ANTIPLATELET AND ANTIHYPERTENSIVE ACTIONS OF RIDOGREL

A Thesis

Submitted to the College of Graduate Studies and Research
in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacology
University of Saskatchewan, Saskatoon

By
Dale William Quest
Fall 1997

© copyright Dale William Quest, 1997. All rights reserved.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-23968-3
UNIVERSITY OF SASKATCHEWAN
College of Graduate Studies and Research

SUMMARY OF DISSERTATION
Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by
Dale William Quest

Department of Pharmacology
University of Saskatchewan

Fall 1997

Examining Committee:
Dr. N.C. Rawlings
Dr. J.S. Richardson
Dr. T.W. Wilson
Dr. M.D. Evered
Dr. J.R. McNeill
Dr. V. Gopalakrishnan
Dr. R.J. Herman
Dr. J. Tuchek

Dean/Associate Dean, Dean's Designate, Chair
College of Graduate Studies and Research
Chair of Advisory Committee, Department of Pharmacology
Supervisor, Department of Pharmacology & Medicine
Department of Physiology
Department of Pharmacology
Department of Pharmacology
Department of Pharmacology & Medicine
Department of Pharmacology

External Examiner:
Dr. R.Z. Lewanczuk, M.D., Ph.D., F.R.C.P.C.
362 C Clinical Wing
Heritage Medical Research Centre
Division of Endocrinology and Metabolism
University of Alberta
Edmonton, Alberta  T6G 2S2
Antiplatelet and Antihypertensive Actions of Ridogrel

Thromboxane plays a principal role in platelet aggregation, and is a potent vasoconstrictor. It is now understood, that the receptor for thromboxane (TP receptor) responds to multiple endogenous ligands. By itself, inhibiting the synthesis of thromboxane does not exclude functional responses mediated by TP receptors. Ridogrel is an investigational drug with dual activities as an antagonist of TP receptors, and an inhibitor of thromboxane synthase.

The experiments reported in this thesis demonstrate that, while single doses of ridogrel ranging 1.5 to 125 milligrams per kilogram reduced serum (platelet-derived) thromboxane B\textsubscript{2} synthesis, they did not affect urinary thromboxane B\textsubscript{2} excretion or blood pressure, in twelve-week-old Spontaneously Hypertensive Stroke-Prone Rats (SHR-SP). In contrast, repeated doses of ridogrel 12.5 milligrams per kilogram every twelve hours for seven days, reduced renal thromboxane B\textsubscript{2} synthesis without affecting renal prostacyclin synthesis. Blood pressure was reduced despite an increase in plasma renin activity, and evidence of depressed natriuresis. During the peak stage at which hypertension develops in the male SHR-SP model, an imbalance favouring TP receptor-mediated events over vasodilator and anti-aggregatory prostanoid activity, appears to have a role in maintaining elevated arterial pressure.

The pressor response to the TP receptor agonist U46,619 was shifted to the right following ridogrel pre-treatment, confirming TP receptor antagonism. More importantly, the pressor responses to angiotensin II and noradrenaline were also blunted by ridogrel pre-treatment. The reduction in vasopressor responses to those agents, following repeated administration of ridogrel, suggests modulation of vascular responses by a more generalized mechanism, or that a TP receptor-mediated component contributes to the contractile responses associated with a number of vasopressor agents. The combined actions of a TSI-TPA drug such as ridogrel, have the potential to improve endothelial dysfunction, and attenuate TP receptor-mediated contraction. Furthermore, the redirection of arachidonic acid metabolism toward the synthesis of prostacyclin, and the enhancement of intracellular cAMP generation, would favour vasorelaxation, potentially reduce shear stress and increase erythrocyte deformability in the micro-circulation. It remains a matter of speculation whether any or all of these effects culminate in a net lowering of vascular resistance, sufficient to explain the antihypertensive response to extended treatment with ridogrel.

Untreated twelve-week old SHR-SP excretes less urinary thromboxane B\textsubscript{2} than normotensive Wistar-Kyoto or Sprague-Dawley rats, suggesting that reduced renal thromboxane A\textsubscript{2} synthesis is a compensatory response to hypertension: a compensatory response which ridogrel augments.

An especially attractive prospect is that TSI-TPA drugs may provide anti-platelet activity while lowering blood pressure. A subset of patients may derive additional benefit from the potential antiasthmatic, uricosuric and gastric-protective effects of ridogrel.
PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Pharmacology
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5E5
ABSTRACT

Thromboxane plays a principal role in platelet aggregation, and is a potent vasoconstrictor. It is now understood, that the receptor for thromboxane (TP) responds to multiple endogenous ligands. Inhibiting the synthesis of thromboxane does not prevent functional responses mediated by TP receptors. Ridogrel is an investigational drug with dual activities as an antagonist of TP receptors, and an inhibitor of thromboxane synthase.

Single doses of ridogrel ranging 1.5 to 125 milligrams per kilogram reduced serum (platelet-derived) thromboxane B_2 synthesis, they did not affect urinary thromboxane B_2 excretion or blood pressure, in twelve-week-old Spontaneously Hypertensive Stroke-Prone Rats (SHR-SP). In contrast, repeated doses of ridogrel 12.5 milligrams per kilogram every twelve hours for seven days, reduced renal thromboxane B_2 synthesis without affecting renal prostacyclin synthesis. Blood pressure was reduced despite an increase in plasma renin activity.

The pressor response to the TP receptor agonist U46,619 was shifted to the right following ridogrel pre-treatment, confirming competitive TP receptor antagonism. More importantly, the pressor responses to angiotensin II and noradrenaline were also blunted by ridogrel pre-treatment. The reduction in vasopressor responses to those agents, following repeated administration of ridogrel, suggests modulation of vascular responses by a more generalized mechanism, or that a TP receptor-mediated component contributes to the contractile responses caused by a number of vasopressor agents. The dual actions
of a thromboxane synthase inhibitor/TP receptor antagonist (TSI-TPA) drug such as ridogrel, have the potential to improve endothelial dysfunction, and attenuate TP receptor-mediated contraction. Furthermore, the redirection of arachidonic acid metabolism toward the synthesis of prostacyclin, and the enhancement of intracellular cAMP generation, would favour vasorelaxation, reduce shear stress and increase erythrocyte deformability in the micro-circulation. It remains to be proven whether any or all of these effects explain the antihypertensive response to extended treatment with ridogrel.

Untreated twelve-week old SHR-SP excrete less urinary thromboxane B₂ than normotensive Wistar-Kyoto or Sprague-Dawley rats, suggesting that reduced renal thromboxane A₂ synthesis is a compensatory response to hypertension; a compensatory response which ridogrel augments.

An especially attractive prospect is that TSI-TPA drugs may provide anti-platelet activity while lowering blood pressure. A subset of patients may derive additional benefit from the potential antiasthmatic, uricosuric and gastric-protective effects of ridogrel.
ACKNOWLEDGEMENTS

I have many to thank for their support. Ms. Fran McCauley is a dear friend, and her moral support was as indispensable as her technical expertise. Mr. R. I. (Bob) Wilcox deserves due credit for the proper performance of every mechanical and electronic device in the department — he is a master of improvisation, and a nice guy. I respectfully acknowledge the support and guidance provided by my thesis advisory committee. My sincere thanks to Dr. M. Evered (cognate advisor), Dr. R. J. Herman, Dr. J. R. McNeill, Dr. V. Gopalakrishnan (departmental advisors), and Dr. J. S. Richardson (Graduate Chairman). Financial support was gratefully received from The Heart & Stroke Foundation of Saskatchewan and the College of Medicine Graduate Scholarship Awards Committee. Thank-you Ortho-Janssen Pharmaceutica Inc., for generously supplying ridogrel. Thanks to Debbie Brown for taking very good care of the animals, whose lives we are so deeply indebted to.

Most of all, I have my supervisor to thank. Dr. Thomas W. Wilson has made a career of facilitating the careers of others who have come to him with interests in pharmacology and medicine. He has been a friendly, patient and generous mentor; and has endured personal expense and sacrifice to advance my scholarship. I am much indebted to him. This simple acknowledgement can only begin to express the depth of my gratitude for his commitment, encouragement and example. At the least, I will champion his pride and respect for the reputation and future of the University of Saskatchewan. It is to Dr. Tom Wilson and the University of Saskatchewan that I dedicate this work.
DEDICATION

This thesis is dedicated to

Dr. Tom Wilson — The University of Saskatchewan
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERMISSION TO USE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABREVIATIONS</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xxii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Eicosanoids</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Biochemistry of arachidonic acid metabolism</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Pharmacology of prostanoid inhibition</td>
<td>10</td>
</tr>
<tr>
<td>1.1.2.1 General introduction</td>
<td>10</td>
</tr>
<tr>
<td>1.1.2.2 TP receptors</td>
<td>11</td>
</tr>
<tr>
<td>1.1.2.3 PGHS inhibitors: aspirin</td>
<td>15</td>
</tr>
<tr>
<td>1.1.2.3.1 Aspirin pharmacology</td>
<td>15</td>
</tr>
<tr>
<td>1.1.2.3.2 Aspirin in cardiovascular disease</td>
<td>17</td>
</tr>
<tr>
<td>1.1.2.3.3 Factors which limit the utility of aspirin</td>
<td>18</td>
</tr>
<tr>
<td>1.1.2.4 Other therapeutic agents considered for preventing vascular occlusive events</td>
<td>19</td>
</tr>
</tbody>
</table>
1.1.2.5 Specific inhibitors of thromboxane synthase ...... 21
1.1.2.6 Specific antagonists of TP receptors .................. 21
1.1.2.7 Combined or dual-acting TSI-TPA agents .......... 22

1.1.3 Renal prostanoid physiology ................................ 25
1.1.4 Hypertension .................................................. 27

1.2 Experimental models of essential hypertension ............ 29
1.2.1 Stroke-prone spontaneously hypertensive rat (SHR-SP) .. 29
1.2.2 Considerations in using the Wistar Kyoto rat and the Sprague-Dawley rat as normotensive controls ............... 36

1.3 Therapeutic potential for drugs with anti-thrombotic and anti-hypertensive activity .................................. 37

1.4 Ridogrel ............................................................. 44
1.4.1 Structure and activity ......................................... 46
1.4.2 Pharmacokinetic/pharmacodynamic profile ............... 46
1.4.3 Summary of in vitro and animal research findings .......... 47
1.4.4 Summary of clinical experience .............................. 51

