



**LABILE ZINC AND ITS ROLE IN
REGULATION OF PRO-CASPASE-3
AND NF- κ B ACTIVATION
IN MAST CELLS**

**A thesis submitted to the University of Adelaide as the
requirement of the Degree of Doctor of Philosophy**

by

Lien Ha Ho B. Health. Sc. (Hons).

Department of Medicine
The University of Adelaide
The Queen Elizabeth Hospital

June 2003

TABLE OF CONTENTS

TABLE OF CONTENTS	ii
SUMMARY	viii
DECLARATION	xii
ACKNOWLEDGMENTS	xiii
PUBLICATIONS ARISING FROM THESIS	xvii
CONFERENCE PRESENTATIONS	xviii
LIST OF FIGURES	xix
LIST OF TABLES	xxvi
ABBREVIATIONS	xxvii
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction	2
1.2 Zinc (Zn)	3
1.2.1 Historical Background	3
1.2.2 Chemistry	4
1.2.3 Bioavailability And Absorption	4
1.2.4 Zn Transporters	5
1.2.5 Other Zn Transporters	8
1.2.6 ZnT ₄ Transporter	9
1.2.7 Two Distinct Pools Of Cellular Zn	10
1.2.8 Detection of Zn	11
1.2.9 Techniques For Manipulating Intracellular Zn Levels.....	13
1.2.10 Zn Deficiency.....	14
1.2.11 Biological Functions	16
1.3 Apoptosis	20
1.3.1 Cellular Changes in Apoptosis.....	20
1.3.2 Apoptosis Signalling Pathways.....	21
1.3.3 Caspases	23
1.3.4 Activation Of Caspases	29
1.3.6 Inducers Of Apoptosis: Butyrate, Staurosporine	31
1.3.7 Zn and Apoptosis	32
1.4 Nuclear factor kappa-B (NF-κB)	36
1.4.1 Activation Of NF-κB	37
1.4.2 NF-κB and Zn	39
1.4.3 NF-κB And Apoptosis	41
1.5 Types of Cells Studied	47
1.6 Mast Cells	48
1.6.1 Heterogeneity	49
1.6.2 Growth and Maturation	51

1.6.3 Mast Cell Activation	52
1.6.5 Zn And Mast Cells	58
1.6.6 Mast cell apoptosis.....	61
1.6.7 Recovery Of Mast Cells Following Degranulation.....	65
1.7 Neuronal Cells	65
1.7.1 Zn and Neuronal cells	66
1.7.2 Zn and Neuronal cell apoptosis.....	67
1.8 Project Hypotheses And Aims	71
CHAPTER TWO: MATERIALS AND METHODS.....	72
2. General Methods	73
2.1 Mast Cell Cultures	73
2.1.1 Isolation Of Human Umbilical Cord Blood Mast Cells.....	73
2.1.2 Isolation Of Rat Peritoneal Mast Cells (RPMC).....	75
2.1.3 HMC-1 Human Mast Cell Line And Its Maturation.....	75
2.1.4 Culturing of Human Bone Marrow Mast cells and RBL-2H3 Basophilic Mast cells	76
2.1.5 Toluidine Blue Staining Of Mast Cells.....	76
2.1.6 Degranulation Assays	77
2.1.7 Confirmation Of Mast Cell Degranulation	77
2.1.8 Epithelial Cell Cultures.....	78
2.1.9 Neuronal Cell Cultures.....	79
2.2 Zinc Studies	79
2.2.1 Basal Zinc Measurement.....	79
2.2.2 Zinc Manipulation Assays.....	80
2.2.3 Fluorescence Image Analysis.....	81
2.3 Apoptosis Assays	82
2.3.1 Induction Of Apoptosis By Butyrate And TPEN.....	82
2.3.2 Caspase Assay	82
2.2.3 Protein Measurements.....	83
2.2.4 Morphological Criteria And Chromatin Fragmentation	83
2.4 Immunofluorescence Labelling.....	84
2.4.1 Pro-Caspase-3, -4 And ZnT ₄ Labelling.....	84
2.4.2 NF-κB Labelling	85
2.5 Electron Microscopy	86
2.5.1 Ultra Structural Visualization Of Cells.....	86
2.5.2 Immunogold Labelling Of Cells	87
2.6 ZnT₄ And Pro-caspase-3 mRNA Expression by Northern Hybridization..	89
2.6.1 Isolation Of RNA From Cells	89
2.6.2 RNA Transfer To Hybridization Filter	91
2.6.3 Preparation Of ZnT ₄ DNA Probe For Northern Hybridization	92
2.6.4 Preparation of Pro-caspase-3 DNA Probe For Northern Hybridization.....	95
2.6.5 Northern Hybridization/Blotting.....	97

