textsubscript{2}-treated TRPM2 KO hepatocytes, introduction of 1.3 mM Ca\textsuperscript{2+} to the bath also resulted in [Ca\textsuperscript{2+}]\textsubscript{c} rise, however, to the significantly lower levels, compared to TRPM2 WT hepatocytes, no more than 500 nM (P < 0.001). [Ca\textsuperscript{2+}]\textsubscript{c} rise in H\textsubscript{2}O\textsubscript{2}-treated TRPM2 KO hepatocytes was prevented by the application of 50 µM clotrimazole with H\textsubscript{2}O\textsubscript{2}. However, ACA showed little or no effect on that Ca\textsuperscript{2+} rise, which confirms that the Ca\textsuperscript{2+} entry activated by H\textsubscript{2}O\textsubscript{2} in TRPM2 KO mouse hepatocytes is not mediated by TRPM2 channels (Figure 3.3b).

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**Figure 3.2: TRPM2 channel expression in mice**

**a.** TRPM2 expression in mouse hepatocytes by Western blotting. Western blot analysis was performed using a polyclonal antibody to TRPM2 (1:750; ab63015) and an HPR secondary antibody (1:5,000; ab97130) following standard procedures. Note the absence of the TRPM2 band in the KO mouse. 

**b & c.** TRPM2 expression in mouse hepatocytes by PCR. Presence of Nudix-Box located on the C terminus of TRPM2L was detected in WT mouse hepatocytes using the P6 and P7 primers listed in Table 3.1. The left lane shows part of the ladder (b). Presence of the sequence corresponding to the TRPM2 pore region using P8 and P9 primers listed in Table 3.1 was detected in hepatocytes isolated from WT and TRPM2 Het mice, but not in TRPM2 KO mice (c).
To investigate the effect of acetaminophen on $[Ca^{2+}]_c$ in TRPM2 WT and KO mice, isolated hepatocytes plated on glass cover slips were incubated with 10 mM acetaminophen for 60 minutes in a KRH bath solution containing 1.3 mM Ca$^{2+}$. Treated hepatocytes were then loaded with Fura-2AM in a nominally Ca$^{2+}$ free KRH and, after washing, were transferred to the microscope stage. Introduction of 1.3 mM Ca$^{2+}$ to the bath caused an increase in $[Ca^{2+}]_c$ in TRPM2 WT hepatocytes to levels above 1,500 nM. This Ca$^{2+}$ rise was prevented by application of clotrimazole or ACA to the bath. In acetaminophen-treated TRPM2 KO hepatocytes, no rise in $[Ca^{2+}]_c$ was apparent after the introduction of 1.3 mM Ca$^{2+}$, which confirmed that acetaminophen-induced Ca$^{2+}$ entry in WT mouse hepatocytes is mediated entirely by TRPM2 channels (Figure 3.3c). To investigate further the nature of the Ca$^{2+}$-permeable channels responsible for H$_2$O$_2$ and acetaminophen-induced Ca$^{2+}$ entry in mouse hepatocytes, we used whole cell patch clamping. Similar to rat hepatocytes (see Chapter 2, section 2.3.2), WT mouse hepatocytes developed a non-selective cation current in response to the treatment by H$_2$O$_2$ (1 mM for 30 min) and acetaminophen (10 mM for 1 h) (figure 3.4). Compared to WT hepatocytes, hepatocytes isolated from TRPM2 KO mice showed much smaller, or no non-selective current development in response to the treatment by H$_2$O$_2$ and acetaminophen (figure 3.4). It should be noted here that the isolation and patch clamping of mouse hepatocytes presented a significant challenge. Due to relatively small size, and possibly other reasons, mouse liver was more susceptible to damage during collagenase perfusion. Therefore mouse hepatocytes were, generally, of a lesser quality, compared to rat hepatocytes. Following isolation mouse hepatocytes attached to the glass and spread much faster than rat hepatocytes. As a result, it was not possible to use low resistance patch pipettes (below 2 MΩ), which was necessary to introduce ADPR into the cell. Therefore, we were unable to record ADPR-activated TRPM2 current in mouse hepatocytes. Although we believe that it should be possible to find the right conditions for recording of ADPR-activated TRPM2 current in mouse hepatocytes, we did not pursue it further due to time constraints.
Figure 3.3: H$_2$O$_2$- and acetaminophen-activated Ca$^{2+}$ entry is attenuated in TRPM2 KO mouse hepatocytes

a & b. Ca$^{2+}$ entry in TRPM2 WT (A) and KO (B) mouse hepatocytes activated by the addition of H$_2$O$_2$ to the bath solution (n=3). c. Ca$^{2+}$ entry in TRPM2 WT and KO mice hepatocytes treated with 10 mM acetaminophen for 60 min (n=3). Both clotrimazole (50 μM) and ACA (10 μM) were applied to the bath 5 min before the addition of Ca$^{2+}$. 
Figure 3.4: Acetaminophen and H$_2$O$_2$ activate a nonselective cation current in mouse hepatocytes
Averaged current–voltage plots of membrane currents measured in untreated WT (a & d) and KO (b & e) hepatocytes (Control), and hepatocytes treated with either 0.5 mM H$_2$O$_2$ for 30 min (a & b) or 10 mM acetaminophen for 60 min (d & e). Insets show the amplitude of the cation current (at −100 mV) inhibited by the replacement of 140 mM NaCl in the bath solution with 140 mM NMDG-Cl in WT and KO mouse hepatocytes treated with either H$_2$O$_2$ (0.5 mM for 30 min) or acetaminophen (10 mM for 60 min) (n=17–20 for each condition), (c & f). Data in insets and in panels c and f are presented as means ± SEM.

3.3.4 The effect of TRPM2 channel knock-out on acetaminophen-induced liver damage

To investigate liver toxicity of acetaminophen in vivo, mice were divided into 6 experimental groups as outlined in the Methods section. IP injection of 500 mg/kg acetaminophen resulted in a significant elevation of the liver blood enzymes (ALT and AST) in TRPM2 WT and heterozygote mice, compared to control untreated animals, or mice injected with vehicle (Figure 3.5). There was no significant difference in the mean AST and ALT concentrations between the control and vehicle groups. There was also no significant difference detected in AST or ALT between the acetaminophen-treated TRPM2 WT and heterozygote groups.

The levels of AST in the acetaminophen-treated TRPM2 KO mice were significantly lower, compared to acetaminophen-treated TRPM2 WT mice (1,557±443 and 11,993±2,660 U/l, respectively; P < 0.0001). Similarly, there was a 6-7 fold decline in the blood concentration of ALT in acetaminophen-treated TRPM2 KO mice, compared to TRPM2 (P = 0.0006) (Figure 3.5).

The acetaminophen-induced liver damage was further investigated histologically in tissue slices. Haematoxylin/Eosin (H&E) staining of liver sections of acetaminophen-treated TRPM2 WT and Het mice revealed widespread hepatocellular damage (Figure 3.6d & e). This was characterized by areas of necrosis, infiltration by lymphocytes, and haemorrhage, and was prominent in zones 2 and 3 (hepatocytes around the hepatic vein) (Figure 3.6d). Liver damage was substantially reduced in TRPM2 KO mice treated with acetaminophen compared with the treated WT and Het mice (Figure 3.6f). The area of necrotic damage was much smaller in TRPM2 KO mice and was localized
to zone 3. In some sections of livers from acetaminophen-treated TRPM2 WT and Het mice, haemorrhagic necrosis and extravasations of blood were detected along with necrotic damage. Because the prominent and consistent effect of acetaminophen was necrotic damage, the area of necrotic tissue was quantified. The results indicate that the area of necrosis in the livers of TRPM2 KO mice treated with acetaminophen was substantially smaller than in the livers of acetaminophen-treated TRPM2 WT and Het mice (Figure 3.7).

However, the proportion of the liver necrotic areas in the acetaminophen-treated TRPM2 KO mice, was still significantly larger than in the untreated TRPM2 KO controls (P < 0.003) (Figure 3.7).

3.4 Discussion

In this study, we found that inhibition of TRPM2 channels in isolated hepatocytes and ablation of TRPM2 channels expression in TRPM2 KO mouse considerably attenuates acetaminophen-induced hepatocellular damage. The protective effect of TRPM2 ablation in vivo was evident from the significant reduction in blood liver enzymes in the KO mice compared to WT mice, following acetaminophen injection. In acute liver diseases, damaged hepatocytes release their intracellular content into the bloodstream. AST and ALT are two major liver enzymes, which concentrations in the blood are used as indicators of liver injury (Rumack & Bateman 2012). Amounts of AST and ALT, in particular, higher than 1,000 U/l indicate a marked acute liver damage (Rumack & Bateman 2012; Batt & Ferrari 1995). Although concentrations of both AST and ALT in KO mice following acetaminophen treatment were higher than 1000 U/l, indicating some liver damage, they were 5-6-fold lower than in WT mice after similar treatment.

The prediction of the liver damage by the blood liver enzymes correlated really well with the actual liver damage measured as the areas of necrosis in H&E stained liver slices. The pattern of acetaminophen-induced liver necrosis in WT mice was consistent with previous findings and extended to Zones 3 and 2, and sometimes to Zone 1 (Deleve et al. 1997). In contrast, in TRPM2 KO mice the areas of necrosis were limited to Zone 3. Consistent with 5-fold reduction in blood liver enzymes, there was 4.5-fold reduction in the total size of the necrotic area in TRPM2 KO mouse livers, compared to WT livers.
Figure 3.5: The acetaminophen effect on AST and ALT in the experimental groups

TRPM2 KO mice are substantially protected against acetaminophen-induced damage. Blood concentrations of the liver enzymes AST (a.) and ALT (b.) in TRPM2 KO, TRPM2 Het, and WT mice pretreated with acetaminophen or vehicle for 24 h. The results are the means ± SEM of the number of mice indicated. The degree of significance, determined using the one way ANOVA test was P < 0.007 for comparison of TRPM2 KO with each of TRPM2 WT and TRPM2 Het.
Figure 3.6: Histopathology of the liver slices obtained from acetaminophen-treated WT and TRPM2 KO mice

Representative bright field images of H&E-stained liver sections at 20× magnification. The light coloured areas (pointed by arrows) in the WT acetaminophen and KO acetaminophen images represent areas of necrosis. a. WT-control b. WT-vehicle c. KO-control d. WT-acetaminophen e. Heterozygote-acetaminophen f. KO-acetaminophen.
Figure 3.7: The acetaminophen-induced necrotic areas in liver sections

The percentage of necrosis in TRPM2 WT, KO, and Het liver sections is expressed as a percentage of the total area. The data in each group was averaged from six mice. Data are presented as means ± SEM.
TRPM2 channels are expressed not only in hepatocytes but also in cells of immune system, including macrophages (Yamamoto et al. 2008; Knowles et al. 2011). It is possible that activation of TRPM2 channels in Kupffer cells, liver resident macrophages, or other immune cells contribute to acetaminophen liver damage. The protective effect of TRPM2 inhibitor ACA against acetaminophen-induced hepatocellular death in vivo, suggest that the role of TRPM2 in acetaminophen toxicity is largely limited to hepatocytes.

Ca\(^{2+}\) imaging and patch clamping showed that H\(_2\)O\(_2\)-induced Ca\(^{2+}\) entry and a non-selective cation current are reduced in TRPM2 KO mouse hepatocytes, compared to WT cells, however, some Ca\(^{2+}\) entry remains, and it is mediated by a channel different from TRPM2, as it is not inhibited by ACA. It is likely that hepatocytes express not only TRPM2, but also other oxidative stress-activated cation channels. Activation of one type of such channels, TRPM7, has been shown previously to contribute to oxidative stress-induced neuronal death (Aarts & Tymianski 2005). Recent investigations using HEK293 heterologous expression system, however, showed that TRPM7 is inhibited by H\(_2\)O\(_2\), rather than activated (Inoue et al. 2014).

Treatment of TRPM2 KO mouse hepatocytes with acetaminophen, in contrast to H\(_2\)O\(_2\), caused no measurable Ca\(^{2+}\) entry or development of a non-selective cation conductance. This suggests that H\(_2\)O\(_2\) has more than one pathway through which it activates Ca\(^{2+}\) entry in hepatocytes – one is through generation of APDR, (the same as for acetaminophen), and another, possibly, through direct oxidation of cysteine residues of yet unknown channel. At this stage, however, this suggestion is speculative, and requires further investigation.

Our study also found that the TRPM2 KO mouse liver, although protected to a large degree, still had clear signs of acetaminophen-induced damage. This is consistent with previous findings that showed involvement of multiple mechanisms in acetaminophen-mediated liver damage, including lipid peroxidation, DNA fragmentation, mitochondrial dysfunction and others (Bessem & Vermeulen 2001; Jaeschke, Cover & Bajt 2006; Hirano et al. 1996; Sinha et al. 2013).

One unusual feature of the Ca\(^{2+}\) entry activated by the H\(_2\)O\(_2\) and paracetamol treatment that needs to be discussed, is the slow rate of [Ca\(^{2+}\)]\(_c\) rise in hepatocytes when Ca\(^{2+}\) was added to the bath in Ca\(^{2+}\) imaging experiments using Fura2. Assuming that TRPM2 channels are activated by these treatments, one could expect that [Ca\(^{2+}\)]\(_c\) would rise very quickly when Ca\(^{2+}\) is added to the bath. Unexpectedly, the Ca\(^{2+}\) rise was slow and
continued for 10 min or longer. This can be explained as follows. Ca\(^{2+}\), both intracellular and extracellular, is an important co-factor in the mechanism of TRPM2 activation. Removal of Ca\(^{2+}\) inhibits TRPM2 (Starkus et al, 2007). Therefore, at the moment when Ca\(^{2+}\) is added, there are few active TRPM2 channels, but there is enough ADPR in the cytoplasm to open more channels when Ca\(^{2+}\) is introduced. Re-addition of Ca\(^{2+}\) stimulates TRPM2 activation. As TRPM2 is a non-selective channel mainly conducting Na\(^{+}\), the rate of Ca\(^{2+}\) entry through TRPM2 channels is relatively low. Furthermore, rising intracellular Ca\(^{2+}\) provides positive feedback mechanism, increasing the number of active TRPM2 channels. In addition, ADPR concentration continues to rise during this time. Taken together, this explains why it takes some time before maximum Ca\(^{2+}\) level is reached.