2. PRESENT INVESTIGATION ........................................ 55
2.1 General introduction ............................................. 55

2.2 Aim of this study .................................................. 55
2.2.1 Hypotheses, predictions and approaches ................ 55

3. MATERIALS .......................................................... 58
3.1 Subjects .................................................................................................................. 58

3.1.1 In vitro whole blood studies ........................................................................... 58

3.1.2 In vivo rat experiments ...................................................................................... 58

3.2 Equipment .............................................................................................................. 59

3.2.1 Tail-cuff systolic blood pressure apparatus ................................................. 59

3.2.2 Direct blood pressure measurement apparatus ......................................... 60

3.2.3 Osmolality equipment ...................................................................................... 60

3.2.4 Autoanalyzer for electrolyte and creatinine assay .................................... 60

3.2.5 Scintillation counter for quantifying beta decay ........................................ 60

3.2.6 Scintillation counter for quantifying gamma emission .................................. 61

3.2.7 Whole blood platelet aggregometer ............................................................... 61

3.2.8 Metabolism cages ............................................................................................ 61

3.2.9 Inhalational anesthetic apparatus .................................................................... 61

3.2.10 Gravimetric equipment ................................................................................... 62

3.2.11 Numbered animal identification equipment ............................................. 62

3.3 Chemicals ............................................................................................................... 62

4. ANALYTICAL METHODS .......................................................................................... 64

4.1 Radioimmunoassay of thromboxane B₂ in serum and urine .......................... 64

4.2 Radioimmunoassay of urinary 6-keto-PGF₁α ................................................... 64

4.3 Radioimmunoassay to determine plasma renin activity .................................. 64

4.4 Urine and plasma osmolality .............................................................................. 65

4.5 Urine and serum sodium and creatinine determinations ............................ 65
5. BASELINE COMPARISONS BY AGE AND STRAIN ..................... 70

5.1 Introduction ......................................................................... 70
5.2 Protocol .............................................................................. 70
5.3 Statistical methods ............................................................ 72
5.4 Results .............................................................................. 72
5.5 Discussion ......................................................................... 75

6. WHOLE BLOOD STUDIES OF THROMBOXANE B₂ AND PLATELET AGGREGATION ........................................ 78

6.1 Introduction ......................................................................... 78
6.2 Protocols ............................................................................ 78

6.2.1 Inhibition of whole blood capacity to generate thromboxane B₂ during spontaneous clotting ...................... 78

6.2.2 Inhibition of whole blood platelet aggregation ............... 79
6.2.3 Statistical methods ............................................................ 80
6.2.4 Results ............................................................................ 81
6.2.5 Discussion ....................................................................... 84

7. SINGLE DOSE RIDOGEREL: DOSE-RESPONSES IN RATS .... 89

7.1 Introduction ......................................................................... 89
7.2 Protocol .............................................................................. 89
7.3 Statistical methods ............................................................ 92
7.4 Results .............................................................................. 93
7.5 Discussion ......................................................................... 98
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8.1</td>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td>8</td>
<td>8.2</td>
<td>Protocol</td>
<td>103</td>
</tr>
<tr>
<td>8</td>
<td>8.3</td>
<td>Statistical methods</td>
<td>104</td>
</tr>
<tr>
<td>8</td>
<td>8.4</td>
<td>Results</td>
<td>104</td>
</tr>
<tr>
<td>8</td>
<td>8.5</td>
<td>Discussion</td>
<td>110</td>
</tr>
<tr>
<td>9</td>
<td>9.1</td>
<td>Introduction</td>
<td>119</td>
</tr>
<tr>
<td>9</td>
<td>9.2</td>
<td>Protocols</td>
<td>119</td>
</tr>
<tr>
<td>9</td>
<td>9.2.1</td>
<td>Maximum subpressor dose-response to infused angiotensin II in rats</td>
<td>119</td>
</tr>
<tr>
<td>9</td>
<td>9.2.2</td>
<td>Effect of ridogrel on pressor responses to a graded dose infusion of angiotensin II</td>
<td>122</td>
</tr>
<tr>
<td>9</td>
<td>9.2.3</td>
<td>Ridogrel effects on pressor responses to bolus injections of the TP receptor agonist U46,619, norepinephrine and angiotensin II, in anaesthetized rats</td>
<td>123</td>
</tr>
<tr>
<td>9</td>
<td>9.3</td>
<td>Statistical methods</td>
<td>124</td>
</tr>
<tr>
<td>9</td>
<td>9.3.1</td>
<td>Maximum subpressor dose-response to infused angiotensin II</td>
<td>124</td>
</tr>
<tr>
<td>9</td>
<td>9.3.2</td>
<td>Effect of ridogrel on pressor responses to a graded dose infusion of angiotensin II</td>
<td>124</td>
</tr>
<tr>
<td>9</td>
<td>9.3.3</td>
<td>Ridogrel effects on pressor responses to bolus injections of the TP receptor agonist U46,619, norepinephrine and angiotensin II, in anaesthetized rats</td>
<td>125</td>
</tr>
</tbody>
</table>
9.4 Results .................................................................................................................. 125

9.4.1 Maximum suppressor dose-response
to infused angiotensin II ........................................ 125

9.4.2 Effect of ridogrel on pressor responses
to a graded dose infusion of angiotensin II .............. 127

9.4.3 Ridogrel effects on pressor responses
to bolus injections of the TP receptor agonist U46,619,
norepinephrine and angiotensin II, in anaesthetized rats ... 127

9.5 Discussion regarding responses to pressor agents
in anaesthetized rats ......................................................... 130

10. CONCLUSIONS ................................................................................................. 134

11. FUTURE DIRECTIONS ...................................................................................... 137

12. REFERENCES ...................................................................................................... 138

12. APPENDICES ..................................................................................................... 176
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Age-dependent strain differences in body weight, food intake and water consumption: SHR-SP v. WKY rats</td>
</tr>
<tr>
<td>5.2</td>
<td>Urinary prostanoid profile @ age 6-weeks and 16-weeks: SHR-SP v. WKY rats</td>
</tr>
<tr>
<td>5.3</td>
<td>Strain differences in urine concentration @ 16-weeks: SHR-SP v. WKY rats</td>
</tr>
<tr>
<td>6.1</td>
<td>Results of probit analysis: inhibition of clot thromboxane B₂ generation, comparing the effects of ridogrel and indomethacin</td>
</tr>
<tr>
<td>6.2</td>
<td>Results of probit analysis: inhibition of clot thromboxane B₂ generation, comparing the effects of ridogrel and furegrelate</td>
</tr>
<tr>
<td>6.3</td>
<td>Results of probit analysis: inhibition of platelet aggregation, comparing the effects of ridogrel and indomethacin</td>
</tr>
<tr>
<td>7.1</td>
<td>Results of acute ridogrel doses on <em>ex vivo</em> serum thromboxane B₂ generation</td>
</tr>
<tr>
<td>7.2</td>
<td>Results of acute ridogrel doses on plasma renin activity</td>
</tr>
<tr>
<td>8.1</td>
<td>Comparison of baseline with post-treatment indirect systolic blood pressure measurements between groups</td>
</tr>
<tr>
<td>8.2</td>
<td>Ridogrel dose-dependent inhibition of <em>ex vivo</em> clot generation of thromboxane B₂</td>
</tr>
<tr>
<td>8.3</td>
<td>Ridogrel dose-dependent enhancement of plasma renin activity</td>
</tr>
<tr>
<td>8.4</td>
<td>Comparison of sodium intake, sodium excretion, and cumulative sodium balance in SHR-SP rats pretreated with ridogrel 12.5 mg/kg v control</td>
</tr>
</tbody>
</table>
Table 9.1  Results of strain-dependent maximum subpressor angiotensin II dose-finding experiments .................................. 126

Table 9.2  Mean arterial pressure dose-response to angiotensin II infusions in SHR-SP rats pretreated with ridogrel 12.5 mg/Kg v. control SHR-SP given vehicle alone ........................................... 128
## LIST OF FIGURES

| Fig. 1.1 | Major pathways of the arachidonic acid cascade .......... | 2 |
| Fig. 1.2 | Nonenzymatic free radical-catalzed rearrangements of arachidonic acid to form isoprostanes [8-epi-prostaglandins D, E and F] .................................................. | 3 |
| Fig. 1.3 | The cellular acyltransferase system ..................... | 4 |
| Fig. 1.4 | Specific and nonspecific stimuli affecting acylhydrolase-mediated release of free arachidonic acid from membrane glycerophospholiids ..................... | 6 |
| Fig. 1.5 | Phospholipase pathways involved in release of arachidonic acid from membrane glycerophospholiids .. | 7 |
| Fig. 1.6 | Some biologically active metabolites of arachidonic acid, their synthesis results from metabolism by multiple pathways ............................................................... | 10 |
| Fig. 1.7 | Interactions between platelets and other platelets, platelets and the endothelium, and endothelium-mediated vascular smooth muscle tone ........................................ | 14 |
| Fig. 1.8 | Platelet-vessel interactions mediating focal vasomotion and thrombo-embolism .......................... | 20 |
| Fig. 1.9 | Synergism between thromboxane synthase inhibition and TP receptor antagonism in vivo ................ | 23 |
| Fig. 1.10a | Localization of eicosanoid production along the length of the nephron ........................................ | 26 |
| Fig. 1.11 | Age-related salt retention in the SHR-SP strain .......... | 30 |
| Fig. 1.12 | Effects of dietary protein and salt on blood pressure development and stroke incidence in the SHR-SP strain .. | 33 |
| Fig. 1.13a | Comparison of blood pressure development and stroke incidence between stroke-resistant and stroke-prone strains of SHR .......................................................... | 35 |
Fig. 1.14a Eicosanoid pathways: Pharmacologically unattenuated situation ................................................................. 38
Fig. 1.14b Eicosanoid pathways: Effects of PGHS inhibition .......................... 39
Fig. 1.14c Eicosanoid pathways: Effects of thromboxane synthase inhibition ......................................................... 40
Fig. 1.14d Eicosanoid pathways: Effects of TP receptor antagonism ................................................................. 41
Fig. 1.14e Eicosanoid pathways: Effects of combination or dual-acting TSI-TPA drugs ......................................................... 42
Fig. 1.15 Chemical structures of ridogrel and examples of other dual-acting TSI-TPA compounds ......................................................... 45
Fig. 5.1 Systolic blood pressure comparisons by age and strain:
SHR-SP v. WKY rats ................................................................. 77
Fig. 6.1a,b,c Whole blood platelet aggregation responses, as they appear in the software graphic display window ......................................................... 82
Fig. 6.2 Inhibition of thromboxane B₂, and inhibition of platelet aggregation: concentration-response curves comparing the effects of ridogrel and indomethacin ......................................................... 83
Fig. 6.3 Comparing in vitro concentration-response curves for ridogrel inhibition of clot thromboxane B₂ generation, between rat and human whole blood ......................................................... 86
Fig. 7.1 Skeleton protocol for single dose ridogrel experiments ........ 91
Fig. 7.2 Results from single dose studies with ridogrel:
(a) pretreatment body weight, (b) pretreatment Na⁺ intake .......... 94
Fig. 7.3 Results from single dose studies with ridogrel:
(a) group means for systolic blood pressure,
(b) group means for heart rate ......................................................... 95
Fig. 7.4 Results from single dose studies with ridogrel:
(a) group means for urine thromboxane B₂,
(b) group means for urine flow rate ......................................................... 95
Fig. 7.5 Results from single dose studies with ridogrel:
(a) group means for ex vivo serum thromboxane B₂,
(b) group means for plasma renin activity ................................. 97