2.6.6 Quantification Of Northern Bands	100
2.7. Expression of ZnT₄ By RT-PCR.....	100
2.8 Statistical Analysis.....	102
CHAPTER THREE: ZINC AND ZnT₄ IN MAST CELLS PRIOR TO AND FOLLOWING ACTIVATION.....	103
3.1 Introduction.....	104
3.2 Methods.....	106
3.2.1 Distribution Of Intracellular Labile Zn.....	106
3.2.2 Distribution And Expression Of ZnT ₄	106
3.2.3 Statistical Analysis.....	107
3.3 Results	108
3.3.1 Basal Zinquin Fluorescence Of Mast Cells.....	108
3.3.2 Morphology And Zinquin Fluorescence In Immature And Mature HMC-1 Cells	108
3.3.3 Effect Of Activation On Morphology And Zinquin Fluorescence	109
3.3.4 Repletion Of Zn After Degranulation	111
3.3.5 Expression of ZnT ₄ mRNA in mast cells.....	111
3.3.6 Localization And Levels Of ZnT ₄ In Mast Cells.....	113
3.3.7 Dual Labelling Of ZnT ₄ And Zn In Mast Cells	114
3.3.8 Immunogold Labelling Of ZnT ₄ In Mast Cells.....	114
3.4 Discussion.....	116
CHAPTER FOUR: LOCALISATION OF PRO-CASPASE-3 AND -4 IN MAST CELLS AND EFFECTS OF ACTIVATION.....	119
4.1 Introduction.....	120
4.2 Methods.....	123
4.2.1 Expression Of Pro-Caspase-3 By Northern Hybridization	123
4.2.2 Activation Of Mast Cells	123
4.2.3 Localization Of Pro-Caspase-3 And -4 By Immunofluorescence.....	123
4.2.4 Localization Of Pro-Caspase-3 And -4 By Electron Microscopy.....	123
4.2.5 Statistical Analysis.....	124
4.3 Results	125
4.3.1 Expression Of Pro-Caspase-3 mRNA In Mast Cells	125
4.3.2 Expression Of Pro-Caspase-3 And -4 In Mast Cells By Immunofluorescence.....	125
4.3.3 Effects Of Mast Cell Activators On Pro-Caspase-3 And -4 In Immature And Mature HMC-1 Cells And RPMC	126
4.3.4 Localization Of Pro-Caspase-3 And -4 In Mast Cells By Electron Microscopy.....	127
4.3.5 Quantification Of Gold Labelling	129
4.4 Discussion.....	131

CHAPTER FIVE: INTERACTIONS BETWEEN ZINC DEPLETION AND APOPTOTIC INDUCERS ON CASPASE ACTIVATION IN MAST CELLS 134

5.1 Introduction	135
5.2 Methods.....	137
5.2.1 Activation Of Mast Cells	137
5.2.2 Treatment With Apoptotic Inducers (Butyrate, Staurosporine) Or TPEN	137
5.2.3 Fluorogenic Substrate Assay For Active Caspases.....	137
5.2.4 Statistical Analysis.....	138
5.3 Results	139
5.3.1 Basal Levels Of Active Caspases In Mast Cells.....	139
5.3.2 Concentration-Dependent Induction Of Caspase-3 (DEVD-Caspase) Activity By Butyrate In Mast Cells.....	139
5.3.3 Concentration-Dependent Induction Of Caspase-3 (DEVD-Caspase) Activity By TPEN In Mast Cells	140
5.3.4 Interaction Between Butyrate And TPEN In Induction Of Caspase-3 (DEVD-Caspase) And -6 (VEID-Caspase) Activity In Mast Cells.....	140
5.3.5 The Effect Of Zn Depletion By Degranulator On Caspase Activity In Mast Cells	142
5.3.6 Interaction Between Staurosporine And Compound 48/80 On Levels Of Active Caspases In Mast Cells.....	143
5.3.7 Effects Of Degranulator And TPEN On Chromatin Fragmentation In Immature HMC-1 Cells.....	144
5.4 Discussion.....	145