In conclusion, the experiments conducted in this work show that the ablation or inhibition of TRPM2 channels significantly attenuates acetaminophen-induced liver injury. These data provide a rationale for a future development of the TRPM2 inhibitors and modulators that can be used for the prevention and treatment of acetaminophen toxicity and possibly other oxidative stress–related liver diseases.
Chapter 4: H$_2$O$_2$- and Acetaminophen-induced Oxidative Stress Initiates TRPM2 Channel Trafficking to the Plasma Membrane in Rat Hepatocytes

4.1 Introduction

The Transient Receptor Potential Melastatin 2 (TRPM2) channel is a Ca$^{2+}$ permeable, non-selective cation channel, which belongs to the TRP superfamily of ion channels (Nagamine et al. 1998). The TRPM2 is composed of six transmembrane (TM) domains and cytoplasmic N- and C-termini, and it has a specific enzymatic activity in its C-terminus, where NUDT9-H motif binds and hydrolysates ADPR. ADPR binding to the TRPM2 C-terminus causes opening of the channel pore (Perraud et al. 2001; Kuhn, Heiner & Luckhoff 2005). ADPR is currently considered the main physiological ligand of TRPM2, however, other cellular messengers, including NAD$^+$, NAADP, cADPR and Ca$^{2+}$ have been shown to activate TRPM2 current as well (Sano et al. 2001; Hara et al. 2002; Heiner et al. 2003; Beck et al. 2006; Togashi et al. 2006; Du, Xie & Yue 2009). Furthermore, TRPM2 channel is activated by H$_2$O$_2$, both directly and indirectly (Hara et al. 2002; Perraud et al. 2005; Toth & Csanady 2010). H$_2$O$_2$ and other oxidants can cause elevation of ADPR in the cell through a range of mechanisms, thus activating TRPM2 current. However, it can also act on TRPM2 directly, possibly through cysteine oxidation, as H$_2$O$_2$ has been shown to activate a current mediated by an isoform of TRPM2 channel that lacks NUDT9-H motif and therefore insensitive to ADPR (Kuhn, Heiner & Luckhoff 2005; Fonfria et al. 2004). TRPM2 channels are expressed in a in a range of tissues, including brain, cells of immune system, vasculature, liver, pancreas and prostate (Fonfria et al. 2006; Yang et al. 2006; Wang, Pu & Wang 2007). When expressed heterologously in HEK293 cells, TRPM2 channels localise primarily on the plasma membrane (Zhang et al. 2007). However, in primary cell types, where TRPM2 channels are expressed endogenously, their localisation varies (Lange et al. 2009). In neuronal cells, TRPM2 is mostly expressed on the plasma membrane (Fonfría et al. 2004; Zhang et al. 2007; Naziroglu et al. 2011), whereas in monocytes, TRPM2 is localised on the membranes of intracellular organelles, mainly lysosomes (Sumoza-Toledo et al. 2011; Knowles, Li & Perraud 2013). The TRPM2 expressed in lysosomes may play a role of a Ca$^{2+}$ release channel,
however, its exact function at this location remains unclear (Sumoza-Toledo et al. 2011).

Oxidative stress that causes activation of TRPM2 channels has multiple effects in the cell, which includes lysosomal trafficking towards plasma membrane (Sumoza-Toledo et al. 2011; Perraud, Schmitz & Scharenberg 2003; Bari et al. 2009; Hecquet & Malik 2009; Li, Gulbins & Zhang 2012). It has been previously shown that, oxidative stress modifies the expression of several proteins on the plasma membrane, including acid sphingomyelinase, which is normally located on the lysosomal membrane (Li, Gulbins & Zhang 2012; Carini et al. 2006; Aguilar-Gaytan & Mas-Oliva 2003; Cheng & Vieira 2006). This suggests that oxidative stress triggers lysosomal exocytosis (Li, Gulbins & Zhang 2012). It has been shown that treatment of Jurkat T cells with H$_2$O$_2$ causes lysosomal trafficking and fusion with the plasma membrane. This process is triggered by the rise in [Ca$^{2+}$]$_{c}$, and the buffering of [Ca$^{2+}$]$_{c}$ using EGTA-AM prevents it (Li, Gulbins & Zhang 2012).

In the experiments described in the previous two chapters, we have shown that hepatocytes express functional TRPM2 channels; and the oxidative stress caused by treating hepatocytes with H$_2$O$_2$ and acetaminophen promotes activation of TRPM2 current and Ca$^{2+}$ entry across the plasma membrane. Interestingly, [Ca$^{2+}$]$_{c}$ rise induced by pre-treating cells with H$_2$O$_2$ and acetaminophen developed slowly over several minutes, reaching micromolar concentrations above saturation point of Fura-2. On the other hand, our preliminary data on TRPM2 immunofluorescence in rat and mouse hepatocytes suggest that most of TRPM2 protein is localised intracellularly. Based on these observations, we propose that during oxidative stress in hepatocytes the TRPM2 channels localised in the lysosomes translocate to the plasma membrane, increasing the plasma membrane TRPM2 numbers, leading to sustained rise in [Ca$^{2+}$]$_{c}$, which provides a positive feedback mechanism promoting further lysosomal trafficking and finally cell death.
4.2 Methods and Materials

4.2.1 Chemicals

BSA, DMSO, protease inhibitor, Bicinchoninic acid kit for protein determination and H$_2$O$_2$ were purchased from Sigma-Aldrich (Rockville, Maryland, US). DMEM, penicillin/streptomycin, and trypsin-EGTA were purchased from GIBCO (Grand Island, New York, US). ProLong Gold Antifade reagent was purchased from Invitrogen (Carlsbad, California, US). FBS was purchased from Bovogen (Melbourne, Australia), and collagenase was purchased from Worthington (Lakewood, New Jersey, US). A Pierce Cell Surface Protein Isolation Kit was purchased from Thermo Fisher Scientific (Rockford, US).

4.2.2 Animals

Hooded Wistar rats were housed and bred in the controlled environment with a 12-hour light–dark cycle at least three weeks before the experiment. Male rats aged eight to 12 weeks were used for the experiments.

4.2.3 Solutions

Perfusion media for hepatocyte preparation (mM): 136 NaCl, 4.7 KCl, 0.85 Na$_2$HPO$_4$, 0.45 KH$_2$PO$_4$, 24 NaHCO$_3$, 20 glucose and phenol red (0.001% W/V). Wash media for hepatocyte preparation (mM): 136 NaCl, 4.7 KCl, 0.85 Na$_2$HPO$_4$, 0.45 KH$_2$PO$_4$, 24 NaHCO$_3$, 20 glucose, 1.3 CaCl$_2$, 0.8 MgSO$_4$ + 7 H$_2$O, BSA (10% W/V), penicillin (100 U/ml), streptomycin (100 µg/ml) and phenol red (0.001% W/V). KRH solution (mM): 136 NaCl, 4.7 KCl, 1.3 CaCl$_2$, 1.25 MgCl$_2$, 10 glucose and 10 Na-HEPES with pH adjusted to 7.4 by NaOH. TBS solution (mM): 150 NaCl and 25 Tris with pH adjusted to 7.4 by NaOH. TBST solution: TBS solution plus 0.1% Tween-20. PBS solution (mM): 137 NaCl, 2.7 KCl, 10 Na$_2$HPO$_4$ and 1.8 KH$_2$PO$_4$. 
4.2.4 Hepatocyte Isolation and Culture

The rat was briefly anesthetised with inhaled isoflurane, and then, was immediately injected with ketamin/xylazine (50 and 8 mg/kg, respectively) into the intra-peritoneal (IP) space. After the rat was fully anesthetised, it was moved to the 37°C incubator and fixed on a plate. The abdomen was opened medially and, by two lateral incisions near the ribs (in an inverted T-shape), was fully exposed. The portal vein was cannulised and the liver perfused with the perfusion media warmed to 37°C and bubbled with 2 L/min carbogen [95% O$_2$ and 5% CO$_2$]. The rat was exsanguinated by cutting the inferior vena cava (IVC) below the kidneys. The anterior part of the chest was then removed completely and the upper part of the IVC was cannulised. The abdominal part of the IVC was clamped and the liver was first perfused with media containing EGTA 0.5 mM and then (for around 15 minutes) with the perfusion media containing CaCl$_2$ 1.3 mM and collagenase (25 mg/100 ml). After being fully digested, the liver was harvested, placed in the sterile container on ice and chopped using scissors. The hepatocytes were isolated from the mixture by several washings with the pre-chilled (4°C) wash media, followed by 80 s centrifugation at 500 rpm.

The isolated hepatocytes were cultured on glass coverslips at 37°C in 5% CO$_2$ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v) for 16 to 96 hours before the experiments.

4.2.5 Detection of TRPM2 Protein on the Plasma Membrane Using Cell Surface Biotinylation

Two hours post isolation, the hepatocytes were incubated in a KRH solution at 37°C for 45 minutes with or without 1 mM H$_2$O$_2$. The surface proteins were then extracted using a Pierce Cell Surface Protein Isolation Kit. The manufacturer protocol was used with some modifications to the lysis buffer. After incubation, the hepatocytes were pelleted by centrifuging at 500 g for 2 min. The supernatant was discarded, the pellet was re-suspended and washed twice with cold PBS. Then, 2×10$^6$ cells from each group were incubated with 25 ml of Sulfo-NHS-SS-Biotin containing solution (prepared according to manufacturer protocol) for 30 min at 4°C, with slow shaking. After centrifuging, the cells in the pellet were lysed for 30 min on ice using 500 µl lysis buffer. The lysis buffer contained 50 mM Tris, 150 mM NaCl, 1% Triton-X, 3 mM EDTA and 10 µl of
protease inhibitor. Then the mixture was centrifuged at 12,000 g for 15 min, and 500 µl of the supernant containing protein was gathered and the rest containing the pellet was discarded. One hundred µl of the protein solutions was used to measure the Total protein; the remaining 400 µl were incubated in NeutrAvidin Agarose column for 1 h at room temperature. The column was then centrifuged at 1000 g for 1 min. The protein solution passed through the column was used for measuring of the remnant protein. The protein attached to NeutrAvidin Agarose was eluted using 400 µl of SDS-Page solution containing 50 mM DTT, and used to measure the surface protein (biotinylated protein). The total, surface and remnant protein concentrations were measured using a Bicinchoninic acid kit. The same amounts of total protein and the remnant protein solutions and 10 times of that amount of the corresponding surface protein solution were loaded and separated on the gel using electrophoresis.

After transferring the protein from the gel to the nitrocellulose membrane, the membrane was blocked with 5% skim milk in TBST solution. The membrane was then incubated with primary antibody against the TRPM2 channel (ab63015, Abcam, UK) 1/750 in TBST containing 1% skim milk overnight at 4°C. After washing four times with TBST, the membrane was incubated with HRP anti-sheep antibody (ab6747, Abcam, UK). The TRPM2 protein bands were visualised using H2O2 containing ECL western blotting substrate. It should be noted here that, for detection of the TRPM2 channel in the surface protein extract, the loading volume of the surface protein sample was 10 times greater than that of the volume of the total and the remnant proteins. GAPDH protein was used as a standard (anti-GAPDH antibody (ab8245, Abcam, UK) and HRP anti-mouse antibody (ab6728, Abcam, UK)). The experiment was repeated 3 times using different preparations of hepatocytes.

The autoradiographs were scanned using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, California, US), and the band densities quantified using Quantity One software version 4.3.1 (Bio-Rad Laboratories).

4.2.6 Confocal Microscope Imaging

Isolated hepatocytes plated on glass coverslips were treated with 10 mM acetaminophen for one hour, or 1 mM H2O2 for 45 minutes. The hepatocytes were then fixed with -20°C methanol 100% and blocked with 20% FBS in PBS. Afterwards, the cells were incubated with anti-sheep TRPM2 and anti-mouse Pan-Cadherin (used to
visualise plasma membrane) antibodies for two hours. After washing three times with PBS, the cells were incubated with FITC anti-sheep and Cyan-5 anti-mouse antibodies separately, for one hour each. The coverslips were then mounted on slides using Gold Antifade, and dried.

Images were captured using Leica Spectral SP5 confocal microscope at 100x magnification. Laser-generated, excitation wavelengths of 496 and 633 nm were used to detect FITC (TRPM2 channel) and Cyan-5 (plasma membrane), respectively. After capturing more than 50 images in each group (control, H₂O₂-treated and acetaminophen-treated hepatocytes), the images were processed by Image J using JACoP Plugin to calculate Pearson’s and Manders’ (M1, fraction of cellular plasma membrane merged with TRPM2 channel) coefficients.

4.2.7 Statistical Analysis

Data are presented as means ± SEM. Statistical significance was assessed using a paired t-test.

4.3 Results

To determine the plasma membrane fraction of the total TRPM2 protein expressed in rat hepatocytes we first used Thermo Scientific™ Pierce™ Cell Surface Protein Isolation Kit, which utilises Sulfo-NHS-SS-Biotin for labelling plasma membrane surface proteins for further western blot analysis, as described in the Methods.