Fig. 7.6 Changes in plasma renin activity following furosemide
in rats pre-treated with vehicle or the thromboxane
synthase inhibitor furegrelate .................................................. 101

Fig. 7.7 Relative changes in blood pressure, urinary prostanoids
and plasma renin activity by ridogrel dose ............................... 102

Fig. 8.1 Group comparisons of indirect systolic blood pressures
before and after 7 days of treatment .......................................... 106

Fig. 8.2 Comparison of blood pressure changes (Day#7 SBP minus
Day#0 SBP) across treatment groups, normalized to the
within group baselines ............................................................ 108

Fig. 8.3 Blood pressure changes for individual rats in
the control and ridogrel 12.5 mg/kg groups ......................... 109

Fig. 8.4 Treatment group comparison of ex vivo whole blood
clot generation of thromboxane B₂ at Day#7 ...................... 111

Fig. 8.6 Treatment group comparison of ex vivo plasma
angiotensin I generation rate (Plasma Renin Activity) ........ 113

Fig. 8.7 Superimposed boxplots showing the change in 24 hour
urinary 6-keto-PGF₁α excretion over the 7 day treatment
period, comparing vehicle and ridogrel treated rats ........... 114

Fig. 8.8 The ratio of urinary sodium excretion
over sodium intake, in relation to change in blood
pressure, comparing baseline v. Day#7 in one set of vehicle
and one set of ridogrel 12.5 mg/kg treated rats ................. 116

Fig. 9.1 In addition to their direct actions, several vasoactive
hormones stimulate arachidonic acid turnover .................. 120

Fig. 9.2 Dose-response curves showing the effect of
angiotensin II on mean arterial pressure in SHR-SP
rats pretreated with ridogrel v. control .............................. 129
Fig. 9.3 Areas under the systolic pressor-response curves, comparing the responses to three agonists in ridogrel-pretreated vs vehicle-treated SHR-SP ................................................................. 131

Fig. 9.4 Example of approach to measuring the area under the systolic pressor-response to a bolus injection of norepinephrine 10 µg/kg ................................................................. 133
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine; serotonin</td>
</tr>
<tr>
<td>20:4,ω6</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>20:5,ω3</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>20:3,ω6</td>
<td>dihomo-γ-linolenic acid</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid; arachidonate; 20:4,ω6 5,8,11,14-eicosatetraenoic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adeny1-5’t-pyrophosphoric acid; adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adeny1-5’t-triphosphoric acid; adenosine triphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin; antidiuretic hormone</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>β-TG</td>
<td>beta thromboglobulin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-monophosphate; cyclic adenosine monophosphate.</td>
</tr>
<tr>
<td>COX</td>
<td>cyclo-oxygenase</td>
</tr>
<tr>
<td>cps</td>
<td>centipoise, unit of viscosity</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P₄₅₀</td>
</tr>
</tbody>
</table>
CYP4A  cytochrome P\textsubscript{450-4A}

DiHET\textsubscript{E}  dihydroxyeicosatrienoic acid

DNA  deoxribonucleic acid

DP  prostaglandin D-type receptor designation (IUPHAR 1994)

EDHF  endothelium-derived hyperpolarizing factor

EDRF(NO)  endothelium-derived relaxing factor; putatively nitric oxide (NO)

ELAM  E-selectin cell adhesion molecule

EP  prostaglandin E-type receptor designation (IUPHAR 1994)

EpETrE  epoxyeicosatrienoic acid

FP  prostaglandin F-type receptor designation (IUPHAR 1994)

g  relative centrifugal force

gp\textsubscript{IIb/IIIa}  integrin glycoprotein platelet receptor for fibrinogen and calcium channel

GMP-140  integrin of P-selectin family

GTP  guanosine-5'-triphosphoric acid; guanosine triphosphate

HETE  hydroxyeicosatetraenoic acid

HHT  12-hydroxy-heptadecatrienoic acid

HpE\textsubscript{TE}  hydoperoxysicosatetraenoic acid

HUVEC  human umbilical vein endothelial cells

ICAM  intracellular adhesion molecule

i.p.  intraperitoneal injection
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>intravenous route of administration</td>
</tr>
<tr>
<td>IP</td>
<td>prostaglandin I-type (prostacylin) receptor designation (IUPHAR 1994)</td>
</tr>
<tr>
<td>kg.</td>
<td>kilogram</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>LX</td>
<td>lipoxin</td>
</tr>
<tr>
<td>mg.</td>
<td>milligram</td>
</tr>
<tr>
<td>min.</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mL.</td>
<td>millilitre(s)</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>ng.</td>
<td>nanogram(s)</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide; putative endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>oxo-ETE</td>
<td>ketoecosatetraenoic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor (group of related substances)</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>prostaglandin F$_{2\alpha}$; 15-trihydroxyecosatetraenoic acid</td>
</tr>
<tr>
<td>PGG$_2$</td>
<td>prostaglandin G$_2$; 9,11-epidioxy-15-hydroperoxyecosatetraenoic acid</td>
</tr>
<tr>
<td>PGH$_2$</td>
<td>prostaglandin H$_2$; 9,11-epidioxy-15-hydroxyecosatetraenoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PGHS</td>
<td>prostaglandin H synthase; cyclooxygenase-hydroperoxidase enzyme complex</td>
</tr>
<tr>
<td>PGHS&lt;sub&gt;1&lt;/sub&gt;</td>
<td>prostaglandin H synthase type 1</td>
</tr>
<tr>
<td>PGHS&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin H synthase type 2</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RVR</td>
<td>renal vascular resistance</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley rat</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SHR-SP</td>
<td>stroke-prone spontaneously hypertensive rat</td>
</tr>
<tr>
<td>TP</td>
<td>thromboxane-type receptor designation (IUPHAR 1994)</td>
</tr>
<tr>
<td>TPA</td>
<td>thromboxane receptor antagonist</td>
</tr>
<tr>
<td>TSI</td>
<td>thromboxane synthase inhibitor</td>
</tr>
<tr>
<td>TSI-TPA</td>
<td>dual-acting compound: thromboxane synthase inhibitor and thromboxane receptor antagonist</td>
</tr>
<tr>
<td>TxA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>TxB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>thromboxane B&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>μ</td>
<td>micro quantity</td>
</tr>
<tr>
<td>VCAM</td>
<td>soluble circulating vascular adhesion molecule</td>
</tr>
<tr>
<td>vWf</td>
<td>von Willebrand factor group of multimers (types: plasma, subendothelial and megakaryocyte)</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto rat</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

Appendix A  Nutritional monograph for Prolab R-M-H 3000 rodent chow, Agway Ltd., Syracuse, NY.

Appendix B  Validation of indirect tail-cuff systolic blood pressure technique by comparison with direct carotid arterial catheter systolic blood pressure measurements.

Appendix C  Illustration of the Chrono-Log® Lumi-Aggregation system used for the in vitro whole blood platelet aggregation studies.

Appendix D  Illustration of the Nalgene® metabolic cages, including modifications.

Appendix E  Radioimmunoassay of thromboxane B2 in serum and urine.

Appendix F  Radioimmunoassay of urinary 6-keto-PGF1α.

Appendix G  Radioimmunoassay technique used for determining the rate of angiotensin I generation in plasma (plasma renin activity).
1. INTRODUCTION

This thesis examines the effect of ridogrel, an investigational drug that appears to prevent platelet aggregation and lower blood pressure through its actions on the eicosanoid system.

1.1 Eicosanoids

Eicosanoids are 20-carbon metabolites. Eicosanoid synthesis involves the oxygenation of polyunsaturated fatty acids. Virtually any polyunsaturated fatty acid which contains a 1,4-pentadienyl structural element is a potential substrate for one or more enzymatic pathways (Figure 1.1), or non-enzymatic rearrangement catalyzed by reactive free radical interactions (Figure 1.2). However, arachidonic acid represents by far the major substrate for eicosanoid synthesis in omnivorous species, including rats and humans. Only exceptional or deficient diets incorporate for example, 20:3, ω6 (dihomo-γ-linolenic acid) and 20:5, ω3 (eicosapentaenoic acid) substrates, relative to 20:4, ω6 (arachidonic acid) in proportions sufficient to cause a significant shift toward alternative, and generally less biologically active series of eicosanoids (Dyerberg et al, 1978; Bordet, Guichadant & Lagarde, 1990; Horrobin, 1995).

1.1.1 Biochemistry of Arachidonic Acid Metabolism

Basal cytosolic concentrations of free arachidonic acid are vanishingly low. Once liberated, it is rapidly re-esterified into membrane phospholipids by an avid cellular acyltransferase system (Figure 1.3)(Irvine, 1982; Piomelli & Greengard, 1990). Factors such as hypoxia can delay reincorporation of arachidonate into phospholipids, because acyltransferase activity requires hydrolysis of adenyln-5′-triphosphoric acid (ATP)(Schlondorff
Figure 1.1 Major Pathways of the Arachidonic Acid Cascade

Arachidonic acid is a common substrate for three major metabolic pathways:

I. Prostaglandin H synthase (PGHS) catalyzes formation of cyclic endoperoxides which can subsequently be metabolized to the classic prostaglandins D, E and F, or to thromboxanes and prostacyclins.