CHAPTER SIX: EFFECTS OF ZINC DEPLETION AND SUPPLEMENTATION ON ACTIVATION OF NF- κ B 149

6.1 Introduction	150
6.2 Methods.....	153
6.2.1 Activation Of NF- κ B By TNF- α In Mast Cells And NCI-H292 Human Epithelial Cells.....	153
6.2.2 Activation Of Immature And Mature HMC-1 And RBL-2H3 Mast Cells By Degranulators	153
6.2.3 Detection And Quantification Of NF- κ B By Immunofluorescence Labelling	153
6.2.4 Measurement Of Cell Size By Image Analysis	154
6.2.5 Depletion And Supplementation Of Zn In Mast Cells.....	154
6.2.6 Statistical Analysis.....	154
6.3 Results	155
6.3.1 Activation Of NF- κ B In NCI-H292 Human Epithelial Cells	155
6.3.2 Activation Of NF- κ B In Immature And Mature HMC-1 And RBL-2H3 Mast Cells	155
6.3.3 Effect Of Zn Depletion By TPEN On The Activation Of NF- κ B In Mast Cells	156
6.3.4 Effect Of Degranulators On The Activation Of NF- κ B In Mast Cells.....	157

6.3.5 Effect Of Zn On Changes In Cell Size During Mast Cell Stimulation	158
6.4 Discussion	159
CHAPTER SEVEN: SUPPRESSION OF CASPASE-3 ACTIVATION IN NEUROBLASTOMA CELLS BY INTRACELLULAR LABILE ZINC	164
7.1 Introduction	165
7.2 Methods	167
7.2.1 Zn Supplementation And Depletion Assays	167
7.2.2 Induction Of Apoptosis	167
7.2.4 Statistical Analysis	168
7.3 Results	169
7.3.1 Distribution Of Labile Zn In Neuroblastoma Cells	169
7.3.2 Effect Of Depleting Intracellular Zn In BE(2)-C Cells On DEVD-Caspase Activation	170
7.3.3 DEVD-Caspase Activity In BE(2)-C Cells Treated With Butyrate Plus Staurosporine	170
7.3.4 Adherent Versus Non-Adherent Cells	171
7.3.5 The Effect Of Priming With Butyrate On Induction Of DEVD-Caspase Activity By TPEN	172
7.3.6 Time Course Of TPEN Effects On DEVD-Caspase Activity In Butyrate-Primed BE(2)-C Cells	173
7.3.7 Effects Of Zn Supplementation With Pyrithione On DEVD-Caspase Activity In BE(2)-C Cells	174
7.3.8 Concentration Dependence Of Pyrithione	175
7.4 Discussion	176
CHAPTER EIGHT: GENERAL DISCUSSION AND FUTURE STUDIES	182
8.1 Introduction	183
8.2 Labile Zn And ZnT ₄ In Mast Cells	183
8.3 Localisation Of Pro-Caspases-3 And -4 In Resting And Activated Mast Cells	185
8.4 Interactions Between Zn Depletion And Mast Cell Apoptosis	186
8.5 The Effect Of Zn On NF- κ B In Mast Cells And Relationship To Apoptosis	189
8.6 Zn and caspase activation in neuroblastoma cells	190
8.7 General Model	192
REFERENCES	197
APPENDIX 1A: CELL CULTURES AND BUFFERS	257

<i>APPENDIX 1B: ANTIBODIES, ENZYMES, SUBSTRATES AND KITS.....</i>	<i>261</i>
<i>APPENDIX 1C: CHEMICALS AND EQUIPMENT</i>	<i>266</i>
<i>APPENDIX 1D: JOURNAL PAPERS.....</i>	<i>273</i>

SUMMARY

The main aim of this thesis was to further investigate the relationship between Zinquin-detectable intracellular pools of labile Zn and caspase activation since caspases have now been shown to be important effector enzymes in apoptosis. This was largely studied in mast cells and some findings confirmed in neuronal cells, a cell type with quite different function and cell physiology.