Measurements of the densities of the TRPM2-corresponding bands on the western blots suggested that TRPM2 channel expression on the plasma membrane increased in hepatocytes treated with H₂O₂. If in the control group only 3.57±0.47% of the total TRPM2 protein was detected on the plasma membrane, in hepatocytes treated with H₂O₂, this increased to 16.17±0.73% (n=3; P=0.0077), (Figure 4.1).To confirm the findings of biotinylation experiments we used confocal microscopy with immunofluorescence. In the control, untreated hepatocytes most of the TRPM2-specific labelling was intracellular (Figure 4.2). The representative images in Figure 4.2 (top row) suggest that there is very little overlap between the plasma membrane and TRPM2 in control hepatocytes, whilst in hepatocytes treated with H₂O₂ (middle row) and acetaminophen (bottom row) there is a clear TRPM2 fluorescence on the plasma
membrane. Using ImageJ software, we determined Pearson’s and Manders’ coefficients to quantify the co-localisation of TRPM2- and plasma membrane-specific fluorescence. Both coefficients increased significantly in the cells treated with either H$_2$O$_2$ or acetaminophen, compared to the control (figure 4.2 b,c).

**4.4 Discussion**

The main finding of this study is that oxidative stress in hepatocytes caused by H$_2$O$_2$ or acetaminophen treatment promotes trafficking of TRPM2 channels from intracellular compartments to the plasma membrane. It is likely that this trafficking of TRPM2 channels to the plasma membrane is responsible for the sustained [Ca$^{2+}$]$_c$ rise observed in hepatocytes treated with either H$_2$O$_2$ or acetaminophen (Chapter 2). The TRPM2 channel is one of the members of TRP channel superfamily that is activated during oxidative stress (Hara et al. 2002; Fonfria et al. 2004; Takahashi et al. 2011). TRPM2 channels are responsible for the rise of [Ca$^{2+}$]$_c$, which activates apoptotic and necrotic pathways, leading to cell death (Perraud, Schmitz & Scharenberg 2003; Hecquet et al. 2008; Bari et al. 2009). Initially TRPM2 channel has been characterised as a plasma membrane non-selective Ca$^{2+}$ permeable cation channel (Fonfria et al. 2004), however, later it became apparent that TRPM2 is also expressed in lysosomes (Lange et al. 2009). At least in one cell type, the pancreatic β-cells, the lysosomal TRPM2 channels have been shown to function as Ca$^{2+}$ release channels, contributing to H$_2$O$_2$-induced apoptosis (Lange et al. 2009).
Figure 4.1: The effect of H$_2$O$_2$ on TRPM2 channel expression on hepatocyte plasma membrane

**a.** Detection of TRPM2 expression on the plasma membrane. Total, remnant (total minus surface) and surface protein extracts of control and H$_2$O$_2$-treated hepatocytes were obtained as described in Methods. GAPDH protein was used as a loading control.

**b.** The plasma membrane/total TRPM2 channel fraction ratio (surface/total TRPM2 expression) in control and H$_2$O$_2$-treated hepatocytes from three different experiments was calculated using GS-800 densitometer.

* $P = 0.0077$
Figure 4.2: Effect of H$_2$O$_2$ and acetaminophen on TRPM2 channel trafficking in hepatocytes

a.

b. * P < 0.0001

c. ** P < 0.0001
a. Confocal images of control, H₂O₂- and acetaminophen-treated hepatocytes. Green colour corresponds to the TRPM2- and red colour to the cadherin(plasma membrane)-specific immunofluorescence. Yellow colour signifies the overlap between green and red fluorescence (TRPM2 and plasma membrane, correspondingly). b. Pearson’s coefficient of TRPM2 and plasma membrane co-localisation in control (0.35±0.01, n=51), H₂O₂- (0.75±0.01, n=50) and acetaminophen-treated (0.73±0.01, n=53) hepatocytes. There was a significant difference between the control and H₂O₂- or acetaminophen-treated hepatocytes (P < 0.0001). c. Manders’ coefficient (M1) (fraction of the plasma membrane merged with TRPM2 protein) in control (6.63±1.17, n=51), H₂O₂- (45.14±2.06, n=50) and acetaminophen-treated (41.5±2.65, n=53) hepatocytes. There was a significant difference in Manders’ coefficient between the control and H₂O₂- or acetaminophen-treated hepatocytes (P < 0.0001).

TRPM2 is not the only TRP family member located in the intracellular organelles, other TRP channels, including TRPML1, TRPML2, TRPML3, TRPM7, TRPM8, TRPP1, TRPV1, TRPV2, TRPV4, TRPA1 and TRPC3-6 were found in lysosomes, intracellular vesicles, ER and Golgi (Dong, Wang & Xu 2010). Physiological roles of some of these channels are quite clear, but for most intracellular TRP channels, their function remains to be identified. TRPML, for example, are the best candidates for lysosomal Ca²⁺ release channels, which play a critical role in lysosomal and endosomal trafficking, membrane fusion and fission (Piper & Luzio 2004; Chen, Bach & Pagano 1998; Kim et al. 2009; Martina, Lelouvier & Puertollano 2009). Mutations in human TRPML1 gene cause type IV mucolipidosis, characterised by neurodegeneration, mental retardation and iron deficiency (Chen, Bach & Pagano 1998). Another example is TRPM8 channels expressed in the ER of prostate cancer cells, where they function as agonist-activated Ca²⁺ release channels in store-operated Ca²⁺ entry pathway (Thebault et al. 2005). In contrast, the physiological roles of lysosomal TRPV channels are not entirely clear.

Activation pathway for some TRP channels includes insertion of intracellular vesicles into the plasma membrane (Bezzerides et al. 2004). It has been shown that TRPC5 channels localised in intracellular vesicles in neuritis of hippocampal neurons are rapidly inserted into the plasma membrane in response to stimulation with a growth
factor (Bezzerides et al. 2004). Similarly, vesicular TRPC3 channels are trafficked to the plasma membrane in response to G-protein coupled receptor stimulation (Singh et al. 2004).

Endolysosomal trafficking and fusion requires elevations in [Ca$^{2+}$]$_{c}$ (Stenmark 2009; Roth 2004), and it is likely that the local Ca$^{2+}$ rise which plays a critical role in this process is provided by Ca$^{2+}$ release from endolysosomes themselves (Stenmark 2009; Roth 2004). Oxidative damage caused by acetaminophen or H$_2$O$_2$ treatment of hepatocytes leads to a rise in ADPR concentration in cytoplasmic space (Chapter 2). ADPR can activate TRPM2 channels localised on both, the lysosomes and the plasma membrane. The pH in lysosomal lumen is normally maintained between 4 and 5 by V-type ATPase (Knowles, Li & Perraud 2013), so the lysosomal TRPM2 channels under normal conditions are likely to be blocked (Knowles, Li & Perraud 2013; Yang et al. 2010). However, under oxidative stress when cellular ATP is depleted due to mitochondrial dysfunction, the proton gradient on the lysosomal membrane cannot be longer maintained. The pH in lysosomal lumen rises and TRPM2 channels can now release lysosomal Ca$^{2+}$ and promote lysosomal fusion with the plasma membrane. As a result, the number of functional TRPM2 channels on the plasma membrane increases, causing further increase in [Ca$^{2+}$]$_{c}$. This mechanism has several inbuilt positive feedback loops, which explains a long lasting rise in [Ca$^{2+}$]$_{c}$ causing cell death. Rise in [Ca$^{2+}$], increases open probability of TRPM2 channels (Csanady & Torocsik 2009), causing further increase in [Ca$^{2+}$]$_{c}$, damage to mitochondria, depletion of ATP, Ca$^{2+}$ release from lysosomes, lysosomal fusion with plasma membrane and further increase in the number of functional TRPM2 channels.

In conclusion, the data presented in this work shows for the first time that oxidative stress-mediated damage in hepatocytes results in increased expression of TRPM2 channels on the cell surface, possibly due to lysosomal trafficking and fusion with the plasma membrane. The exact mechanism of this process, however, and whether TRPM2 mediated release of Ca$^{2+}$ from lysosomes plays any role in it require further investigation.
Chapter 5: TRPM2 Channel Inhibition—A Novel Property of Chlorpromazine and Curcumin

5.1 Introduction

Ca\(^{2+}\) signalling is critical for many cellular processes, ranging from growth and division to apoptosis and cell death. Regular cell functioning is regulated by the brief periods of free \([\text{Ca}^{2+}]_c\) rise mediated by the release of intracellular \(\text{Ca}^{2+}\) stored in several compartments, including ER/SR, mitochondria and Golgi apparatus, and inward movement of extracellular \(\text{Ca}^{2+}\) through various channels (Clapham 2007). Tight control of resting \([\text{Ca}^{2+}]_c\) at concentration at around 100 nM is necessary for cell survival (Clapham 2007). In oxidative stress, the continuous rise of \([\text{Ca}^{2+}]_c\) is one of the initial factors responsible for the disruption of mitochondrial function and activation of cell-destructive enzymes, leading to cell death (Gorlach, Klappa & Kietzmann 2006). This rise is mostly mediated by the inward movement of \(\text{Ca}^{2+}\) from the extracellular medium through various channels and transporters on the plasma membrane (Sato et al. 2009).

Previous research has suggested that some members of Transient Receptor Potential (TRP) channel family may provide a \(\text{Ca}^{2+}\) entry pathway under oxidative stress (Miller & Zhang 2011; Gees, Colsoul & Nilius 2010; Miller 2006). The TRP channel superfamily contains a diverse variety of cation channels expressed in mammalian cells, and consists of six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ANKTM), P (polycystin) and ML (mucolipin) (Gees, Colsoul & Nilius 2010; Montell 2005). Members of TRP family that may contribute to \([\text{Ca}^{2+}]_c\) rise during oxidative stress include TRPC3, TRPC4, TRPV1, TRPM2 and TRPM7 channels (Miller & Zhang 2011; Gees, Colsoul & Nilius 2010; Miller 2006; Sumoza-Toledo & Penner 2011). Of these, TRPM2 are likely to play the most significant role in the persistent \([\text{Ca}^{2+}]_c\) rise (Miller & Zhang 2011; Gees, Colsoul & Nilius 2010; Miller 2006).

Recently we have shown that TRPM2 channels are expressed in high numbers in rodent hepatocytes and are responsible for H\(_2\)O\(_2\)- and acetaminophen-induced persistent \(\text{Ca}^{2+}\) rise, leading to hepatocellular damage (Kheradpezhouh et al. 2014). Furthermore, experiments using TRPM2 KO mice have demonstrated that lack of TRPM2 channels
significantly protects the liver from acetaminophen-induced damage (Kheradpezhouh et al. 2014). Contribution of TRPM2 channels to oxidative-stress induced cell damage makes them a potential therapeutic target for treatment of a range of oxidative stress-related diseases.

There are several known inhibitors of TRPM2 channel, including ACA (anthranilic acid), clotrimazol, econazol and FFA (Flufenamic acid), (Bari et al. 2009; Hill, McNulty & Randall 2004; Hill et al. 2004). None of these has been used clinically to inhibit TRPM2 channels because the concentrations needed to achieve even partial block are beyond the safety margins of the drugs.

In this study, we investigated the effects of two compounds on TRPM2 channels: chlorpromazine and curcumin. The rationale for investigating these chemicals as potential TRPM2 blockers is that both, chlorpromazine and curcumin, were shown to protect liver from acetaminophen toxicity (Saville et al. 1988; Kheradpezhouh et al. 2010).

Chlorpromazine, an approved antipsychotic drug and a CaM antagonist, has been shown to protect liver at 6 mg.kg\(^{-1}\) from acetaminophen-induced damage (Saville et al. 1988). Furthermore, chlorpromazine has been shown to prevent liver damage induced by other toxins, including D-galactosamine (Tsutsui et al. 2003). Curcumin, another liver protective agent, is the main active compound of turmeric derived from the rhizome of *Curcuma longa*. It has been widely investigated during the past two decades, and its therapeutic effect in a number of diseases has been demonstrated. Its therapeutic action is mediated through various mechanisms, including free radical scavenging activity, anti-inflammatory properties and modulation of some signalling pathways (Zhu et al. 2004; Zuccotti et al. 2009; Tomita et al. 2006; Zong et al. 2012). Previous research has found that the level of protection afforded by curcumin against acetaminophen-induced liver damage is comparable to that of NAC (Kheradpezhouh et al. 2010). Currently NAC is the main treatment for acetaminophen overdose in humans (Lauterburg, Corcoran & Mitchell 1983).
5.2 Methods and Materials

5.2.1 Chemicals

Acetaminophen, ADPR, pleuronic acid, curcumin, Chlorpromazine, BSA and NAC were purchased from Sigma-Aldrich (Rockville, Maryland, US). DMEM, penicillin/streptomycin, and trypsin-EGTA were purchased from GIBCO (Grand Island, New York, US). Fura2-AM was purchased from Invitrogen (Carlsbad, California, US). FBS was purchased from Bovogen (Melbourne, Australia). Tris and glycerin were purchased from Amresco (Solon, Ohio, US).

5.2.2 Animals

Hooded Wistar rats were housed and bred in the controlled environment with a 12-hour light–dark cycle at least three weeks before beginning the experiments. Male rats aged eight to 12 weeks were used for the experiments. All experiments were approved by the Animal Ethics Committees of the University of Adelaide and Flinders University of South Australia.