II. Cytochrome P450s generate monoxygenase and epoxygenase metabolites, e.g., hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids.

III. Lipoxygenases produce several metabolites, e.g., HETEs, diHETEs, di-OH-HETEs, leukotrienes, lipoxins and hepoxilins. Many are mediators of inflammation and hypersensitivity.
Figure 1.2  Nonenzymatic free radical-catalyzed rearrangements of arachidonic acid to form isoprostanes [8-epi-prostaglandins of the D, E and F series]

I.  The initial step in the formation of an isoprostane from arachidonic acid, is the generation of a lipid free radical, by the abstraction of a proton from one of the methylene-interrupted carbon-carbon bonds (C7, C10 or C13).

II.  The above example, shows a free radical attack at C10.  The relative proportion of isomers formed, depends at which of the three alternative positions proton abstraction occurs.

III.  The lipid radical is converted to a peroxyl radical by reaction with molecular oxygen, to yield an endoperoxide.

IV.  Until quenched by an antioxidant molecule, the free radical chain reaction can continue to propagate isoprostane formation in situ from the hormone-responsive pool of arachidonate.  Isoprostanes can remain stable acylated at the sn-2 position of membrane phospholipids.

R* symbol represents any free radical

From Lawson & Maxey (1996) with permission, ©Cayman Currents.
Figure 1.3  The cellular acyltransferase system

Two mechanisms for release of free arachidonic acid are shown:

1). Phospholipase C (PL-C) cleaves the phosphate-ester bond to release diacylglycerol (DAG) from phosphoinositol. Sequential lipases mediate release of free arachidonic acid.

2). Phospholipase A2 (PLA2) catalyzes direct hydrolysis to free arachidonic acid from the sn-2 position of membrane phospholipids.

The cellular acyltransferase system is the opposing mechanism. It is responsible for the rapid reacylation of free arachidonic acid, which restores the basal cytosolic concentration of arachidonic acid to zero.

et al, 1985). The availability of free arachidonate is a rate-limiting step for eicosanoid biosynthesis, and is dependent upon deacylation from its esterified state. Phospholipase activation mediates its transient mobilization from membrane phospholipids, cholesterol esters, triglycerides, diglycerides or monoglycerides (Dennis, 1987; Habenicht et al, 1990; Dennis et al, 1991; Wellner et al, 1991). Specific stimuli to evoke phospholipase activity include certain neurotransmitters, and endocrine and local hormones which activate GTP-binding-protein-linked receptors. Activation can also be triggered by non-specific membrane perturbations of physiologic relevance eg., hæmodynamic shear-stress, or in response to an ischaemic or traumatic insult (Figure 1.4). A substantial amount of arachidonic acid is esterified to glycerophospholipids at the sn-2 position. Membrane phospholipids are thought to be a major source of mobilizable arachidonic acid. However, only a small amount is liberated even after maximal hormone stimulation; thus it appears that the hormone responsive pool is small compared to the total phospholipid-bound content of arachidonate (Needleman et al, 1979; Schwartzman et al, 1981).

Some pools of arachidonate are invulnerable to activated lipase. Lipocortins are endogenous peptide inhibitors of arachidonate release. They are synthesized in response to glucocorticoids. Lipocortins sterically hinder phospholipase activity, by binding to the phospho-lipid substrate (Blackwell et al, 1983; Davidson et al, 1987). Arachidonate avidly binds to both intra-cellular and extracellular protein. Fractions bound by other than ester-linkage do not comprise a hormone-responsive source of substrate for eicosanoid synthesis (Lands, 1979).

There are three major pathways for hormone stimulated release of membrane-bound arachidonate (Figure 1.5). It has been proposed that for different tissues, the activation of specific acylhydrolase systems correspond to different stimuli. Accordingly, each system may subsequently liberate arachidonate from discrete lipid pools (Schwartzman et al, 1981; Monaco, 1982). In several cell types, alternate pathways for eicosanoid metabolism co-exist, and may be linked to a specific phospholipase. For example, platelets synthesize thromboxane
Figure 1.4 Specific and nonspecific stimuli affecting acylhydrolase-mediated release of free arachidonic acid from membrane phospholipids

Specific stimuli include endocrine, paracrine and autocrine mediators, e.g., angiotensin II, AVP, thromboxane, arachidonic acid itself and drugs such as furosemide.

Nonspecific stimuli include shear stress, trauma and ischaemic insult.

Inhibitors include 15-hydroperoxyeicosatetraenoic acid (15-HpETE) and lipocortins synthesized in response to glucocorticoids.
Figure 1.5  Phospholipase pathways involved in release of arachidonic acid from membrane glycerophospholipids

Note: For illustrative purposes, different phospholipids are shown as substrate for each acylhydrolase pathway. However, each has a range of substrate specificities. Furthermore, there are multiple isoforms of these phospholipases. Among them, the tissue distribution, mechanisms of activation and preferential substrates vary.
A₂ via the prostaglandin H synthase (PGHS₁) pathway, and 12-hydroperoxyeicosatetraenoic acid via the 12-lipoxygenase pathway. Platelet agonists are known to activate the phospholipase C/glyceride lipase, or the phospholipase A₂ pathways differentially. Since both pathways liberate arachidonate, it is likely that as yet undefined mechanisms function at the subcellular level to integrate the respective pathways for cycling and metabolism of arachidonic acid (Neufeld & Majerus, 1983).

Some of the final bioactive products of arachidonic acid result from subsequent cross-metabolism by another eicosanoid pathway (Figure 1.6). Accordingly, inhibitors that are highly selective for either pathway can limit the synthesis of these mediators. The effects on renal, vascular and immune function, of metabolites formed by PGHS-CYP450 and PGHS-lipoxygenase cross-metabolism of arachidonic acid, are emerging themes in eicosanoid research (Neufeld & Majerus, 1983; Escalante et al, 1989; Schwartzman et al, 1989; Romero et al, 1991; Sakair et al, 1993; Woodward et al, 1993).

Arachidonate mobilized in one cell, can serve as precursor for eicosanoids formed in neighbouring cells. Adipocytes synthesize prostacyclin from arachidonate released from endothelial cells (Parker et al, 1989). Likewise, isolated renal collecting tubules require exogenous arachidonate for arginine vasopressin-stimulated prostaglandin E₂ production (Kirschenbaum et al, 1982). Thus, the in vivo response may depend upon cooperative endothelial or interstitial cell cycling of arachidonate (Jaisser et al, 1989). More important than arachidonate itself, in regard to the vascular actions of thromboxane synthase inhibitors, is the diffusion of the cyclic endoperoxide intermediates of platelet PGHS origin to the vascular wall. Vascular synthesis of prostaglandins and prostacyclin has been shown to rely significantly, upon this platelet-dependent source of substrate (Needleman, Wyche & Raz, 1979; Marcus et al, 1980; Bertele & De Gaetano, 1982; Deckmyn et al, 1983; Schafer, Crawford & Gimbrone, 1984; Chesterman et al, 1986; Datar, McCauley & Wilson, 1987; Gresele et al, 1987; Nowak & FitzGerald, 1989; Badahman & Wilson, 1994; Yang et al, 1994).
Figure 1.6 Some biologically active metabolites of arachidonic acid, their synthesis results from metabolism by multiple pathways

Example shows sequential action by both prostaglandin H synthase and cytochrome P450 enzymes, to form active metabolites. Several arachidonic acid metabolites remain potential substrates for subsequent further metabolism by other eicosanoid pathways. Research interest and current knowledge regarding the biological actions of these cross-metabolites of arachidonic acid is in its infancy.

cytochrome P450 (CYP), cortical collecting ducts (CCD), 5,6-epoxyeicosatrienoic acid [5,6-EpETRE] (5,6-EET), prostaglandin E-type receptors (EP), prostaglandin H synthase [cyclooxygenase/peroxidase complex] (PGHS)
Generally, eicosanoids are synthesized and immediately released in response to a stimulus, and are not stored. Exceptions include isoprostanes, isoleukotrienes, Bisothromboxanes and the cytochrome P_{450}-4A (CYP4A) ω-hydroxylation, cytochrome P_{450}-2C (ω/ω-1)-oxidation products. The formation of isoprostanes can occur \textit{in situ}, and the monooxygenase products can be reacylated. Both are relatively stable esterified at the sn-2 position, and can be cleaved preformed following phospholipase activation. The isoprostanes are biologically active at prostanoid receptors, thus even following extended prostaglandin H synthase (PGHS) inhibition, or in the presence of thromboxane synthase inhibitors, neurohormonal stimuli could potentially release mediators which produce prostanoid actions. Their prostanoid activity could however, be abrogated by prostanoid receptor antagonists (Morrow et al, 1990, 1992, 1994; Takabashi et al, 1992; Fukunaga et al, 1993a, 1993b; Kayganich-Harrison et al, 1993; Yura et al, 1993; Gopaul et al, 1994; Longmire et al, 1994; Lynch et al, 1994; Proudfoot et al, 1995).

Apart from localized neurohormonal and non-specific release of substrate, physiologic changes e.g., sharp increases in renal perfusion pressure, have been shown to evoke mass release (mainly arachidonate and adrenic acid) from the lipid-rich storage granules of renal medullary interstitial cells, into the prehepatic circulation. This mechanism has been shown to subserve a renal-hepatic vasodepressor system, which requires both renal and hepatic cytochrome P_{450} metabolism (Muirhead et al, 1989; 1991a; 1991b; 1994; Karlström et al, 1990; Göthberg et al, 1991; Cowley, 1994).

1.1.2 Pharmacology of Prostanoid Inhibition

1.1.2.1 General introduction

Interventions which attenuate the function of the prostaglandin H synthase complex, thromboxane synthase enzyme and the TP receptor, will be considered along side the mechanistic consequences of these interventions.
With regard to arachidonic acid metabolism, the term *prostanoid* is encountered frequently in the literature, but an adequate definition is not. The term seems often to pertain to eicosanoids that are synthesized directly from arachidonate via the cyclooxygenase (PGHS) pathway. Alternatively, structural isomers are catalyzed by free radicals, resulting in concentrations which may have both physiological and pathological implications, and which activate receptors previously thought to be specific for metabolites derived from the cyclooxygenase pathway (Morrow et al, 1990, 1992, 1994; Takabashi et al, 1992; Fukunaga et al, 1993a, 1993b; Kayganich-Harrison et al, 1993; Yura et al, 1993; Gopaul et al, 1994; Longmire et al, 1994; Lynch et al, 1994; Proudfoot et al, 1995).

