Vascular reactivity in sepsis and platelet dysfunction in septic shock

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THESIS DECLARATION

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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Signed,

Benjamin Reddi
I am grateful to Dr David Wilson for his unstinting guidance and support, an inspiring teacher and scientist. Thank you to Prof. John Beltrame for his expert advice and A/Prof. Robert Young for his encouragement and practical support.

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**THESIS ABSTRACT**

Sepsis remains an important global cause of morbidity and mortality. Sepsis can be complicated by pathological vasodilation causing cardiovascular septic shock. The present study identifies that dysfunction of the RhoA/Rho-kinase (ROK) signaling pathway in vascular smooth muscle cells contributes to vasomotor dysfunction in sepsis. ROK inhibits myosin light chain phosphatase (MLCP) through Thr855 phosphorylation of MYPT, the 130 kDa myosin binding regulatory subunit of MLCP. MLCP dephosphorylates myosin light chain (LC20) inhibiting the acto-myosin cross-bridge cycling underpinning vasoconstriction or platelet contraction. ROK dependent MLCP inhibition therefore favours vasoconstriction and can be indexed by Thr855-MYPT phosphorylation. Western blot analysis identified that Thr855 phosphorylation of MYPT was reduced in arterial segments isolated from a murine caecal ligation and puncture model of sepsis. Wire myography yielded data consistent with reduced contractile responses to thromboxane A2 receptor stimulation, high [K+] mediated depolarisation and direct PKC stimulation. α1-adrenergic receptor mediated vasoconstriction was similar in septic and non-septic animals, possibly reflecting the multiple mechanisms by which α1- adrenergic agonists elicit vasoconstriction. Certain bacterial toxins and inflammatory mediators have the potential to attenuate ROK signaling; our data suggest therapeutic benefit of agents that promote MLCP inhibition or which vasoconstrict independent of the RhoA/ROK pathway.

Current vasopressor strategies for septic shock primarily rely upon catecholamine therapy. However, there is interest in administration of vasopressin, an
endogenous vasopressor inappropriately suppressed in septic shock. It is proposed that vasopressin mediates Ca$^{2+}$ sensitisation through ROK mediated inhibition of MLCP, however, neither vasopressin dependent Ca$^{2+}$ sensitisation nor Thr855 MYPT phosphorylation have been directly identified. In permeabilised rat caudal artery Ca$^{2+}$ sensitisation was observed and found to depend at least partly upon PKC signaling. In contrast, stimulation with arginine vasopressin (AVP) was not associated with Thr855 MYPT phosphorylation despite the ROK inhibitor Y27632 attenuating vasopressin dependent vasoconstriction. These data support clinical evaluation of vasopressin therapy targeted to cases of septic shock arising from organisms capable of producing toxins, which neutralise RhoA/ROK. Furthermore, the data suggest either an MLCP independent vasoconstrictor role for ROK or ROK independent action of Y27632.

Sepsis is also complicated by coagulopathy promoting both thrombosis and haemorrhage. Data regarding platelet function in sepsis is equivocal and absent in the specific subset of patients with septic shock. Recognising the importance of platelet contraction in thrombus formation and suggested similarities between vascular smooth muscle and platelet contraction we aimed to identify whether platelet contractile dysfunction contributed to impaired platelet aggregation in septic shock. Whole blood impedance aggregation was impaired in patients suffering from septic shock; deficits in aggregation correlated with illness severity. Impaired platelet aggregation was not associated with biochemical evidence of contractile dysfunction: neither Ser19-LC$_{20}$ nor Thr855-MYPT phosphorylation differed between septic shock and non-septic patients. These data indicate that
therapeutic strategies to restore platelet function in septic shock might more profitably focus on platelet adhesion and secretion.

These studies identify MLCP inhibition as a potential therapeutic avenue to ameliorate vascular smooth muscle, but not platelet, function in septic shock. Vasopressin might provide particularly effective vasoconstriction when targeted to cases of septic shock associated with disrupted ROK/MLCP integrity.
### LIST OF COMMON ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>(c)AMP</td>
<td>(cyclic) adenosine monophosphate</td>
</tr>
<tr>
<td>(c)GMP</td>
<td>(cyclic) guanosine monophosphate</td>
</tr>
<tr>
<td>[Ca^{2+}]</td>
<td>calcium concentration</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine trisphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CLP</td>
<td>caecal ligation and puncture</td>
</tr>
<tr>
<td>CPI-17</td>
<td>PKC-potentiated inhibitory protein of 17 kDa</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>effective concentration for 50% response</td>
</tr>
<tr>
<td>Emax</td>
<td>maximal effective concentration</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine trisphosphate</td>
</tr>
<tr>
<td>IP_{3}(R)</td>
<td>inositol trisphosphate (receptor)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N\textsubscript{ω}-Nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LC_{20}</td>
<td>20kDa myosin light chain</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type (Ca\textsubscript{v}1.2) calcium channel</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
</tr>
<tr>
<td>MYPT</td>
<td>myosin phosphatase targeting protein</td>
</tr>
<tr>
<td>NO(S)</td>
<td>nitric oxide (synthase)</td>
</tr>
<tr>
<td>NSCC</td>
<td>non-selective cation channel</td>
</tr>
<tr>
<td>PKA/B/C</td>
<td>protein kinase A/B/C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TBS (- T)</td>
<td>tris buffered saline (with Tween 20)</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TRAP</td>
<td>thrombin receptor activating protein</td>
</tr>
<tr>
<td>TxA\textsubscript{2}</td>
<td>thromboxane A\textsubscript{2}</td>
</tr>
<tr>
<td>VSM(C)</td>
<td>vascular smooth muscle (cell)</td>
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Calcium desensitisation is associated with loss of vasopressor sensitivity in a murine model of polymicrobial sepsis
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<th>Contributed to study design, supervised data acquisition, analysis of results and editing of manuscript</th>
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Attenuated platelet aggregation in patients with septic shock is independent from the activity state of myosin light chain phosphorylation or a reduction in the Rho kinase-dependent inhibition of myosin light chain phosphatase

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