5.2.3 Solutions

Wash media for hepatocyte preparation (mM): 136 NaCl, 4.7 KCl, 0.85 Na₂HPO₄, 0.45 KH₂PO₄, 24 NaHCO₃, 20 glucose, 1.3 CaCl₂, 0.8 MgSO₄ + 7 H₂O, BSA (10% W/V), penicillin (100 U/ml), streptomycin (100 µg/ml) and phenol red (0.001% W/V). Krebs-Ringer-Hepes (KRH) solution (mM): 136 NaCl, 4.7 KCl, 1.3 CaCl₂, 1.25 MgCl₂, 10 glucose and 10 Na-HEPES with pH adjusted to 7.4 by NaOH. Control bath solution for patch clamping (mM): 140 NaCl, 4 CsCl, 2 CaCl₂, 2 MgCl₂ and 10 Na-HEPES, adjusted to pH 7.4 with NaOH. Pipette solution for patch clamping (mM): 130-caesium glutamate, 5 MgCl₂, 5 CaCl₂ and 10 EGTA, adjusted to pH 7.3 with NaOH. Tris-buffered saline (TBS solution) (mM): 150 NaCl and 25 Tris with pH adjusted to 7.4 by NaOH. TBST solution: TBS solution plus 0.1% Tween-20. Phosphate buffered saline (PBS) solution (mM): 137 NaCl, 2.7 KCl, 10 Na₂HPO₄ and 1.8 KH₂PO₄.
5.2.4 Hepatocyte Isolation and Culture

The rat was briefly anesthetised with inhaled isoflurane, and then, was immediately injected with ketamin/xylazine (50 and 8 mg/kg, respectively) into the intra-peritoneal (IP) space. After the rat was fully anesthetised, it was moved to the 37°C incubator and fixed on a plate. The abdomen was opened mediially and, by two lateral incisions near the ribs (in an inverted T-shape), was fully exposed. The portal vein was cannulised and the liver perfused with the perfusion media warmed to 37°C and bubbled with 2 L/min carbogen [95% O₂ and 5% CO₂]. The rat was exsanguinated by cutting the inferior vena cava (IVC) below the kidneys. The anterior part of the chest was then removed completely and the upper part of the IVC was cannulised. The abdominal part of the IVC was clamped and the liver was first perfused with media containing EGTA 0.5 mM and then (for around 15 minutes) with the perfusion media containingCaCl₂ 1.3 mM and collagenase (25 mg/100 ml). After being fully digested, the liver was harvested, placed in the sterile container on ice and chopped using scissors. The hepatocytes were isolated from the mixture by several washings with the pre-chilled (4°C) wash media, followed by 80 s centrifugation at 500 rpm. The isolated hepatocytes were cultured on glass coverslips at 37°C in 5% CO₂ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v) for 16 to 96 hours before the experiments.

5.2.5 HEK 293T Cell Line Culture and Transfection

HEK 293T cells were cultured in 75 cm² flasks at 37°C in 5% CO₂ in air (v/v) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (v/v). Cells were harvested using PBS containing EGTA. The harvested cells were plated onto glass coverslips and 8 h later transfected with pCIneo expression vector containing human TRPM2 cDNA (generously provided by Professor Yasuo Mori, Japan) using TrueFect transfection reagent (United BioSystems Inc., US). Sixteen hours later, the transfected HEK 293T cells were used for the patch-clamp experiments.
5.2.6 Calcium Imaging

Fura2-AM was dissolved in 5 µl of 20% pluronic acid in DMSO (w/v) and diluted in KRH buffer to the final concentration of 5 µM. Sixteen hours after plating on glass coverslips, the hepatocytes were loaded with Fura2-AM for 30 minutes, washed and incubated in KRH solution for 10 minutes in the CO₂ incubator at 37°C. The fluorescence of Fura-2 was measured using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, Omega XF04 filter set for Fura-2, Photonic Science ISIS-3 ICCD camera and UIC Metafluor software. Fluorescence images were obtained every 10 seconds using a 20× objective. Fluorescence ratio values (340/380 nm) were transformed to [Ca²⁺]ₐ using the equation derived by Grynkiewicz, Poenie & Tsien (1985); a Kₐ of 224 nM for binding of Fura-2 to Ca²⁺; and ionomycin and EGTA to determine Rₘₐₓ and Rₘᵟᵳₐ, respectively.

5.2.7 Patch-clamp Recording

Membrane currents were measured at room temperature (23°C) using standard patch clamping in a whole-cell mode, and a computer-based EPC-9 patch-clamp amplifier run by PULSE software. In order to monitor the development of membrane currents, voltage ramps between -120 to +120 mV were applied every two seconds following the achievement of whole-cell configuration. The holding potential was -40 mV. The data were analysed using PULSEFIT software. The TRPM2 current of hepatocytes was activated by adding 1 mM ADPR to the pipette solution, respectively. Patch pipettes were pulled from borosilicate glass and fire-polished to a resistance between 1.5 and 2.5 MΩ. The series resistance did not exceed 7.5 MΩ and was 50 to 70% compensated.

5.2.8 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA), followed by the Bonferroni post hoc test and Student’s t-test (two tailed).
5.3 Results

To investigate whether chlorpromazine blocks TRPM2 channels we used isolated rat hepatocytes and whole cell patch clamping. As we showed previously (Chapter 2), addition of 1 mM ADPR to the pipette solution caused activation of a large non-selective cation current in hepatocytes, mediated by TRPM2 channels. Application of 100 µM chlorpromazine to the bath inhibited this current to the baseline. The amplitude of the inward current at -100 mV in the presence of 100 µM chlorpromazine was comparable to that when Na+ in the bath was replaced with NMDG, signifying a virtually complete block (Figure 5.1a). Half-maximum block of TRPM2 current was achieved by applying 5-10 µM of chlorpromazine (not shown).

To ascertain whether chlorpromazine also blocks acetaminophen-induced cation current, plated hepatocytes were incubated with 10 mM acetaminophen for 60 minutes prior to transferring them to the bath for patch clamping. Similar to ADPR-activated current, cation current activated in hepatocytes by the treatment with acetaminophen was inhibited by 100 µM chlorpromazine (Figure 5.1a & b). The time courses of inhibition for the currents activated by ADPR and acetaminophen were very similar (Figure 5.1b). However, the block of acetaminophen-induced current was incomplete, suggesting that in hepatocytes acetaminophen activates not only TRPM2 but also some other non-selective cation channels.

Similar results were obtained when we used HEK293 cells heterologously expressing human TRPM2 channels. Chlorpromazine (100 µM) inhibited both, the ADPR- and acetaminophen-induced TRPM2 currents in HEK293 cells, with the similar time course (Figure 5 c,d,e,f). The inhibition of acetaminophen-induced current in HEK293 cells was complete, unlike in rat hepatocytes, confirming that the remaining current not blocked by chlorpromazine in hepatocytes is mediated by a different channel.
Figure 5.1: Chlorpromazine blocks ADPR- and acetaminophen-activated TRPM2 current in rat hepatocytes and HEK293T cells.
a. The time course of inhibition of ADPR- and acetaminophen-activated TRPM2 current in rat hepatocytes by 100 μM chlorpromazine. Each data point represents amplitude of the current at −100 mV. b. The same data as in (a.) normalized to the maximum current. c. The amplitude of the current inhibited by the replacement of NaCl in the bath solution by NMDG+ in hTRPM2 transfected cells. Control: untreated cells (n=6). Acetaminophen: cells were treated with 10 mM acetaminophen for 40 min before current measurements (n=6). ADPR: cells were patch clamped using pipette solution containing 100 μM ADPR (n=7). Cells treated with acetaminophen exhibited significantly larger nonselective cation current compared with untreated cells (P<0.002). d. Current-voltage (I-V) plots recorded in a representative cell treated with acetaminophen under control conditions (Acetaminophen), after replacement of NaCl in the bath solution with NMDG-Cl (NMDG) and in the presence of 100 μM chlorpromazine in the bath. e. & f. The time course of chlorpromazine-induced (100 μM) inhibition of the inward current activated by either intracellular ADPR or preincubation with 10 mM acetaminophen. Each data point represents current amplitude measured at −100 mV. Data presented as means±SEM.

Previously, we have shown that TRPM2 KO mice are essentially protected against acetaminophen-induced liver damage (Kheradpezhouh et al. 2014). At the same, others and we have demonstrated that acetaminophen toxicity can be ameliorated in rodents by intraperitoneal injection of curcumin prior to administration of acetaminophen (Kheradpezhouh et al. 2010; Girish et al. 2009). It has been suggested that curcumin works through scavenging of free radicals (Agnihotri & Mishra 2011). Here, we investigate whether liver-protective properties of curcumin are related, in any way, to its effects on TRPM2.

First, we used Ca\(^{2+}\) imaging to ascertain the effect of curcumin on acetaminophen-induced [Ca\(^{2+}\)]\(_c\) rise. Isolated rat hepatocytes plated on glass cover slips were incubated with 10 mM acetaminophen in the presence or absence of 5 μM curcumin for 60 minutes in KRH bath solution containing 1.3 mM Ca\(^{2+}\). Treated hepatocytes were then loaded with Fura-2AM in a nominally Ca\(^{2+}\) free KRH and, after washing, were transferred to the microscope stage. Introduction of 1.3 mM Ca\(^{2+}\) to the bath resulted in [Ca\(^{2+}\)]\(_c\) increase to levels above 3 μM in the acetaminophen-treated hepatocytes, indicating that acetaminophen activated Ca\(^{2+}\) entry across the plasma membrane through Ca\(^{2+}\) permeable channels. In contrast, hepatocytes treated with acetaminophen
in the presence of curcumin showed no change in $[Ca^{2+}]_c$, similarly to the control (untreated) hepatocytes (Figure 5.2a,b).

Similarly, the hepatocytes treated with 0.5 mM H$_2$O$_2$ for half an hour, showed a significant rise in $[Ca^{2+}]_c$ after the introduction of 1.3 mM Ca$^{2+}$ to the bath. The control, untreated hepatocytes, and hepatocytes treated simultaneously with H$_2$O$_2$ and curcumin exhibited no rise in $[Ca^{2+}]_c$ after Ca$^{2+}$ add back to the bath (Figure 5.2c,d). These results indicate that, in hepatocytes, curcumin prevents acetaminophen- and H$_2$O$_2$-induced increase in $[Ca^{2+}]_c$.

Whole-cell patch-clamp recordings confirmed the findings of Ca$^{2+}$ imaging experiments. As we showed previously (Chapter 2), isolated rat hepatocytes treated with H$_2$O$_2$ or acetaminophen exhibited large non-selective cation current, mediated mostly by TRPM2 channels (Figure 2.9). Addition of 5 µM curcumin into incubation medium together with H$_2$O$_2$ or acetaminophen prevented development of any current above background conductance seen in control hepatocytes. Interestingly, curcumin inhibited development of not only TRPM2 current, but also of a non-specific leakage (see Figure 2.9, Chapter 2), suggesting that Na$^+$ and Ca$^{2+}$ entry mediated by TRPM2 may contribute to the development of non-specific leakage in hepatocytes (Figure 5.3).

In the experiments described above curcumin was present in the medium during treatments (H$_2$O$_2$ or acetaminophen) that cause oxidative stress-induced cell damage. In both cases, curcumin could work as a reactive species scavenger, preventing oxidative stress, and therefore, generation of ADPR and activation of TRPM2 channels. It is possible, however, that curcumin interacts with TRPM2 directly, as it does with some other channels, including TRPA1 and TRPV1 (Leamy et al. 2011; Martelli et al. 2007; Yeon et al. 2010). To investigate such possibility, TRPM2 current was activated by intracellular application of 1 mM ADPR through patch pipette, in hepatocytes pre-incubated with for 15 minutes of 5 µM curcumin in the bath., Whole-cell patch clamping showed that activation of TRPM2 current by ADPR was almost completely inhibited by curcumin. It should be noted here that curcumin had an inhibitory effect on TRPM2 only if applied to the bath prior to achieving whole-cell configuration. If applied after the development of the current, curcumin had no effect on TRPM2, even at 50 µM. For comparison, we investigated whether NAC, which is used in therapy for acetaminophen overdose, has any effect on TRPM2. In contrast to curcumin, NAC applied to the bath at 100 µM had no effect on activation of TRPM2 currents by ADPR (Figure 5.4).
Figure 5.2: The effect of curcumin on $[\text{Ca}^{2+}]_c$ in acetaminophen- and H$_2$O$_2$-treated hepatocytes

a. $\text{Ca}^{2+}$ entry activated in hepatocytes treated with 10 mM of acetaminophen in the presence or absence of 5 µM curcumin. b. Peak $[\text{Ca}^{2+}]_c$ in the control, curcumin-, acetaminophen-, and acetaminophen- and curcumin-treated hepatocytes 15 minutes after introducing 1.3 mM $\text{Ca}^{2+}$ to the bath. c. $\text{Ca}^{2+}$ entry activated in hepatocytes treated with 0.5 mM H$_2$O$_2$ in the presence or absence of 5 µM curcumin. d. Peak $[\text{Ca}^{2+}]_c$ in the control, curcumin-, H$_2$O$_2$-, and H$_2$O$_2$- and curcumin-treated hepatocytes 15 minutes after introducing 1.3 mM $\text{Ca}^{2+}$ to the bath. $[\text{Ca}^{2+}]_c$ was calculated using equation derived by Grynkiewicz, Poenie and Tsien (1985).
Figure 5.3: The effect of curcumin on membrane current of acetaminophen- and H$_2$O$_2$-treated hepatocytes
**a.** Current-voltage plots recorded in response to 100 ms voltage ramps between -120 and 120 mV in the control hepatocytes (control), hepatocytes treated with 10 mM acetaminophen for 60 minutes in control bath solution (acetaminophen), and hepatocytes treated with 10 mM acetaminophen and 5 µM curcumin for 60 minutes (acetaminophen+curcumin). **b.** The average amplitude of TRPM2-mediated Na\(^+\) current, obtained by replacing Na\(^+\) in the bath solution with NMDG\(^+\), in the control hepatocytes (control) and hepatocytes treated with 10 mM acetaminophen in the presence or absence of 5 µM curcumin for 60 minutes. **c.** Current-voltage plots recorded in response to 100 ms voltage ramps between -120 and 120 mV in the control hepatocytes (control), hepatocytes treated with 0.5 mM H\(_2\)O\(_2\) for 30 minutes in (H\(_2\)O\(_2\)), and hepatocytes treated with 0.5 mM H\(_2\)O\(_2\) in the presence of 5 µM curcumin for 30 minutes (H\(_2\)O\(_2\)+curcumin). **d.** The average amplitude of TRPM2-mediated Na\(^+\) current, obtained by replacing Na\(^+\) in the bath solution with NMDG\(^+\), in the control hepatocytes (control) and hepatocytes treated with 0.5 mM H\(_2\)O\(_2\) for 30 minutes, in the presence or absence of 5 µM curcumin.