Hydroxy- and epoxy- prostaglandins appear to have an important role in renal function. They are cyclooxygenase-dependent metabolites, formed prior or subsequent to the metabolism of arachidonate by monooxygenase or epoxygenase enzymes (Escalante et al, 1989; Neufeld & Majerus, 1983; Schwartzman et al, 1989; Romero et al, 1991; Sakair et al, 1993; Woodward et al, 1993).

In this thesis, regardless of the mechanism by which the substance has been synthesized, the term *prostanoids* will refer to endogenous mediators which, on a pharmacological basis, appear to activate prostanoid receptors. These have been divided into five types: labelled TP, IP, EP, FP and DP. The EP receptors are of several subtypes (Coleman et al, 1994).

1.1.2.2 TP receptors

Whether or not the TP receptor has subtypes remains controversial (Halushka et al, 1986, 1989; Ogletree & Allen, 1992; Buzzard et al, 1994). Nüsing et al (1993) cloned and characterized a gene for the TP receptor from a human genomic library. It was found to be present as a single copy per haploid. The gene spanned 15kb, and contained three exons, separated by two introns. Reverse transcription and polymerase chain reaction, performed
using primers flanking intron 2, and poly (A)* ribonucleic acid (RNA) from three cell types (1) MEG-01 human megakaryocyte leukemia cells, 2) human placenta, 3) mesangial cells), amplified a single 173bp DNA fragment. This seemed to suggest that no alternative splicing of the coding region occurs, and that a single TP receptor type exists (Coleman et al, 1994). Ware and coworkers (Raychowdhury et al, 1995) have since demonstrated the existence and differential distribution of two splicing variants affecting the cytoplasmic tail of the human TP receptor. A 343 amino acid receptor is expressed predominantly by platelets, whereas the expression of a 369 amino acid receptor predominates in endothelium. Both splice variants abound in placenta. The question remains whether the two variants confer differences in ligand binding, G-protein binding specificity for signal transduction, or differential desensitization in respective tissues.

There are several TP receptor agonists and antagonists. They range from analogues of TXA₂ or PGH₂, to compounds which bear no obvious structural resemblance. Among these, some have shown discrepant agonist-antagonist activity between platelet versus vascular tissue. Some of the compounds are known to have partial agonist activity, and may simply re-demonstrate established knowledge that TP receptors are coupled to more than one G-protein-linked signal transduction pathway (Knezevic et al, 1993), that post-receptor coupling differs between vascular smooth muscle and platelets, and that platelet TP receptor activation can stimulate post-receptor pathways for amplification of the aggregatory process that do not exist in vascular smooth muscle (Siess et al, 1986; Brass et al, 1987; Sage & Rink, 1987; Nakaro et al, 1988; Arita et al, 1989). Takahara et al.(1990) showed that platelet shape change and the secretory/aggregatory responses to TXA₂, are respectively mediated by two or more receptor-effector systems. Webb et al.(1993) showed, that three different TPA radioligands (BMS180,291(02); SQ29,548 and GR32,191), all produced the same Bₘₐₓ, and none influenced the off-rate of the others. Their conclusions largely dismiss the likelihood of two distinct binding sites, and are consistent with the notion that different states of the same receptor are present on human platelets. It has yet to be elucidated, precisely how more than one guanine-nucleotide binding protein or coupling efficiencies, are involved in transducing
the binding event into a signal which ultimately results in platelet shape-change/adhesion, or secretion/aggregation or both. There is some indication that the competitive nature of an agonist elicits both biochemical responses, whereas relatively irreversible antagonism may inhibit aggregation without mediating parallel changes in platelet shape (Okwu et al, 1992).

Tissue-specific responses could also reflect differences in the relative selectivity of the compounds, in the presence of other prostanoid receptors (Coleman, Smith & Narumiya, 1994). It should also be considered, that tissue-specific TP receptor activation can secondarily stimulate synthesis and release of mediators which, by their autocrine or paracrine action, are capable of modifying the TP receptor-mediated response. For example, the TP receptor agonist U-46619 stimulates G_q-coupled activation of phospholipase C, increased IP_3, influx of extracellular calcium, and mobilization of calcium from intracellular stores (Rink & Sage, 1990). Consequently, platelets aggregate, while in vascular smooth muscle the phosphorylation of myosin light chain kinase is coupled to contraction (Dorn et al, 1992; Miki et al, 1992; Yamagishi et al, 1992). *In vivo*, both tissue responses occur in proximity (Figure 1.7), where platelet-vascular interactions are in part dependent on the functional status of the vascular endothelium, which also expresses TP receptors. Paracrine interactions between heterogeneous cell types is manifest, where autacoids including 12- and 15-HpETE, PAF, 5-HT, adenine nucleotides, prostanoids and radicals, are released by activated platelets, and then modify the vascular smooth muscle contractile response. Concurrently, autacoids of leukocyte and vascular origin e.g., LTs, LXs, NO, PGI_2, PGE_2, ET, EDHF and radicals, are capable of modifying platelet activity (Moncada, 1982; Loots & De Clerck, 1993; Ambrosio et al, 1994; Lin et al, 1994).

The major endogenous TP receptor agonists are the endoperoxides prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2), thromboxane A_2 (TxA_2), prostaglandin F_2α (PGF_2α) (Sammuelson, 1987; Coleman et al, 1994), 20-hydroxy endoperoxides (Escalante et al, 1989; Schwartzman et al, 1989; Carroll et al, 1992), and the 8-epi-prostaglandins (Escalante et al,
Figure 1.7 Interactions between platelets and other platelets, platelets and the endothelium, and endothelium-mediated vascular smooth muscle tone

Activated platelets release several factors in addition to those shown. At sites where platelets are activated, thrombin is also formed. Receptors for many of the platelet factors are expressed on the endothelium. When the endothelium is functionally intact, the net effect is anti-aggregatory and vasorelaxant. At sites where the endothelium is dysfunctional or absent, platelet-derived substances may cause contraction and promote thrombosis.

vascular smooth muscle (VSM); endothelial cells (EC), thrombin receptor (T); platelet-activating factor (PAF); serotonin (5-HT); adenylyl-5'-triphosphoric acid (ATP); adenylyl-5'-pyrophosphoric acid (ADP); thromboxane A₂ (TXA₂); cyclic endoperoxides (cyclic Eps); endothelium-derived relaxing factor (EDRF(NO)); prostacyclin (PGI₂); guanylyl cyclase (GC); adenylyl cyclase (AC); cyclic adenylyl-3', 5'-monophosphate (cAMP); cyclic guanosine-3', 5'-monophosphate (cGMP).
1989; Schwartzman et al, 1989; Morrow et al, 1992; Takahashi et al, 1992; Fukunaga et al, 1993; Longmire et al, 1994; Morrow et al, 1994). The synthesis of all but the last can be attenuated by inhibiting the prostaglandin H synthase enzyme (PGHS).

1.1.2.3 PGHS inhibitors: emphasis on aspirin

There are two isoforms of the PGHS glycoprotein complex: PGHS₁ is constitutively expressed in virtually all tissues, whereas PGHS₂ is normally expressed in few tissues, but can be induced in several (Frölich, 1997). The two isoforms share 75% primary structural identity. All of the residues identified as important for their catalytic functions are conserved (Smith & Bell, 1978; Schlondorff & Ardaillou, 1986; Bonvalet, Pradelles & Farman, 1987).

PGHS catalyzes two reactions. The first is the cyclooxygenase reaction, which results in cyclization of C-8 to C-12 of the arachidonic acid backbone, to form 9,11-epidioxy-15-hydroperoxyeicosatetraenoic acid (PGG₂). A distinct, neighbouring catalytic site, having reduced glutathione-dependent peroxidase activity, then converts PGG₂ to the 9,11-epidioxy-15-hydroxy derivative, PGH₂. Both endoperoxides have a half-life of about 5-minutes, and both are about 3-fold more potent agonists than thromboxane A₂ at vascular and platelet TP receptors (Samuelsson, 1987; Mayeux et al, 1988).

1.1.2.3.1 Aspirin pharmacology

Aspirin acetylates a number of proteins, including PGHS. The acetylation of the serine residue, places a bulky group which interferes with arachidonate access to the cyclooxygenase active site. Aspirin is the only nonsteroidal anti-inflammatory drug (NSAID) that irreversibly inactivates cyclooxygenase activity, and so affords an advantage over other NSAIDs as an antiplatelet agent. Given orally in low doses, it is absorbed into the pre-hepatic circulation, where it acetylates proteins. Platelets, which predominantly synthesize thromboxane from the endoperoxide products of PGHS are not nucleated, and thus cannot
synthesize new enzyme. Endothelial cells synthesize mainly prostacyclin, and in further contrast to platelets, are able to synthesize new enzyme to re-establish PGHS activity over approximately 2-6 hours. Aspirin undergoes extensive first-pass hydrolysis, so that very little of the acetylated form of the drug reaches the systemic circulation. All of the metabolites (which are relatively weak reversible inhibitors of PGHS) are rapidly cleared in urine and bile. The low-dose strategy seeks to suppress platelet PGHS, while sparing as much as possible endothelial PGHS. Effective antiplatelet therapy requires 70-95% inhibition of the maximal capacity of platelets to produce TXA₂ (Patscheke, 1990; Buerke et al, 1995). Endothelial PGHS is unavoidably suppressed, generally by 10-30%, in patients receiving doses of 70-80mg per day. Within this dose range, ex vivo platelet aggregation was reduced by only 15-70% (Weksler et al, 1985). It is important to recognize that up to 80 per cent of the substrate from which endothelial cells synthesize prostacyclin, and perhaps other endothelium-derived mediators of vasorelaxation, are endoperoxides of platelet origin (Schafer, Needleman, Wyche & Raz, 1979; Marcus et al, 1980; Bertele & De Gaetano, 1982; Deckmyn et al, 1983; Crawford & Gimbrone, 1984; Chesterman et al, 1986; Datar, McCauley & Wilson, 1987; Gresele et al, 1987; Nowak & FitzGerald, 1989; Badahman & Wilson, 1994; Yang et al, 1994). Clinical studies have shown that combined TSI-TPA, increases bleeding time more than TSI or TPA alone, or low dose aspirin (Patrono, 1990; Gresele et al, 1991); and indeed that low dose (300mg.) aspirin increases bleeding time more than high dose (3g.) aspirin (O'Grady & Moncada, 1978; Amezcu et al, 1979). The reduction in vascular prostacyclin synthesis by aspirin, probably reflects a reduction in substrate availability to prostacyclin synthase, as well as inhibition of vascular PGHS.