Mast cells were chosen because these cells are important inflammatory cells that have been shown to contain Zn rich granules. However, there have been no previous studies of labile Zn in these cells, the mechanisms by which it is accumulated and its function in relationship to apoptosis. Labile Zn was shown to be rich in mast cells by Zinquin fluorescence with a granular like staining pattern and the fluorescence decreased during degranulation. A major issue is how mast cells regulate their uptake of Zn from their environment. The experiments reported here are the first to describe the localization of a Zn transporter, ZnT₄ in mast cells. There was high expression of both the mRNA and its protein in mast cells, suggesting that it is likely to be involved in the transportation of Zn either across the plasma membrane or into the granules. The absence of overlap between Zn and ZnT₄ may imply that only a subset of granules require ZnT₄ as their major Zn transporter. Other transporters need to be investigated.

The distribution of pro-caspases, important components in apoptosis signaling has not been a focus of mast cell biologists. It was important to determine whether these were

present in mast cells and their relationship to mast activation. In mast cells, expression of pro-caspase-3 mRNA was shown and the detection of its protein by immunocytochemistry indicated that it was translated. One unexpected finding was that pro-caspase-3 (and -4) were localized within granules of the mast cells as shown by immunoelectron microscopy. This was confirmed by their loss during degranulation.

Explanations for why pro-caspases are localized in mast cell granules include: 1) pro-caspases may be released and activated during degranulation and cleave extracellular substrates, 2) caspases may be released and induce apoptosis in other cells, 3) caspases may have a special function in cleaving proteins within the granules and 4) in order to prolong their survival during activation, mast cells may shed their caspases during degranulation and thereby become more resistant to noxious stimuli.

Depletion of Zn by one mechanism (TPEN chelation) but not by degranulation increased the activation of pro-caspase-3, either spontaneously or in cells treated with the apoptotic inducer butyrate. The experiments described in this thesis show that at least under *in vitro* conditions, degranulated mast cells can recover their granular content of Zn and do not undergo apoptosis spontaneously. Similar results were obtained for a range of degranulators and various mast cell types. These findings suggest that there are two labile pools of Zn; one regulates caspase activation and apoptosis while the other (the granular pool) has other functions.

To further understand the mechanism by which Zn may regulate apoptosis in mast cells, the effects of Zn supplementation and depletion on activation of NF- κ B were

investigated. NF- κ B is thought to be an anti-apoptotic factor prolonging the survival of inflammatory cells. Zn supplementation by the ionophore pyrithione blocked nuclear translocation of NF- κ B, an important step in the activation of this transcription factor. Furthermore, Zn depletion by TPEN was found to be an effective inducer of nuclear NF- κ B translocation. This has not previously been demonstrated. These findings suggest that Zn is a regulator of NF- κ B translocation and raise questions as to whether abnormalities in NF- κ B activation occur in Zn deficiency.

The studies with neuroblastoma cells confirmed some of these findings. BE(2)-C neuroblastoma cells had cytoplasmic/cytoskeletal pools of intracellular labile Zn which were further increased by Zn ionophore or decreased by Zn chelator TPEN. As for most cells there was synergy between TPEN and butyrate in caspase activation and this was suppressed by Zn supplementation, suggesting that the effect of Zn on caspase regulation is a general phenomenon.

As a summation of the findings reported in this thesis, a general model is proposed describing possible interactions between Zn, pro-caspases and NF- κ B. Some of these findings were reported in Ho et al 2000 and form part of a submitted manuscript Ho et al 2003.