Lack of any blocking effect of curcumin on TRPM2 current after its’ full development suggests that curcumin does not block the TRPM2 pore, but affects the mechanism of channel activation by ADPR. One of the possibilities is that curcumin interferes with ADPR binding to the Nudix motif in the TRPM2 C-terminus. To get some insight into this we used HEK293T cells transfected with hTRPM2 cDNA and whole cell patch clamping. Similarly to TRPM2 current in hepatocytes, current mediated by TRPM2 heterologously expressed in HEK293T cells was completely inhibited by 5 µM curcumin applied to the bath 15 minutes prior to patch clamping (Figures 5.5a,b). To investigate whether curcumin compete with ADPR for the binding site on TRPM2, we obtained dose-response curves for curcumin at two ADPR concentrations (0.1 and 1 mM) (Figure 5.5 c). The IC50 derived from the curves were not significantly different for 0.1 and 1 mM ADPR, suggesting that there is no competition between ADPR and curcumin (Figure 5.5c).
Figure 5.4: The effect of curcumin and NAC on the ADPR-activated TRPM2 current in hepatocytes

a. Development of TRPM2 current in response to intracellular ADPR in control hepatocytes, (control), hepatocytes pre-treated with 5 µM curcumin for 15 minutes (curcumin), and hepatocytes pre-treated with 100 µM NAC 15 minutes (NAC). Current was recorded in response to 100 ms voltage ramps between -120 and 120 mV, applied every two seconds. Current amplitude at -100 mV is plotted against time. b. The amplitude of the ADPR-activated TRPM2 current under conditions indicated on the X-axis, determined by replacing Na\(^+\) with NMDG in normal hepatocytes. The amplitude of Na\(^+\) current in hepatocytes not treated with intracellular ADPR is shown for comparison (untreated).
Figure 5.5: The effect of curcumin on ADPR-activated current in HEK 293T cells expressing TRPM2

a. Activation of TRPM2 current by intracellular ADPR in TRPM2-transfected HEK 293T cells in normal bath solution (control), and after pre-treatment with 5 µM curcumin for 15 minutes (curcumin). Current was recorded in response to 100 ms voltage ramps between -120 and 120 mV, applied every two seconds. Current amplitude at -100 mV is plotted against time. 
b. Current-voltage plots of ADPR-activated current in TRPM2-transfected HEK 293T cells in normal bath solution (control) and cells pre-treated with 5 µM curcumin for 15 minutes (curcumin). c. The dose-response curves for curcumin in TRPM2-transfected HEK 293T cells at two different concentrations of ADPR (0.1 and 1 mM). The amplitude of ADPR-activated current at -100 mV is plotted against curcumin concentrations. The IC₅₀ of for curcumin blocking ADPR-activated current were 53±16 nM and 42±17 nM for 0.1 and 1 mM ADPR, respectively.
The inhibitory effect of curcumin on activation of TRPM2 current could not be washed before losing the gigaseal. Therefore, to determine the reversibility of inhibition of TRPM2 by curcumin, the transfected HEK 293T cells were incubated with 2 µM curcumin for 15 minutes, then washed with normal bath media for 10 minutes and used for patch clamping with 1mM ADPR in the pipette solution. The amplitude of TRPM2 current in cells treated with and then washed of curcumin was 100.57±23.13 pA/pF, compared to 491±132.95 pA/pF in untreated cells, suggesting that curcumin had a long-lasting effect on TRPM2 activation (Figure 5.6).

5.4 Discussion

In this study, we show for the first time that chlorpromazine and curcumin are inhibitors of TRPM2 channel. The time course of TRPM2 inhibition by these compounds suggests that they affect the channel through different mechanisms. Chlorpromazine applied to the bath at 100 µM blocks fully developed TRPM2 current within 20-30 s, whilst curcumin inhibits development of TRPM2 current in response to oxidative damage or intracellular application of ADPR, but does not block active TRPM2 channels.
Figure 5.6: The wash-out the effect of curcumin on ADPR-activated current in TRPM2-expressing HEK 293T cells

The average amplitude of TRPM2 current measured at -100 mV in cells incubated in the normal bath solution (control), pre-incubated with 2 µM curcumin for 15 min (curcumin), and pre-incubated with 2 µM curcumin 15-minutes and then washed out for 10 minutes (curcumin wash-out).
Chlorpromazine is an antipsychotic drug, which inhibits a number of different types of postsynaptic receptors in the CNS, including dopamine, serotonin, histamine, α-adrenergic and muscarinic receptors (Conley & Kelly 2002). In addition, chlorpromazine is a known inhibitor of Ca\textsuperscript{2+}-calmodulin and voltage-gated Ca\textsuperscript{2+} channels (Ray et al. 2001; Choi et al. 2001). Previous findings that chlorpromazine inhibits Ca\textsuperscript{2+} accumulation and DNA fragmentation in hepatocytes, thus protecting liver from acetaminophen overdose, highlighted the role of Ca\textsuperscript{2+} in acetaminophen-induced liver damage. Importantly, chlorpromazine protects hepatocytes from death without affecting bioactivation of acetaminophen, or intracellular GSH (Saville et al. 1988). Both, acetaminophen metabolism into NAPQI (N-acetyl-p-benzoquinone imine) and following depletion of intracellular GSH have been proposed to be most critical steps in development of acetaminophen toxicity (Coles et al. 1988; Green, Dabbs & Tyson 1984; Tarloff et al. 1996). Thus, experiments with chlorpromazine suggested that blocking Ca\textsuperscript{2+} signalling pathways involved in DNA damage alone, is largely sufficient to prevent acetaminophen-induced liver damage. These earlier studies did not investigate the source of Ca\textsuperscript{2+} rise in hepatocytes, although protective effects of verapamil, another known inhibitor of Ca\textsuperscript{2+} channels, against acetaminophen-induced liver damage suggested involvement of Ca\textsuperscript{2+} channels on the plasma membrane.

The data presented here show that chlorpromazine blocks TRPM2 channels. Taking this together with our previous findings that the genetic knockdown of TRPM2 expression in mice protects hepatocytes from acetaminophen overdose, we can propose that the liver protective properties of chlorpromazine are due to its inhibition of TRPM2 channels. Our data does not provide a definitive answer about the mechanism of TRPM2 inhibition by chlorpromazine, however, a relatively slow time course (tens of seconds) and a very slow and incomplete wash-out (not shown) suggest that the binding site for chlorpromazine is intracellular. Furthermore, taking in the account that another CaM inhibitor, calmidazolium, also blocks TRPM2 currents, and that CaM plays an essential role in regulation of TRPM2 channels activity, it can be suggested that chlorpromazine inhibits TRPM2 channels by inhibiting CaM (Starkus et al. 2007).

The mechanism of TRPM2 inhibition by another agent investigated here, curcumin, is likely to be different from that of chlorpromazine, or any other known TRPM2 blocker. Curcumin is known as a powerful antioxidant and free-radical scavenger, but it also has anti-carcinogenic, anti-inflammatory, neuroprotective and some other properties ((Zhu et al. 2004; Zuccotti et al. 2009; Tomita et al. 2006; Zong et al. 2012; Zhang et al. 2006;...
Furthermore, curcumin has been shown to inhibit a number of K\(^+\) channels, including Kv1.3, Kv1.4, Kv11.1 and TREK-1, and Ca\(^{2+}\) release activated Ca\(^{2+}\) (CRAC) channels (Choi et al. 2013; Liu, Danthi & Enyeart 2006; Shin et al. 2011). Recently shown liver protective properties of curcumin against acetaminophen overdose have been attributed to its free-radical scavenging (Kheradpezhouh et al. 2010), however, other mechanisms, including inhibition of NF-κB and dependent pro-inflammatory genes may also play a role (Fan et al. 2014). The data presented in this study add inhibition of TRPM2 channels to the list of possible mechanism of liver protection by curcumin. In acetaminophen overdose, *in vivo* curcumin may work as an antioxidant and free-radical scavenger, reducing damage to mitochondria and DNA, thus inhibiting production of ADPR, the main agonist of TRPM2. A similar mechanism is proposed for the protective effect of NAC, as a GSH precursor, for oxidative stress-induced cellular damage (Ozgul & Naziroglu 2012). NAC has been shown to inhibit activation of TRPM2 current by H\(_2\)O\(_2\) (Ozgul & Naziroglu 2012), however, our patch clamping data presented in this study indicate that NAC has no effect on activation of TRPM2 by ADPR. In contrast, curcumin strongly inhibits ADPR-mediated activation of TRPM2. The mechanism of the inhibition is not yet clear; however, it does not involve a direct block of the pore, or competition of curcumin with ADPR for the binding site. Very slow wash-out of curcumin could be the evidence of strong binding, with slow off rate, of curcumin to TRPM2. Alternatively, curcumin may cause TRPM2 modification through yet unknown pathway, which affects its function or localization.

In conclusion, this study showed that chlorpromazine and curcumin are the most potent inhibitors of TRPM2 channels discovered so far. The IC\(_{50}\) for chlorpromazine (~5 μM) is higher than the safe dose that can be used in humans; however, for curcumin, the IC\(_{50}\) of ~50 nM, and even the concentration required for a complete block of TRPM2 (~ 1 μM) are well within the safety margins for its human consumption (Sanmukhani et al. 2014; Kanai et al. 2013; Zuccotti et al. 2009). This makes curcumin a potential therapy for diseases where deleterious effects of oxidative stress, at least in part, are due to aberrant Ca\(^{2+}\) entry mediated by TRPM2 channels. Curcumin has already been suggested as a treatment for some neurodegenerative conditions, and TRPM2 channels are expressed in very high numbers in the brain and have been implicated in neurodegeneration (Zhang et al. 2006; Yanagisawa et al. 2011; Zhu et al. 2004). It is possible that inhibition of TRPM2 channels, in addition to free radical scavenging, is the mechanism by which curcumin protects cells from oxidative stress-induced damage.
Chapter 6: General Discussion

The liver is the largest internal organ, which performs over 500 specific functions ranging from glucose and lipid metabolism to detoxification of xenobiotic compounds (Knell 1980; Herbarth et al. 2004). Most of liver functions require energy to proceed, which makes liver a highly aerobic and oxygen dependent tissue and also makes it susceptible to cell damage due to hypoxia and toxic insults (Mari et al. 2010). Production of reactive oxygen and nitrogen species during metabolism is normal; however, increased formation of ROS and RNS plays a central role in liver damage in many important liver diseases. Liver damage due to oxidative stress is a hallmark of a number of liver diseases, including ischemia-reperfusion injury, liver fibrosis and cirrhosis, non-alcoholic fatty liver disease (NAFLD), viral hepatitis, hepatocellular carcinoma and several drug toxicities (Muriel 2009; Gao & Bataller 2011; Lee 2003). Liver toxicity caused by an overdose of one of the most widely used drugs, acetaminophen, has been in focus of a large number of investigations (Mitchell et al. 1973; Bessems & Vermeulen 2001; Walker, Racz & McElligott 1980; Lores et al. 1995; Gibson et al. 1996; Chan et al. 2001). The main steps in development of acetaminophen-induced liver injury have been reasonably well characterised; however, one aspect of it, the role of intracellular Ca$^{2+}$, has been consistently overlooked. Possibly, due to an early suggestion that intracellular Ca$^{2+}$ rise in acetaminophen poisoning is more likely to be a consequence of hepatocellular damage, than its’ cause (Harman et al. 1992; Grewal & Racz 1993).

The research described in this thesis was started in an attempt to find the source of Ca$^{2+}$ rise in hepatocytes during acetaminophen overdose. Now we can say that the pathway for Ca$^{2+}$ entry in acetaminophen poisoning is mediated by TRPM2 channels, and these channels play a significant role in hepatocellular damage. TRPM2 are non-selective, Ca$^{2+}$ permeable channels, activated by intracellular ADP-ribose, cADPR, NAD$^+$ and NAADP (Perraud et al. 2001; Sano et al. 2001; Beck et al. 2006; Kolisek et al. 2005). Some studies that used heterologous expression of TRPM2 in HEK293 cells suggest that TRPM2 channels are directly activated by H$_2$O$_2$ (Wehage et al. 2002); however, it is likely that activation of TRPM2 by H$_2$O$_2$ in vivo is indirect, and is mediated by ADPR generated in mitochondria and nucleus in response to H$_2$O$_2$-induced oxidative stress (Hara et al. 2002; Perraud et al. 2005). Calcium, both
intracellular and extracellular, plays a role of an important co-factor in activation of TRPM2 channels, acting synergistically with ADPR and thus providing a positive feedback for the TRPM2-mediated Ca\(^{2+}\) entry pathway (Perraud et al. 2001; Csanady & Torocsik 2009).