Aspirin also has an cyclooxygenase-independent antithrombotic action. Aspirin enhances the expression of endothelial apyrase. These ectonucleotidases function to prevent accumulation of proaggregatory concentrations of extracellular adenine nucleotides e.g., ADP and ATP (Cheung, Visser & Bakker, 1994).
1.1.2.3.2 Aspirin in cardiovascular disease

Completed and ongoing trials indicate that aspirin's antiplatelet activity reduces the incidence of fatal and non-fatal vascular-occlusive events (Anti-platelet Trialists' Collaboration, 1988; ISIS-2 Collaborative Group, 1988; U.K. TIA Aspirin Trial, 1988; Physician's Health Study Research Group, 1989; RISC Study, 1990; Stroke Prevention in Atrial Fibrillation Study Group Investigators, 1990; Dutch TIA Trial Study Group, 1991; European Working Group on Critical Leg Ischemia, 1991; Swedish Aspirin Low-dose Trial, 1991). There is no conclusive evidence that chronic aspirin is protective in patients with chronic stable coronary heart disease syndromes, but aspirin does confer risk reduction to patients who have unstable coronary artery disease, including unstable angina and myocardial infarction (ISIS-2, 1988; Carins et al, 1992). Aspirin interferes with the synthesis of thromboxane A₂, but it does not affect the accumulation or actions of other relevant proaggregatory vasospastic mediators of platelet-driven thrombosis. Thromboxane A₂, serotonin, ADP, platelet-activating factor, thrombin, tissue factor and oxygen-derived free radicals, accumulate at sites of endothelial injury, particularly at sites of atherosclerotic plaque fissuring and ulceration. As mentioned above, PGHS inhibition may be too broad a sword, because it impairs the synthesis of prostacyclin. Prostacyclin plays a beneficial role, by its antiaggregatory and vasodilator actions. Furthermore, the benefits of even low-dose aspirin can be off-set by potential gastric-mucosal toxicity. It has been estimated that among 1000 patients treated for a year, low-dose (70-100mg/day) aspirin, given for secondary prevention of cardiovascular complications, prevents five deaths and ten non-fatal cardiovascular events. When given for primary prevention, only three fewer cardiovascular-related deaths, and six non-fatal events are expected among 1000 patients treated with aspirin. In either case, aspirin produced 30 clinically evident gastrointestinal bleeds per 1,000 patients treated. This indicates reduced benefit with the same bleeding risk, when prescribed as a primary preventive approach (Silagy et al, 1993).
1.1.2.3.3 Factors which limit the utility of aspirin

The mechanisms by which low-dose aspirin leads to gastroduodenal mucosal injury are complex. Aspirin has a pKa of about 3, while intra-gastric pH is often less than 3.5. In the stomach, aspirin is predominantly non-ionized, and rapidly diffuses into mucosal epithelial cells, where the cytosolic pH is >7. Consequently, the ions become trapped within the cell, leading to a back-diffusion of protons that undermines the mucous-bicarbonate barrier. Enteric coated aspirin reduces gastric injury, but reduces the amount of non-hydrolyzed aspirin absorbed. This is a Catch-22, since the key is to achieve levels of acetylated aspirin in the pre-systemic circulation, to result in permanent inactivation of platelet PGHS. Furthermore, since platelet inhibition will be greatest in blood vessels underlying the gastric epithelium, that is where protracted bleeding time effects will be greatest. Duodenogastric reflux of bile containing active salicylate metabolites may also contribute to gastric mucosal injury. PGE₂ and PGI₂ inhibit, while thromboxane modestly stimulates, gastric acid production (Bunce & Spraggs, 1988; Clayton et al, 1988). Inhibition of gastric mucosal PGHS will decrease gastric pH. Because PGE₂ and PGI₂ enhance cAMP-mediated mucous-bicarbonate secretion, they defend against mucosal injury at concentrations below those required to inhibit gastric acid secretion (Prichard et al, 1989; Nascitz et al, 1990; Silagy et al, 1993).

Aspirin triggers bronchospasm in about ten per cent of asthmatics (Hoigné & Szczeklik, 1992). Before considering all of these limitations, aspirin is so inexpensive that no alternative therapy could compare favourably on the basis of cost:benefit alone (Wilson et al, 1996). Limitations considered, there is an impetus to develop agents that can be used chronically, and interfere with multiple mediators of vasospasm and thrombus development (Willerson, 1996).
Other therapeutic agents considered for preventing vascular occlusive events

Orally active thrombin receptor antagonists such as agatroban are being investigated as one such alternative (Lefkovits & Topol, 1994). Dipyridamole is an antiplatelet and vasodilator agent which acts to enhance intracellular cAMP. Currently, it is used in combination with warfarin to prevent embolization from vascular synthetic surfaces (Antiplatelet Trailists Collaboration, 1988). Sulfinpyrazone is a uricosuric agent which inhibits the platelet release reaction, platelet-vascular adhesion and prostanoid synthesis, and was shown to reduce the incidence of sudden death after myocardial infarction (ANTURANE Reinfarction Trial Research Group, 1980). Orally bioavailable antagonists of platelet-glycoprotein IIb/IIIa receptors (anti-integrins), xemlofiban for example, are also being evaluated (Lefkovits, Plow & Topol, 1995). Unlike ticlopidine's interactions with gp IIb/IIIa, the mechanism of the anti-integrins is specific, and more precisely understood (DiMinno et al, 1985). Irrespective of the metabolic pathway responsible for initiating platelet activation, xemlofiban targets the final common pathway of platelet aggregation, by preventing the interplatelet bridging of fibrinogen between gp IIb/IIIa receptors and the associated calcium transient. It does not, however, prevent platelet release of adeny1-5'-pyrophosphoric acid (ADP), serotonin or endoperoxides, which can exert potentially detrimental effects on vascular tone, regardless of whether platelet aggregation per se is inhibited (Figure 1.8a,b). In contrast to TSI-TPA drugs such as ridogrel and DT-TX30SE, such anti-integrins also fail to enhance the vascular synthesis of thromboresistant PGI2/PGD2 (Cairns, 1996). A long-acting orally bioavailable agent, with comparable and combined inhibitory activities for thromboxane synthase (IC50 1.2 μM) and human platelet cAMP phosphodiesterase (IC50 6.4 μM), has been reported to produce a synergistic enhancement of the antiaggregatory and antithrombotic actions of prostacyclin, along with a significant blood pressure reduction (Martinez et al, 1992).
Platelet attachment and release of mediators

**Figure 1.8** Platelet-vessel interactions mediating focal vasomotion and thromboembolism

(a) Localized vascular injury, particularly at sites of subendothelial exposure caused by balloon injury or atherosclerotic plaque fissuring and ulceration, causes platelets to adhere and degranulate. Degranulating platelets release high concentrations of thromboxane A$_2$ (TXA$_2$), serotonin (5HT), adenosine-5'-pyrophosphoric acid (ADP) and cyclic endoperoxides. Local accumulation of these potent agonists produce vasoconstriction, and amplify platelet aggregation.

From Hirsh, Campbell, Willerson & Hillis (1981) with permission ©Excerpta Medica Inc.

(b) Vital tissue micrograph, showing a small artery in which focal vasospasm produces a sausage-link effect. Platelet activation and vasoconstriction are localized to sites where the endothelium is absent. At site where the endothelium remains intact, the capacity to generate prostacyclin (PGI$_2$) from the endoperoxides released by activated platelets, and production of EDRF(NO), appear to prevent constriction and the extension of platelet thrombus beyond the site of vascular injury.

1.1.2.5 Specific inhibitors of thromboxane synthase

Selectively inhibiting thromboxane synthase, not only leaves vascular prostaglandin synthesis intact, but may further enhance its concentration, given that platelet endoperoxides are a putative substrate for vascular prostaglandin synthesis (Vermyleen et al, 1981; Vermyleen & Deckmyn, 1992; Gresele et al, 1994). However, the endoperoxide intermediates PGG₄ and PGH₂ are more potent TP receptor agonists than thromboxane A₂ itself (Kd 43nM vs 125nM for vascular smooth muscle, and Kd 45±2nM vs 163±21nM for washed human platelets), and the endoperoxides have a 10-fold longer half-life (5 minutes vs 30 seconds for thromboxane A₂)(Hamberg & Samuelsson, 1975; Samuelsson, 1987; Mayeux et al, 1988). This has been suggested to limit the benefits of only attenuating thromboxane synthase (Bertelé et al, 1981; Hornby & Skidmore, 1982; FitzGerald, Reilly & Pederson, 1985; Patscheke, 1985; Gresele et al, 1991).