Physiologically, TRPM2 channels are essential for normal immune response to bacterial infection through their control of the cytokine release in monocytes and the maturation and chemotaxis of dendritic cells (Yamamoto et al. 2008; Knowles et al. 2011). There is also evidence that TRPM2 regulate insulin secretion from pancreatic β-cells, as glucose-induced insulin secretion from the islets isolated from TRPM2 KO mouse seems to be impaired (Uchida et al. 2011). The current list of pathologies where TRPM2 channels might be involved is much longer than the list of known physiological functions of TRPM2. It has been shown, albeit with different degree of certainty for different diseases, that (i) TRPM2 channels are responsible for H\(_2\)O\(_2\)-induced damage of neurons and pancreatic β-cells (Kaneko et al. 2006; Bari et al. 2009); (ii) permanent salivary gland damage by the radiation therapy of head and neck cancers leading to a loss of salivary fluid secretion is mediated by TRPM2 channels (Liu et al. 2013); (iii) TRPM2 channels are required for DSS-induced experimental colitis (Yamamoto et al. 2008); (iv) TRPM2 are over-expressed in cerebral cortex and hippocampus in response to traumatic brain injury, and in glial cells in response to ischaemic injury (Cook et al. 2010; Yuruker, Naziroglu & Senol 2014); (v) TRPM2-mediated Ca\(^{2+}\) entry is involved in neurodegeneration in Alzheimer’s disease and Amyotrophic lateral sclerosis (Fonfria et al. 2005; Hermosura et al. 2008); and etc. Research presented in this thesis adds to this list ‘acetaminophen overdose-induced liver damage’.

In Chapter 2 of this thesis we show, using both RT-PCR and western blotting, that TRPM2 channels are expressed in high numbers in rat hepatocytes. Treatment of hepatocytes with acetaminophen or H\(_2\)O\(_2\) results in activation of a large non-selective cation current and Ca\(^{2+}\) entry, that can be inhibited using the known blockers of TRPM2 channels, clotrimazole and ACA. The properties of acetaminophen and H\(_2\)O\(_2\)-induced non-selective cation currents are similar to those activated by intracellular application of ADPR, however, pre-treatment of hepatocytes with acetaminophen or H\(_2\)O\(_2\) causes membrane blebbing, which makes patch clamping of treated cells, and separation of TRPM2 current from non-specific leakage somewhat difficult. Importantly, knock down of TRPM2 expression by specific siRNA results in similar levels of reduction,
approximately 60%, corresponding to the level of TRPM2 knockdown, in the amplitudes of all, ADPR-, H$_2$O$_2$-, and acetaminophen-induced cation currents. Interestingly, the extent of Ca$^{2+}$ entry reduction by TRPM2 knock down, measured by using Fura-2 and Ca$^{2+}$ imaging, is greater than 60% (so it is greater than the level of TRPM2 knock down). This can be explained by the difference in the experimental conditions used in patch clamping and Ca$^{2+}$ imaging. In patch clamping intracellular Ca$^{2+}$ is strongly buffered to ~120 nM with EGTA, and Ca$^{2+}$ entering through TRPM2 channels cannot fully contribute to the positive feedback mechanism. In contrast, in Ca$^{2+}$ imaging intracellular Ca$^{2+}$ is allowed to rise, which results in recruitment of additional TRPM2 channels. Reduction in the number of channels due to siRNA-mediated knock down also results in the weakening of the positive feedback mechanism due to a reduction in Ca$^{2+}$ entry and longer distances between the adjacent channels. Reduction in both, the channels numbers and the strength of the feedback between them, could be the reason for a stronger than expected effect of TRPM2 knockdown on Ca$^{2+}$ entry measured by Fura-2.

Regarding the pathway of TRPM2 channels activation, our data does not support the notion that TRPM2 can be activated directly by H$_2$O$_2$ (Kheradpezhouh et al. 2014). Both treatments, by acetaminophen or H$_2$O$_2$, result in a marked increase in the production of poly-ADPR in hepatocytes, which can be quickly converted by Poly-ADPR-glycohydrolase into ADPR that is free to diffuse from the nucleus to the plasma membrane and to bind to the Nudix box on the C-terminus of TRPM2 channel (Perraud et al. 2001). Almost complete inhibition of acetaminophen- and H$_2$O$_2$-induced Ca$^{2+}$ entry by the inhibitor of PARP-1, the enzyme catalyzing synthesis of poly-ADPR from NAD in the nucleus, suggest that ADPR is the main activator of TRPM2 channels in hepatocytes.

The fact of activation of TRPM2 current in hepatocytes by acetaminophen treatment per se does not prove that TRPM2 channels contribute to liver damage in acetaminophen overdose. To address this issue, we used TRPM2 KO mouse. As expected, hepatocytes isolated from TRPM2 KO mice showed greatly reduced Ca$^{2+}$ entry in response to H$_2$O$_2$ treatment, and virtually no Ca$^{2+}$ entry in response to acetaminophen treatment, compared to WT hepatocytes. Calcium imaging data was well supported by the patch clamping results. Importantly, compared to WT mice, TRPM2 KO mice treated with toxic dose of acetaminophen exhibited significantly lower blood levels of liver enzymes, ALT and AST, which are used as markers of liver
damage (Rumack & Bateman 2012; Batt & Ferrari 1995). Furthermore, histological examination of liver slices showed that areas of necrosis in the livers of acetaminophen-treated TRPM KO mice were considerably smaller than in the livers of WT mice subjected to a similar treatment. The results of this Chapter allowed us to conclude that activation of TRPM2 channels contributes to acetaminophen-induced damage. Significant reduction in hepatocellular death induced by acetaminophen in the presence of TRPM2 inhibitor, ACA, in culture suggests that TRPM2 expressed in hepatocytes plays the main role. However, this data does not exclude a possibility that TRPM2 expressed in Kupffer cells also play some role in acetaminophen-induced liver damage. Cells of immune system, including macrophages and monocytes express TRPM2 channels, and both, Kupffer cells (liver resident macrophages) and blood-derived monocytes, were implicated in liver inflammation and hepatocyte damage (Wehrhahn et al. 2010; Knowles et al. 2011; Yamamoto et al. 2008; Zigmond et al. 2014). Currently nothing is known about the role of TRPM2 channels in Kupffer cell mediated liver inflammation and this topic requires investigation in a separate study.

As some other TRP channels, TRPM2 is expressed not only on the plasma membrane, but also on the membranes of intracellular organelles, primarily, lysosomes (Sumoza-Toledo et al. 2011; Knowles, Li & Perraud 2013). The distribution of TRPM2 between different sites in the cell varies between cell types (Fonfria et al. 2004; Zhang et al. 2007; Sumoza-Toledo et al. 2011; Lange et al. 2009), however, it is not yet known what controls TRPM2 channels localisation. First images of hepatocytes labelled with TRPM2 specific antibodies, obtained by using immunofluorescence microscopy, suggested that most of TRPM2 protein is intracellular, with very little staining on the plasma membrane. This was quite surprising, given the very large amplitude of ADPR-activated TRPM2 currents in hepatocytes. Furthermore, there were some other unusual features of TRPM2, particularly the time course of Ca^{2+} entry activation in hepatocytes treated with H_2O_2 and acetaminophen. As cells were pre-treated with H_2O_2 or acetaminophen before the start of the recording, one could assume that TRPM2 channels were already active when Ca^{2+} was added to the bath. Indeed, there was no delay in the start of Ca^{2+} entry, but [Ca^{2+}]_c continued to rise for 20 min or more, until Fura-2 was saturated.

Activation of TRPM2 current in rat hepatocytes, measured by patch clamping, was also slow, sometimes taking 5-10 minutes, although equilibration of ADPR concentration between patch pipette and the cell would take no longer than 60 s. These observations
led us to a hypothesis that during TRPM2 current activation intracellular TRPM2 channels are incorporated into the plasma membrane. This was investigated in Chapter 4.

The idea that oxidative stress may affect the distribution of certain proteins between the intracellular organelles and the plasma membrane is not new (Li, Gulbins & Zhang 2012; Carini et al. 2006; Aguilar-Gaytan & Mas-Oliva 2003; Cheng & Vieira 2006). This can occur through at least two mechanisms. It has been shown that oxidative stress prevents endocytic trafficking and internalisation of a number of plasma membrane receptors and channels (Aguilar-Gaytan & Mas-Oliva 2003); and more importantly (for our study), it has been previously demonstrated that oxidative stress triggers lysosomal trafficking and fusion with the plasma membrane (Li, Gulbins & Zhang 2012; Carini et al. 2006). Immunofluorescence microscopy, using TRPM2 specific antibody and plasma membrane staining, showed quite a dramatic change in TRPM2 localisation in hepatocytes treated with H₂O₂ or acetaminophen. Whilst in untreated cells there was little overlap between TRPM2-specific fluorescence and the plasma membrane, in treated cells, plasma membrane staining was almost completely covered with TRPM2. These results were confirmed by the surface protein biotinylation experiment. The question remains whether intracellular ADPR, introduced through a patch pipette in the absence of oxidative stress, also promotes TRPM2 trafficking to the plasma membrane. Our patch clamping data on rat hepatocytes suggest that it does, however, this should be further investigated using GFP-tagged TRPM2, heterologously expressed in an appropriate cell system where it mainly localises to the lysosomal membrane.

The ultimate goal of any biomedical research, apart from expanding knowledge, is to find a way of using this knowledge to treat human diseases. In ion channels research, a lot of effort is focused on finding channel modulators, drugs that inhibit or open specific ion channels, which can be used as therapy of certain human conditions. Probable involvement of TRPM2 channels in a wide range of human diseases caused by oxidative stress in different tissues and organs makes TRPM2 a potential target for intervention. Currently, however, there are no specific, high-affinity inhibitors of TRPM2 (Bari et al. 2009; Hill, Mcnulty & Randall 2004; Hill et al. 2004). Before commencing this research we knew that curcumin, a component of spice used in many oriental dishes and also a well-studied anti-oxidant (Yadav et al. 2009; Zhu et al. 2004; Zuccotti et al. 2009), can ameliorate acetaminophen-induced liver damage in rats at least as efficiently as NAC, currently used in clinic to treat acetaminophen overdose.
Curcumin has been found to have numerous potential applications in human diseases, mainly as a powerful anti-oxidant; however, some effects of curcumin cannot be attributed solely to its’ free radical-scavenging properties (Zhu et al. 2004; Zuccotti et al. 2009; Tomita et al. 2006; Zong et al. 2012). Research presented in Chapter 5 show that curcumin prevents development of TRPM2 current in hepatocytes treated with acetaminophen and \( \text{H}_2\text{O}_2 \), but it also prevents activation of TRPM2 by intracellular ADPR with \( \text{IC}_{50} \) of about 50 nM. Therefore, the therapeutic effect of curcumin against acetaminophen overdose is likely to be mediated by both, its anti-oxidant properties and through inhibition of TRPM2. The latter may be more important, as curcumin is quickly metabolised, and it is difficult, if not impossible, to maintain its’ blood levels high enough for curcumin to work as free radical scavenger, but concentrations that would have a significant effect on TRPM2 (50 nM range) are quite feasible (Sanmukhani et al. 2014; Kanai et al. 2013; Zuccotti et al. 2009). The mechanism of TRPM2 inhibition by curcumin is not yet clear, however, we know that it does not block the channel pore. The hypothesis that will be used in future research is that curcumin prevents ADPR-induced lysosomal trafficking/fusion to the plasma membrane.

Another drug that has been shown to reduce acetaminophen-induced liver damage, chlorpromazine (Saville et al. 1988), also turned out to be TRPM2 channel blocker. Unlike curcumin, chlorpromazine blocks TRPM2 current when it has already developed, and it is likely that it works by inhibiting CaM binding to TRPM2. However, again, the mechanism of the block should be further investigated. The concentration of chlorpromazine used to prevent liver damage in mice (Saville et al. 1988) is comparable to the concentration that can affect TRPM2 amplitude (\( \text{IC}_{50} \sim 5 \mu\text{M} \)), suggesting that chlorpromazine works, at least in part, through inhibition of TRPM2 channels. Due to relatively low affinity and potentially serious side effects, chlorpromazine might not be usable for human therapy, but it can be used to design other, more selective TRPM2 blockers.

Prior to this study nothing was known about TRPM2 channels in the liver and very little about the role of \( \text{Ca}^{2+} \) in acetaminophen toxicity. The results presented in this thesis allow construction of a more precise picture of what happens in hepatocytes during acetaminophen overdose with respect to the role of \( \text{Ca}^{2+} \). Excess of acetaminophen that cannot be metabolised through innocuous pathways is metabolised by the cytochromes P450 into highly reactive compound, NAPQI, which leads to
exhaustion of hepatocellular GSH and formation of NAPQI-protein adducts (Jollow et al. 1973; Green, Dabbs & Tyson 1984; Tarloff et al. 1996). This causes initial damage to mitochondria, resulting in formation of ROS, RNS and generation of ADPR. At the same time, ROS-mediated damage to DNA activates PARP-1 and production of poly-ADPR in the nucleus. ADPR activates a small number of TRPM2 channels on the plasma membrane, which mediate a sustained rise in intracellular Na\(^+\) and Ca\(^{2+}\) concentrations. Rising Ca\(^{2+}\) causes further damage to mitochondria with further production of ROS and the release of mitochondrial endonucleases, thus further facilitating DNA damage and ADPR production (Bajt et al. 2003; Hinson, Roberts & James 2010; Kon et al. 2004; Reid et al. 2005; Cover et al. 2005a). At the same time high intracellular Na\(^+\) activates Na\(^+\)-K\(^+\)-ATPase and causes a drop in intracellular concentration of ATP, which cannot be replenished by damaged mitochondria. Low intracellular ATP results in alkalinisation of lysosomal lumen, due to inhibition of H\(^+\)-ATPase. Low pH of lysosomal lumen would normally inhibit TRPM2 channels localised on lysosomal membrane (Knowles, Li & Perraud 2013; Yang et al. 2010), however, when lysosomal pH rises above pH 6.5, opening of TRPM2 channels on the lysosomal membrane is triggered by ADPR, already present at a high concentration in the cytosol. Active TRPM2 channels act as a Ca\(^{2+}\) release pathway from lysosomes, thus contributing to [Ca\(^{2+}\)]\(_c\) rise and promoting lysosomal fusion with the plasma membrane. As a result, the number of active TRPM2 channels on the plasma membrane increases and the whole cycle repeats itself. Eventually, Ca\(^{2+}\) overload causes hepatocellular death through oncotic necrosis. Not every step in this proposed mechanism of hepatocytes damage is yet supported experimentally. Our results are consistent with the notion of TRPM2 trafficking from intracellular compartments to the plasma membrane during oxidative stress, however, the mechanism how this happens, requires further investigation.

In conclusion, TRPM2 may contribute not only to acetaminophen-induced liver damage, but also to some other liver pathologies caused by oxidative stress. From this point of view, TRPM2 could represent an important therapeutic target, and the research into the mechanisms regulating TRPM2 activity together with the search for TRPM2-specific inhibitors could bring new therapies for a range of liver disorders.
Appendix

TRPM2 channels mediate acetaminophen-induced liver damage

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Acetaminophen (paracetamol) is the most frequently used analgesic and antipyretic drug available over the counter. At the same time, acetaminophen overdose is the most common cause of acute liver failure and the leading cause of chronic liver damage regulating liver transplantation in developed countries. Acetaminophen overdose causes a multitude of interrelated biochemical reactions in hepatocytes including the formation of reactive oxygen species, deregulation of Ca2+ homeostasis, covalent modification and oxidation of proteins, lipid peroxidation, and DNA fragmentation. Although an increase in intracellular Ca2+ concentration in hepatocytes is a known consequence of acetaminophen overdose, its importance in acetaminophen-induced liver toxicity is not well understood, primarily due to lack of knowledge about the source of the Ca2+ rise. Here we report that the channel responsible for Ca2+ entry in hepatocytes in acetaminophen overdose is the Transient Receptor Potential Melanostatin 2 (TRPM2) cation channel. We show by whole-cell patch clamping that treatment of hepatocytes with acetaminophen results in activation of a cation current similar to that activated by H2O2 or the intracellular application of ADP ribose. siRNA-mediated knockdown of TRPM2 in hepatocytes inhibits activation of the current by either acetaminophen or H2O2. In TRPM2 knockout mice, acetaminophen-induced liver damage, assessed by the blood concentration of liver enzymes and liver histology, is significantly diminished compared with wild-type mice. The present data strongly suggest that TRPM2 channels are essential in the mechanism of acetaminophen-induced hepaticcellular death.

Acetaminophen (N-acetylsalicylic acid), when used at prescribed doses, is a safe analgesic and antipyretic drug (1). Its overdose, however, can be life threatening, causing severe liver and kidney damage (2–5). In Western countries, acetaminophen-induced hepatotoxicity is a leading cause of acute liver failure requiring liver transplantation (6). Due to a wide spread availability of acetaminophen and potentially lethal consequences of its overdose, the mechanisms of acetaminophen hepatotoxicity have been in focus of a large number of investigations (7). Despite significant progress, the exact pathways of acetaminophen hepatotoxicity that lead to hepaticcellular death are still not completely understood. It is clear, however, that acetaminophen toxicity arises from its metabolic activation (8, 9).

In the liver, therapeutic doses of acetaminophen are metabolized by glucuronidation and sulfation into nontoxic compounds (1). Only a small amount of acetaminophen is converted by hepatic cytochrome P450 (CYP)-dependent mixed function oxidases to the reactive intermediate metabolite N-acetyl-p-benzoquinoneimine (NAPQI). The NAPQI generated by a therapeutic dose of acetaminophen is rapidly metabolized to nontoxic products by conjugation with glutathione (GSH) (1, 10). With large doses of acetaminophen, however, hepatic GSH becomes depleted resulting in the accumulation of toxic amounts of NAPQI. Covalent binding of NAPQI to cellular proteins has previously been considered the main cause of liver cell death under these circumstances. Indeed it has been shown that covalent binding precedes hepaticcellular death, and treatments that prevent covalent binding also prevent liver necrosis (11). More recently, however, it has been suggested that, by itself, the covalent binding of NAPQI is not sufficient to induce apoptosis or necrosis. The toxic signal produced by covalent binding undergoes further amplification through the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), deregulation of Ca2+ homeostasis, and increased intracellular Ca2+ content, causing oxidant stress in mitochondria and inducing the mitochondrial membrane permeability transition (12, 13). Although widely acknowledged, the role of Ca2+ in acetaminophen toxicity is poorly understood and has not been thoroughly investigated.

Nonselective Ca2+ channel blockers chlorpromazine and verapamil have been shown to attenuate liver injury in mice (14, 15), however, the mechanism of their protective properties in acetaminophen overdose is not clear, and it is not known whether it involves any Ca2+-permeable channels on the plasma membrane of hepatocytes.

The only Ca2+-selective channel that has been clearly identified in hepatocytes so far is the Ca2+ release-activated Ca2+ channel activated by the depletion of intracellular Ca2+ stores downstream of phospholipase Cγ and phospholipase Cδ signaling (16, 17). In addition, a number of Ca2+-permeable nonselective cation channels with no clearly defined functions and mostly from the TRP family of channels have been shown to be present in hepatocytes and liver cells (18, 19). One of these channels, Transient Receptor Potential Melanostatin 2 (TRPM2), whose presence in the liver has only been demonstrated on an mRNA level (19), is activated in response to oxidative stress and, potentially, can be involved in acetaminophen-induced Ca2+ rise in hepatocytes.

Significance

Acetaminophen overdose is the most common cause of acute liver failure and the leading cause of chronic liver damage requiring liver transplantation in developed countries. There are limited options for early treatment. Acetaminophen liver toxicity leads to the formation of reactive oxygen and nitrogen species which cause an increase in intracellular Ca2+ and hepaticcellular death. We show that acetaminophen-induced liver toxicity depends on Transient Receptor Potential Melanostatin 2 (TRPM2) cation channels in hepatocytes, which are activated in response to oxidative stress and are responsible for Ca2+ overload. Lack of TRPM2 channels in hepatocytes or their pharmacological inhibition protects liver from acetaminophen toxicity. This provides evidence that TRPM2 may present a potential therapeutic target for treatment of oxidative-stress-related liver diseases.


The authors declare no conflict of interest.

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Results

Acetaminophen and H$_2$O$_2$ Activate Nonselective Cation Current in Hepatocytes. To investigate the role of Ca$^{2+}$-permeable channels in acetaminophen toxicity in the liver, first we examined the effects of acetaminophen on the free cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_c$) in rat hepatocytes. Hepatocytes were incubated with 10-15 mM acetaminophen for 60 min in a bath solution containing 1.3 mM Ca$^{2+}$. Treated hepatocytes were then loaded with Fura-2-acetoxymethyl (AM) in a nominally Ca$^{2+}$-free bath solution, and after washing, were transferred to the microscope stage. After introduction of 1.3 mM Ca$^{2+}$ into the bath, [Ca$^{2+}$]$_c$ increased to micromolar levels, indicating that acetaminophen activates Ca$^{2+}$ entry across the plasma membrane through Ca$^{2+}$-permeable channels. Control cells showed no change in [Ca$^{2+}$]$_c$ (Fig. 1A). Ca$^{2+}$ entry activated in hepatocytes by precubation with acetaminophen was inhibited by 50 μM chlorotriazone and 10 μM N-2-aminoethylisothiourea (hantamonic acid (ACA)) (Fig. 1A). Chlorotriazone and ACA were previously shown to block heterologously expressed TRPM2 channels (20, 21).

To investigate the nature of the Ca$^{2+}$-permeable channels responsible for acetaminophen-induced Ca$^{2+}$ entry, we used whole-cell patch clamping. After isolation, hepatocytes were cultured for 24–48 h on glass coverslips and treated with or without acetaminophen for 60 min. The average density of baseline current at −100 mV in rat hepatocytes normally varies between 2 and 4 pA/pF and the current-voltage (I–V) plot shows some outward rectification due to Cl$^-$ conductance (22) (Fig. 1B). Hepatocytes preincubated with 10 mM acetaminophen for 60 min, however, showed significantly larger currents upon establishing whole-cell configuration, with the average current density of 6–10 pA/pF at −100 mV (Fig. 1B). The virtually linear I–V plot, near-zero reversal potential, and sensitivity of the inward current to the replacement of extracellular Na$^+$ with the large cation N-methylglucamine (NMDG) (Fig. 1D) suggested that treatment with acetaminophen resulted in the activation of nonselective cation channels. Similarly to acetaminophen-induced Ca$^{2+}$ entry, this nonselective cation current was blocked by chlorotriazone and ACA (Fig. 1B). Only chlorotriazone is shown, as ACA produced the same level of inhibition. When we increased the time of pretreatment with acetaminophen, hepatocytes became increasingly damaged with extensive membrane blebbing so that they were not amenable to Fura-2 Ca$^{2+}$ measurements and patch clamping. Direct application of acetaminophen to the bath in patch clamping or Ca$^{2+}$ imaging experiments had no acute effect on membrane current (Fig. 1B).

Oxidant stress caused by ROS and RNS formed in the liver in acetaminophen overdose is considered a major mediator of hepatocellular death (23). To determine whether hepatocytes express ROS-sensitive Ca$^{2+}$-permeable channels which can potentiate toxicity induced by ROS and RNS generated in hepatocytes treated with acetaminophen, we used Ca$^{2+}$ imaging and patch clamping of isolated rat hepatocytes treated with H$_2$O$_2$. Fura-2 experiments indicated a robust rise of ([Ca$^{2+}$]$_c$) in response to incubation with 0.5 mM H$_2$O$_2$ for 25–30 min, whereas patch clamping showed activation of a nonselective cation current similar to that activated by acetaminophen (Fig. 1C and D). Both Ca$^{2+}$ entry and the nonselective cation current activated in hepatocytes by incubation with H$_2$O$_2$ were inhibited by 50 μM chlorotriazone and ACA (Fig. 1C and D). As observed for 10-15 mM acetaminophen, 0.5 mM H$_2$O$_2$ produced some membrane blebbing in hepatocytes. Cells with damaged membranes were not amenable to patch clamping. Therefore, the amplitude of the cation current activated by incubation with 1 mM H$_2$O$_2$ was likely to be underestimated, as only visibly undamaged cells were used. Adding 1 mM H$_2$O$_2$, directly to the bath after achieving the whole-cell configuration with control pipette solution did not result in activation of a noticeable current within 10 min of recording or before the seal between the cell and the pipette was lost. However, application of 10 mM H$_2$O$_2$ to the bath in such experiments resulted in a relatively rapid development of a large cation current with properties similar to those of the currents activated by precubation of hepatocytes with 0.5 mM H$_2$O$_2$ or 10 mM acetaminophen (Fig. 1E and F).
The hallmark of TRPM2-mediated currents is activation by ATP released (ADPR) and H$_2$O$_2$. Several splice variants of TRPM2 channel have been reported (31). One of them, with a deletion in the C terminus (TRPM2CA), lacks the ADPR-binding motif and has been shown to be activated by H$_2$O$_2$, but not ADPR (31). To determine the main splice variants of TRPM2 expressed in hepatocytes we used RT-PCR and showed that rat hepatocytes express only long isoform of TRPM2 (LTPM2) with the ADPR binding motif intact (Table S1 and Fig. 3A). To establish if these channels are functional we used whole-cell patch clamping of hepatocytes cultured for 24 h after isolation and a pipette solution supplemented with ADPR. Addition of 1 mM ADPR resulted in activation of a large nonspecific current (137 ± 27 pA/pF) 1-5 min after establishing the whole-cell configuration (Fig. 2A and B). Lower concentrations of ADPR produced smaller currents (EC$_{50}$ 480 µM) with a longer time course of activation. The ADPR-activated current was inhibited by ACA and clotrimazole at the same concentrations as those that inhibited the currents activated by acetaminophen and H$_2$O$_2$ (Fig. 2A and C).

In addition to ACA and clotrimazole, we investigated the effects of another sodium channel subunit from sodium channel (14, 32). ADPR- and acetaminophen-activated current in rat hepatocytes was fully blocked by 100 µM chlorpromazine (C2D and Fig. 3A), with an EC$_{50}$ of ~5 µM. The time course of inhibition of the current activated by ADPR was very similar to that of the current induced by acetaminophen (Fig. 3A and B). To confirm that chlorpromazine blocks TRPM2 channels we used HEK293T cells transfected with TRPM2 cDNA (Fig. 3C and D). TRPM2 current activated by either acetaminophen or ADPR in transfected HEK293T cells was blocked by chlorpromazine with similar time courses and concentrations as the currents activated in rat hepatocytes (Fig. S2 E and F).