1.1.2.6 Antagonists of TP receptors

TP receptor antagonists prevent PGG₂, PGH₂, TXA₂, PGF₂α, 20-OH PGG₂, PGH₂ and 8-epi-prostaglandin activation of TP receptors, without altering the relative concentrations of eicosanoids synthesized. Tesfamariam and Ogletree (1995) showed that prolonged treatment with the TP receptor antagonist ifetroban, normalized the impaired endothelium-dependent relaxations that distinguished SHR from their WKY controls. Importantly, the authors demonstrated that the improved endothelium-dependent relaxation was not accompanied by a consequent reduction in blood pressure. Unlike TSIs, TPAs fail to enhance endogenous production of antiaggregatory vasodilator prostaglandins. Furthermore, most TPAs are competitive antagonists, such that at localized sites of platelet activation, endogenous agonists could reach overwhelming concentrations (Vermyleen & Deckmyn, 1992).
1.1.2.7 Combined or dual-acting TSI-TPA agents

Combining a thromboxane synthase inhibitor with a TP receptor antagonist offers a theoretical advantage. A TSI promotes redirection of endoperoxides toward potentially antiaggregatory, vasodilatory, natriuretic, gastric protective prostaglandins; while a TPA blocks the receptor activation due to the resultant accumulation of endoperoxides, and the actions of other potential agonists (Bertelé et al, 1981). Combined TSI-TPA treatment results in a significant rise in cAMP levels (Figure 1.9), which may contribute to the ability of these drugs to also inhibit the actions of thromboxane-independent mediators of platelet activation (Gesele et al, 1989; Hoet et al, 1990), and blunt the vascular smooth muscle tension responses to non-specific pressor substances as well. When indomethacin was added to dual TSI-TPA treatment, the rise in cAMP was attenuated, and the prolongation of template bleeding time was reduced. These observations suggest that re-orientation of prostaglandin endoperoxide toward synthesis of inhibitory prostaglandins, contributes to the efficacy of TSI-TPA drugs as antiplatelet agents (Gesele et al, 1987; Yao et al, 1990; Imura, Terashita & Nishikawa, 1990). The benefits of enhancing prostacyclin and PGI3 synthesis goes beyond that of simply balancing the effects of thromboxane. Prostacyclin inhibits aggregation and disperses existing aggregates (platelet-platelet interaction), at a much lower concentration than that required to inhibit adhesion (platelet-vessel wall interaction) (Vane & Botting, 1995). Platelets play an important role in maintaining endothelial integrity and haemostasis in normal individuals, and a fairly fixed requirement of 7-10 billion platelets per litre per day are expended for that purpose (Hanson & Slichter, 1985). Prostacyclin allows platelets to adhere and participate in the process of endothelial repair, while at the same time, preventing or limiting thrombus formation or extension beyond the site of vascular injury (Sixma, 1994).

5-Hydroxytryptamine (5-HT), adenine nucleotides and thromboxane A2 are released by platelets in high concentrations at sites of subintimal exposure, which may induce vasospastic flow reduction even before blood flow becomes mechanically obstructed by the formation of a thrombus (Figure 1.8a,b, on page 20)(De Clerck & Janssen, 1990). Spasm may
Figure 1.9  Synergism between thromboxane synthase inhibition and TP receptor antagonism in vivo

Thromboxane synthase is inhibited, while platelet PGHS (cyclooxygenase) remains intact and able to generate PGD₂ and diffusible endoperoxides, in lieu of thromboxane A₂ (TXA₂). The platelet endoperoxides are substrate for prostacyclin synthesis by adjacent endothelial cells, and since their direct agonist effects are blocked, the net effect is to increase both platelet and vessel wall cAMP generation. As would be expected, these TSI-TPA effects are further enhanced by cAMP-phosphodiesterase inhibition.

From Gresele et al (1991) with permission ©Elsevier Trends Journals
precipitate plaque rupture, or disrupt the dynamics of laminar coronary blood flow, resulting in thrombosis and infarction (Maseri et al, 1978; Zelinger et al, 1982; Cohen, Shepherd & Vanhoutte, 1983; Vincent et al, 1983; Braun & Schrör, 1990; Fiedler et al, 1990; Patscheke, 1990; Treasure et al, 1991; Treasure et al, 1992; Zeiher et al, 1993). Ridogrel, while neither an inhibitor nor an antagonist of 5-hydroxytryptamine, prevented platelet 5-HT-induced vasospasm by preventing the initial platelet activation involved in 5-HT release. This effect was mimicked by a TSI, but not a TP antagonist alone, and the benefits of ridogrel or TSI were prevented by indomethacin. Evidently, the enhancement of antiaggregatory/vasodilatory prostanoids, secondary to thromboxane synthase inhibition, is an important corollary in counter-acting this angiospastic phenomenon (Mayeux, Kadowitz & McNamara, 1989; De Clerck & Janssen, 1990; Loots & De Clerck, 1993).

Taddei & Vanhoutte (1993) demonstrated that endothelium-dependent contractions to endothelin-1 and endothelin-3 occurred in aorta from spontaneously hypertensive rats, and that a significant component of the contractile response was inhibited by a TSI (dazoxiben) or a TPA (SQ-29,548). Escalante et al (1989) showed that contractions of rat aortic rings elicited by 20-HETE, were inhibited completely by indomethacin, and reversed to a relaxation by the TPA agent SQ29,548. Schwartzman et al (1989) further demonstrated that the inhibitory effect of SQ29,548 on 20-HETE-evoked vasoconstriction, was associated with the appearance of labile metabolites with a half-life of approximately six minutes. The authors suggested that the vasoconstrictor metabolites were likely 20-hydroxy-PPG₂ and 20-hydroxy-PGH₂ (PGHS-dependent endoperoxides of 20-HETE). Many have shown that thromboxane synthase inhibition modulates the hypertensive response of acute and chronic increases in angiotensin II, and that concurrent treatment with a TPA further inhibits the contractile response (Purkerson et al, 1986; Welch, Wilcox & Dunbar, 1989; Mistry et al, 1990; Wilcox & Welch, 1990; Badahman & Wilson, 1994; Keen et al, 1997).
1.1.3 Renal Prostanoid Physiology

A principal role of PGHS products of AA metabolism in renal physiology is to modify the actions of other hormones or autacoids involved in the control of renal vascular tone, mesangial and glomerular function, and the handling of salt and water at various segments along the tubule. Renal eicosanoids contribute to blood pressure control by modulating renal sensitivity to renin-angiotensin and adrenergic pressor systems, amplifying the depressor kallikrein-kinin system, and dampening salt and water retentive mechanisms (Schlondorff & Ardaillou, 1986; Rump & Schollmeyer, 1989). Thromboxane inhibits, while prostacyclin stimulates, renin release (Berl et al, 1979; Baylis, 1987; Welch, Wilcox & Dunbar, 1989). Thromboxane potentiates tubuloglomerular feedback in the rat (Welch & Wilcox, 1992). Inhibition of PGHS activity in the absence of renal nerve stimulation, sympathoadrenal hormones, angiotensin II or AVP, has little effect on renal function (Zipser, 1985; Munger & Baylis, 1988). The release of vasodilator prostaglandins in response to renal vasoconstrictors such as angiotensin II, AVP, norepinephrine, platelet-activating factor and serotonin, is a highly relevant biologic response. It has important consequences for the preservation of renal perfusion under conditions of, or conditions that mimic, underperfusion (eg, radiocontrast-evoked release of adenosine, congestive heart failure, renal artery stenosis, intravascular volume depletion and salt depletion) in which the pressor hormone concentrations are enhanced (Baylis & Brenner, 1978; Yared, Kon & Ichikawa, 1985; Foidart & Mahieu, 1986; Goto, Jackson & Ohnishi, 1987; Walker, Brizze & Harrison-Bernard, 1988). NSAIDs which inhibit prostaglandin generation, can cause acute renal insufficiency under these conditions (Badr & Ichikawa, 1988).

The profile of eicosanoids that are generated, differs according to the zone and structure within the kidney, and prostanoid synthesis and metabolism varies along the nephron (Figure 1.10) (Frölich et al, 1978; Whorton et al, 1978; Morrison, 1986; Wilson & Carruthers, 1992). Sodium intake influences prostanoid interactions with intrarenal hormonal systems such as renin-angiotensin-aldosterone and kallikrein-kinin activity (Mullane & Moncada,
Figure 1.10 Localization of eicosanoid production along the length of the nephron

(a) Differential concentrations of PGHS (cyclooxygenase activity)
(b) Localization of lipoxygenase and cytochrome P₄₅₀ enzymes

proximal convoluted tubule (PCT), proximal straight tubule (PST),
thick ascending limb of Henle's loop (TALH), distal convoluted tubule (DCT)

From Morrison (1986) with permission © Excerpta Medica Inc.

1.1.4 Hypertension

The cause of essential hypertension is unknown, but likely involves interactions between genetic and environmental factors. Variations exist in the expression and severity of this disease, and perhaps in its underlying mechanisms. In a subset of humans with essential hypertension, PGHS inhibition results in salt retention, and NSAIDs have been shown to compromise the efficacy of established antihypertensive drug therapies other than calcium antagonists (Timarco, De Simone & Cuocolo, 1985; Stoff, 1986; Patruno & Dunn, 1987). There is growing evidence that endothelial dysfunction and platelet hyperresponsiveness exists, even in patients with uncomplicated hypertension, and contributes to the risk that they will experience cardiovascular complications (Blann et al, 1993; Cadwgan & Benjamin, 1993; Pedrinelli et al, 1994; Kloczko et al, 1995; Andrioli et al, 1996). The platelet-vessel interaction involves the expression of platelet, plasma and tissue adhesion factors (shape change, β-TG, vWF, fibronectin, vitronectin, gpIIb/IIIa and GMP-140) which cause platelets to begin to tumble along the vessel wall, adhere and degranulate. Haemorheological factors (shear and viscosity) are intimately involved in the platelet-vascular interaction. For example, during smooth laminar blood flow, the formed elements of the blood e.g., erythrocytes, are concentrated toward the centre of the lumen. Due to the greater mass of erythrocytes, when flow dynamics are disrupted, they function to disperse platelets, thus increasing platelet contact with the vessel wall (Lowe, 1994). Platelets contain three main types of granules: alpha, dense, and lysosomes. Activated platelets degranulate to release direct vasoconstrictor substances (eg., TXA₂, endoperoxides, serotonin) and mediators which amplify the thrombogenic process (eg., PAF and TXA₂). Chemotactic
factors stimulate adhesion and invasion of white cells (e.g., VCAM, ICAM), as well as substances (e.g., 12- and 15-HpETE), which inhibit production of vasodilator prostanoids (e.g., prostacyclin) (Lin et al, 1994). Severe hypertension is associated with more advanced stages of vascular disease. Advanced vascular disease is characterized by impaired responsiveness to endothelium-dependent vasodilators such as acetylcholine and bradykinin, by increased responsiveness to non-endothelium dependent vasoconstrictors, by platelet clumping and adhesion, by leukocyte infiltration into the myointima, and increased vascular resistance (Weissser et al, 1993; Lüscher & Dubey, 1995; Andrioli et al, 1996).