Acetaminophen overdose has been shown to cause DNA fragmentation in hepatocytes and, as a consequence, activation of poly(ADP-ribos)e polymerase (PARP), which generates polyADPR, the main precursor of cytoplasmic ADPR (33). Using antibodies against polyADPR and immunofluorescence we were able to demonstrate an increase in polyADPR production in hepatocytes treated for 45 min by 10 µM acetaminophen or 1 mM H$_2$O$_2$ (Fig. 3A). Incubation with 10 mM acetaminophen overnight induced much stronger polyADPR production. To investigate whether generation of ADPR in hepatocytes in response to H$_2$O$_2$ and acetaminophen contributes to activation of Ca$^{2+}$ entry we used PARP inhibitor 3,4-dihydro-2H-[1,3]benzodiazepin-2-one (DHP) (34). Measurements of [Ca$^{2+}$]$_{ic}$ showed that DHP strongly inhibited Ca$^{2+}$ entry in rat hepatocytes, supporting the notion that Ca$^{2+}$ rise induced by H$_2$O$_2$ and acetaminophen is mediated by ADPR-activated TRPM2 channels (Fig. 3 B and C).

To confirm that the current activated by ADPR, H$_2$O$_2$, and acetaminophen is mediated by TRPM2 channels we used siRNA-mediated knockdown of TRPM2 in rat hepatocytes. In cells transfected with siRNA against TRPM2, patch clamping showed that membrane currents activated by the application of intracellular ADPR, H$_2$O$_2$, or acetaminophen were each reduced by 65-70% (Fig. 4A). RT-PCR and Western blotting confirmed that by using siRNA-mediated knockdown we reduced TRPM2 expression in primary hepatocytes by about 60% (57 ± 5%, n = 3) within 48 h after transfection (Fig. 4B, Table S2, and Fig. S5). Measurements of [Ca$^{2+}$]$_{ic}$ using Fura-2AM revealed that siRNA-mediated knockdown of TRPM2 expression resulted in a significant reduction of Ca$^{2+}$ entry in hepatocytes treated by H$_2$O$_2$ or acetaminophen (Fig. 4 C and D). These results confirm that the cation current activated by acetaminophen and H$_2$O$_2$ in hepatocytes is mediated by TRPM2.

To further test whether H$_2$O$_2$ and acetaminophen-activated Ca$^{2+}$ entry is mediated by TRPM2 channels, we conducted measurements of [Ca$^{2+}$]$_{ic}$ using Fura-2AM and hepatocytes isolated from TRPM2 knockout (KO) and (wild-type) WT mice, and the inhibitors of TRPM2 channel, ACA and clotrimazole (Fig. 5 A–C). RT-PCR and Western blot analysis confirmed that WT mouse
hepatocytes express the same isoform of TRPM2, LTTRPM2, as rat hepatocytes and that TRPM2 protein is absent in TRPM2 KO mouse hepatocytes (Table S3 and Figs. S4 and S5). In response to H₂O₂ or acetaminophen treatment, hepatocytes isolated from TRPM2 WT mice showed Ca²⁺ entry similar to that of rat hepatocytes, which was blocked by ACA and clotrimazole (Fig. 5 A and C; compare with Fig. 1 A and C). In contrast, hepatocytes isolated from TRPM2 KO mice had a significantly smaller Ca²⁺ entry activated by H₂O₂ and virtually no Ca²⁺ entry in response to the treatment with acetaminophen (Fig. 5 B and C). A relatively small Ca²⁺ rise in TRPM2 KO mouse hepatocytes induced by H₂O₂ was blocked by clotrimazole, but not ACA, suggesting the presence of a minor H₂O₂-activated Ca²⁺ entry pathway not mediated by TRPM2 channels. Results of Ca²⁺ imaging were supported by patch clamping of hepatocytes isolated from TRPM2 WT and KO mice. Treatment of hepatocytes with H₂O₂ or acetaminophen resulted in activation of a significantly larger nonsynaptic cation current in TRPM2 WT hepatocytes compared with hepatocytes from KO animals (Fig. S6 A–D).

Ablation of TRPM2 Channels Protects Against Acetaminophen Toxicity in the Liver. Treatment of isolated rat and mouse hepatocytes in culture with acetaminophen causes progressive cell death through necrotic necrosis, thus mimicking the effects of acetaminophen on the intact liver (35). In the next experiment we investigated whether ablation of TRPM2 channels by ACA affords protection to hepatocytes against high doses of acetaminophen. Indeed, ACA (1 μM) reduced cellular death by ~60% in hepatocytes treated with 10 mM acetaminophen for 16 h compared with hepatocytes treated with acetaminophen alone (n = 3; P < 0.01) (Fig. S6E).

To establish whether activation of TRPM2 channels plays a role in acetaminophen-induced liver damage in vivo we used TRPM2 KO mice (36). Lip injection of acetaminophen (500 mg/kg) to WT mice resulted in a large increase in the blood concentrations of the liver enzymes, alanine transaminase (ALT) and aspartate transaminase (AST) compared with vehicle-injected animals (Fig. 6A). Suggesting severe liver damage 24 h postinjection (cf. ref. 37). In TRPM2 WT mice this dose of acetaminophen also caused a large elevation of liver enzymes. However, in TRPM2 KO animals the blood levels of ALT and AST were ~5–7 times lower than in WT and Het mice (Fig. 6A). Hematoxylin–eosin (H&E) staining of liver sections of acetaminophen-treated TRPM2 WT and Het mice revealed widespread hepatoceleular damage (Fig. 6D). This was characterized by areas of necrosis, infiltration by lymphocytes, and hemorrhage, and was prominent in zones 2 and 3 (hepatocytes around the hepatic vein) (Fig. 6D). Liver damage was substantially reduced in TRPM2 KO mice treated with acetaminophen compared with the treated WT and Het mice (Fig. 6D). The area of necrotic damage was much smaller in TRPM2 KO mice and was localized to zone 3. In some sections of livers from acetaminophen-treated TRPM2 WT and Het mice, hemorrhagic necrosis and extravasations of blood were detected along with necrotic damage. Because the prominent and consistent effect of acetaminophen was necrotic damage, the area of necrotic tissue was quantified. The results indicate that the area of necrosis in the livers of TRPM2 KO mice treated with acetaminophen was substantially smaller than in the livers of acetaminophen-treated TRPM2 WT and Het mice (Fig. S6F).

Discussion

In this study we show that hepatocytes express long isoform of TRPM2 channels. These channels function as a cation entry pathway across the hepatocyte plasma membrane and similarly to TRPM2 channels heterologously expressed in HEK293 cells are activated by ADPβS included in the patch pipette and H₂O₂ added to the bath solution (34, 35). The important finding of this study is that TRPM2 channels mediate a substantial increase in [Ca²⁺]ᵢ, in hepatocytes treated with toxic concentrations of acetaminophen. Furthermore, we show that Ca²⁺ entry through TRPM2 channels plays a significant role in acetaminophen-induced hepatoceleular death both in vitro and in vivo.
The mechanism of acetaminophen hepatotoxicity has been under intensive investigation for several decades (7). It has been established that acetaminophen overdose causes a multitude of interrelated cellular events (7), but the relative importance of each of these events in hepatocellular death is not well understood. Briefly, the main steps that lead to acetaminophen hepatotoxicity can be summarized as follows. Saturation of gluaronic oxidation and sulfation pathways by excessive levels of acetaminophen leads to the increased acetaminophen metabolism by several isoforms of CYP (CYP2E1, CYP2A1, CYP3A4, and CYP2D6) into the reactive metabolite NAPQI (6, 33). NAPQI saturates and depletes intracellular GSH and covalently binds to protein (8, 9). Lack of GSH causes accumulation of ROS and RNS and oxidative stress. Increased oxidative stress, together with covariant binding, causes mitochondrial dysfunction, DNA fragmentation, and derangement of Ca\(^{2+}\) homeostasis (7, 10). Oxidants and increased Ca\(^{2+}\) promote mitochondrial permeability transition, which, in turn, initiates further oxidative stress, loss of mitochondrial potential, and cessation of ATP synthesis (25). Finally, loss of ATP triggers necrosis of hepatocytes.

Two cellular processes in this sequence are likely to result in an increase in cytoplasmic concentration of ADPR, the main ligand of the Ca\(^{2+}\)-permeable TRPM2 channels (29, 40). Opening of the mitochondrial permeability transition pore in the inner membrane releases ADPR from mitochondria, whereas activation of PARP by DNA damage results in generation of ADPR precursor, poly(ADPR) (40, 41). The importance of the events that produce cytoplasmic ADPR is emphasized by the findings that the inhibitors of mitochondrial permeability transition and the PARP inhibitors protect the liver against acetaminophen overdose (33). The results of this study show that the increase in poly(ADPR) production in hepatocytes treated with 10 mM acetaminophen can be detected by immunofluorescence within 45 min of the start of the treatment, and progressively increases with time, which creates favorable conditions for activation of TRPM2 channels. Inhibition of acetaminophen-induced Ca\(^{2+}\) entry in hepatocytes by PARP inhibitor DPQ shows here suggest a mechanism for the known protective effects of PARP inhibitors against acetaminophen toxicity (33).

Derangement of Ca\(^{2+}\) homeostasis in acetaminophen hepatotoxicity has been demonstrated in earlier studies (14, 32); however, it has been suggested that intracellular Ca\(^{2+}\) rise, mainly due to inhibition of Ca\(^{2+}\)-Mediated ATPase, accompanies rather than causes hepatocellular injury (42). Ca\(^{2+}\) channels antagonists, verapamil and chlorpromazine, have been shown to protect the liver against acetaminophen toxicity, but whether they actually block any channels in hepatocytes has never been investigated. We have shown here that chlorpromazine blocked TRPM2 currents activated in rat hepatocytes by ADPR, H\(_2\)O\(_2\), or acetaminophen. It also blocked TRPM2 channels heterologously expressed in HEK293T cells and activated by acetaminophen or ADPR. This suggests that the protective properties of chlorpromazine may be due to its inhibition of TRPM2 channels, although chlorpromazine may have other relevant targets in hepatocytes (14).

More definitive data about the role of TRPM2 mediated Ca\(^{2+}\) entry in acetaminophen toxicity come from TRPM2 KO mice experiments. Lack of TRPM2 channels results in significantly improved blood levels of the liver enzymes ALT and AST and in significantly reduced liver damage 24 h post-acetaminophen injection, suggesting that activation of TRPM2 channels contribute to hepatocellular death. The deleterious effects of TRPM2 activation may not be just due to Ca\(^{2+}\) entry, but also to a very high Na\(^+\) and K\(^+\) conductance through these channels. Accumulation of Na\(^+\) and loss of K\(^+\) leads to a loss of the plasma membrane potential and activation of Na/\(K\) ATPase which contributes to the reduction of cellular ATP levels and promote cell necrosis. These findings add considerably to our current understanding of the mechanism of acetaminophen liver toxicity. Currently, the only clinically available treatment for acetaminophen overdose is N-acetyl-cysteine, a GSH precursor, which has to be administered within 15–16 h after acetaminophen ingestion to be effective (43). If this time window is lost, the efficacy of N-acetyl-cysteine is in preventing liver damage is significantly reduced, and liver failure is the likely outcome (43). The TRPM2 channel offers an alternative therapeutic target which may allow treatment over a wider window of time. Moreover, inhibitors of TRPM2 offer the potential to treat other ROS-mediated liver diseases such as nonalcoholic liver disease, hepatitis, and hepatic cellular carcinoma.

**Materials and Methods**

**Animals.** Hooded Wistar (H/W) rats and TRPM2 KO and WT mice were housed and bred in the controlled environment with a 12:12 light:dark cycle. Animals had access to food and water ad libitum. TRPM2 KO mice were obtained from Yauco Mori’s laboratory (Kyoto University, Kyoto, Japan). All animal studies were approved by the Animal Ethics Committees of the University of Adelaide and Flinders University of South Australia.

**Hepatic Isolation and Culture.** Hepatocytes were isolated from H/W rats by liver perfusion with collagenase using the protocol described previously.
(44). The isolated hepatocytes were cultured on glass coverslips at 37 °C in 5% CO₂ air (v/v) in DMEM containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS (v/v) for 16-18 h before experiments. Isolated mouse hepatocytes were prepared by retrograde perfusion of the liver through the inferior vena cava and cut-open portal vein. The rest of the protocol was similar to that used to prepare isolated rat hepatocytes (44).

Calcium Imaging: Hepatocytes cultured on glass coverslips for 24 h were loaded with Fura-2-AM (1 μM) for 30 min, washed, and incubated in Krebs-Ringer-Hepes solution for 10 min in a CO₂ incubator at 37 °C. The fluorescence of Fura-2 was measured using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-400 wavelength switcher, Omega X401 filter set for Fura-2, Photonic Science Q Interfaced CCD camera, and Universal Image Frame Metallux software. Fluorescence images were obtained every 20 s using a 2× objective. Fluorescence ratio values (480:510 nm) were transformed to [Ca²⁺], using the equation derived by Grynkiewicz et al. (45).

Patch-Clamp Recording: Membrane currents were measured at room temperature (23 °C) using standard patch-clamping in a whole-cell mode and a computer-based EPC-9 patch-clamp amplifier run by PULSE software (HEKA) (22). To monitor the development of membrane currents, voltage ramps between -120 and +120 mV were applied every 2 s following the achievement of whole-cell configuration. The holding potential was -40 mV. The data were analyzed using PULSE software (HEKA). For current measurements, a current pipette was pulled from borosilicate glass and fire polished to a resistance between 1.5 and 2.5 MΩ. Mouse hepatocytes were generally less amenable for patch clamping, therefore to increase the probability of forming a gigaseal, smaller patch pipettes with a resistance between 3 and 5 MΩ were used. Series resistance was 50-70% uncompensated.

Statistical Analysis: Data are presented as means ± SEM. Statistical significance was assessed using ANOVA followed by the Bonferroni post hoc test or using unpaired two-tailed t test with Welch’s correction.

Chemicals, solutions, and methods for RP-FCR, cell transfections, Western blot analysis, cell viability assay, and histopathology are provided in Materials and Methods.

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