It seems remarkable that while aspirin appears to inhibit the synthesis of thromboxane by a theoretically less than optimal mechanism, it can reduce by a quarter the incidence of myocardial and cerebrovascular events occurring in patients with previous vascular disease. The degree to which any unifactorial intervention (blood pressure-lowering, lipid control, lifestyle modifications or diabetic control) impacts on mortality and morbidity, is likewise relative to the aggregate multifactorial risk profile of the population treated. The benefits of each intervention increase along a continuum of increasing clinical risk. The benefits of antiplatelet prophylaxis in apparently healthy subjects may be nonexistent or even negative, yet the benefits in patients with previous events is substantial. The question is whether benefits of an antiplatelet strategy exist intermediate between the healthy population and those with previous vascular events. Programs such as the Cardiovascular Risk Factor Reduction Unit (C.R.F.R.U.) in Saskatoon, may be best equipped to address this question, having the capacity to discern a patient population with known clinical risk associations for vascular disease prior to an event. In that context, the requirement for an agent to be highly efficacious and very well tolerated, becomes increasingly important as the probability of an event becomes more remote.
1.2 Experimental Models of Essential Hypertension

There are several genetically hypertensive rat strains. Spontaneously hypertensive rat models are used extensively for hypertension research, in part because of the contracted time span for development of a hypertensive syndrome that appears similar to human essential hypertension in several respects. The uniformity of body size, isogenetic background, ease of handling and cost, are additional factors contributing to their widespread use in research.

1.2.1 Stroke-prone Spontaneously Hypertensive Rat (SHR-SP)

Through selective inbreeding of offspring from SHR that subsequently succumbed to stroke, the SHR-SP was established in the early 70's at Kyoto University by Yamori and Okamoto (Yamori, 1984). In contrast to other genetically hypertensive rodent models, SHR-SP spontaneously develop hypertensive vascular lesions similar to human disease, e.g., they invariably develop hypertensive retinopathy (Ruchoux et al, 1990), left ventricular hypertrophy (Kihara et al, 1993), and cerebrovascular disease (Yamori, 1984). High blood pressure develops in SHR-SP in the presence of reduced plasma and blood volume. Packed cell volume is 5-6% higher than WKY, which in addition to the increased relative dynamic viscosity of plasma, is thought to contribute to flow resistance at the microvascular level. SHR-SP rats exhibit some degree of insulin resistance and glucose intolerance which correlates with weight gain. SHR-SP have low baseline serum cholesterol levels. This is due to reduced cholesterol synthesis, owing to impaired liver mevalonate 5-pyrophosphate decarboxylase activity (Sawamura, Nara & Yamori, 1992). If SHR-SP are maintained on a diet rich in fat and cholesterol, they develop sudanophilic myointimal fat deposits in the mesenteric, renal, coronary and cerebral-basilar arteries (Yamori, 1984). While sodium retention is greater in pre-hypertensive SHR-SP than age matched WKY, fractional sodium excretion approaches equilibrium, along with the age-related rise in blood pressure (Figure 1.11). Plasma renin, angiotensin II and aldosterone levels are normal or reduced, although it
Figure 1.11  Age-related salt retention in the SHR-SP strain

During the early phase of hypertension development, SHR-SP animals retained more salt than WKY rats. With increasing age and blood pressure, sodium excretion reaches equilibrium with sodium intake. Chemical renal sympathectomy actually increased sodium retention, suggesting that increased renal sympathetic tone is not a salt-thrifty mechanism.

has been demonstrated that intrarenal angiotensin II content is elevated (Taugner et al, 1982; Navar et al, 1986; Opie, 1994). Dietary salt-loading leads to a marked rise in blood pressure without intravascular volume expansion. While a rightward shift in the renal function curve is apparent, there remains controversy over whether increased renal vascular resistance is primary (leading to a compensatory increase in blood pressure), or whether it is an elicited response that parallels a primary rise in perfusion pressure, and thus protects the kidney (Cowley, 1992). Consistent with the hypothesis that central sympathoneuronal overactivity may be a primary pathogenetic determinant of the increasing vascular resistance that precedes the onset of hypertension, chronic moxonidine (a central sympatholytic I1-imidazoline selective agonist) completely normalized arterial blood pressure in SHR (Buccaefisco et al, 1995). In the obese spontaneously hypertensive rat (SHROB; Koletsky rat), which has been characterized as a model for human syndrome X, moxonidine not only moderated blood pressure, but also reduced the extent of renal injury, glucose intolerance, body weight, hyperinsulinemia, and moderated both cholesterol and triglycerides (Ernsberger et al, 1996). It is likely that the manner in which the kidneys participate as victim or villain varies over the course of developing hypertension. Renal vascular smooth muscle hyperplasia and microvascular rarefaction may be delayed, but progress nonetheless in the wake of the reduced blood pressure that follows attenuation of central sympathetic outflow or renal denervation (Kashgarian, 1990). Identification of a point mutation generating an amino acid substitution in the signal peptide of low affinity nerve growth factor receptor, provides further evidence that an abnormality of the sympathetic nervous system might be pathogenetic (Nemoto et al, 1994).

Our colony of SHR-SP originated from a SHR-SP/A3N breeding nucleus obtained from Dr. Carl Hansen, at the National Institutes of Health, Bethesda, MD. In contrast to the normative data published for this strain (Ogata et al, 1982; Yamori, 1984; IFFA CREDO, 1985; Vacher, Richer & Guidicelli, 1996), our rats seemed to follow a less malignant course of hypertension development, with a delayed onset, and with lower blood pressures in relation to age. A lower incidence of stroke was surmised, since no rats appeared to succumb by the
age of 20 weeks; the age at which our breeding stocks were routinely retired and euthanized. This raised concern that we had not sufficiently maintained selective pressure for the hypertensive phenotype. However, nutritional factors offer a more likely explanation (Figure 1.12a,b). Notably, the low sodium (0.44% w/w) and higher protein (22.5%) content of the commercial formula we provided as standard ration, can be compared to studies involving salt and protein manipulations by others (Matsuo & Nagaoka, 1981; Yamori, 1991). In rats receiving one per cent sodium chloride in drinking water, and/or a protein restricted diet of less than ten per cent, the onset of hypertension occured by the fourth week. These diet manipulations also provoked an accelerated slope of blood pressure development, and installation of the stabilized phase by 12 to 14 weeks of age at an average systolic pressure of 245 mm Hg. The near certainty of stroke by the twentieth week, limited the average life-span to 126±17 days. In contrast, SHR-SP on a reduced (<1%) sodium and high (24%) protein diet had an average life-span of 277±26 days. Sodium restriction delayed the onset of hypertension until the sixth to eighth week. Hypertension developed at a slightly reduced slope, and stabilized by 20 weeks at 212 ± 3 mm Hg. (Monograph supplied by Iffa Credo, 1984).

SHR-SP have a lower body weight in relation to age than SHR. In both strains, the initial (pre-hypertensive) vascular changes comprise reversible functional vasoconstriction of peripheral resistance vessels. Increased central sympathetic tone, altered regulation of intracellular cation concentrations and enhanced rate of vascular smooth muscle growth have been implicated. Although hyperplastic growth can be further accelerated by neurohormonal e.g., β-adrenergic stimulation, there is strong evidence that the arterial smooth muscle cells from SHR-SP harbour a primary genetic predisposition toward hypertrophic and proliferative growth. Yamori et al (1981) compared growth of aortic smooth muscle cells obtained from 1-month old rats. Cultured under conditions devoid of any blood pressure or humoral influence, Yamori et al (1981) found that the doubling time for SHR-SP cells was sixty percent of that required for cells obtained from normotensive Wistar Kyoto (WKY) rats. Markers for DNA and protein synthesis were elevated accordingly in cells from the
Figure 1.12  Effects of dietary protein and salt on blood pressure development and stroke incidence in the SHR-SP strain

(a) Salt-loading augments the development of hypertension in SHR-SP, but when potassium is substituted for sodium, the development of hypertension is attenuated. Neither treatment influences blood pressure in WKY rats.

(b) The combination of a low protein diet and modest salt supplementation, provoked development of higher blood pressure and greater incidence of stroke.

From Yamori (1984) with permission © Elsevier Science Publishers BV.
spontaneously hypertensive strain.

Endothelial-dependent vasorelaxation is impaired, and the vasculature is hyper-reactive to vasopressor stimulation (Brayden, Halpern & Brann, 1983; Mayhan, Faraci & Heistad, 1988; Mayhan, Faraci & Heistad, 1987; Tesfamariam & Halpern, 1988; Chatziantoniou, Daniels & Arendshorst, 1990; Diederich et al, 1990; Mayhan, 1990; Yang et al, 1991; Contard et al, 1993). Structural changes (vascular hypertrophy and hyperplasia) lag behind functional vasoconstriction, in their relative contribution to increased peripheral vascular resistance. By 12 to 14 weeks of age, the maximum potential for a vasodilator to attenuate resistance, is limited by the morphological changes, giving rise to the more stabilized hypertensive plateau which defines the established phase (Dietz, Schömig & Rascher, 1984; Tesfamariam & Halpern, 1988; Contard et al, 1993).

The incidence of stroke is closely related to the grade of development of hypertension (Figure 1.13a,b), and survival is extended by treatments effective at modulating blood pressure (Yamori & Horie, 1977; Gries, Kretzschmar & Neumann, 1989; Perzborn et al, 1990; Camargo et al, 1991). The predilection for cerebrovascular lesions to occur in the rheologically sluggish and mildly chronically hypoxic recurrent arterial branches of the anteromedial and occipital cortex or the basal ganglia, is similar to human studies. The medial smooth muscle cells of these small intracerebral arteries weaken, and allow extensive plasma leakage through the damaged endothelial cells. Thrombosis develops at sites of fibrinoid necrosis or within microaneurysms, thus a common pathogenetic process conspires toward cerebral infarct or hemorrhage (Yamori, 1984). SHR have increased platelet reactivity relative to normotensive WKY rats (as evidenced by higher basal and stimulated platelet \([\text{Ca}^{2+}]\), and higher sensitivity to thrombin-induced platelet aggregation). In contrast, SHR-SP, despite development of higher levels of blood pressure, have basal and stimulated platelet \([\text{Ca}^{2+}]\), and sensitivity to thrombin-induced aggregation that is similar to WKY (Ono et al, 1996) or even hyporesponsive (Tomita et al, 1989). Vascular fragility and susceptibility to rupture has been attributed in part, to defective cross-link formation in collagen and elastic
Figure 1.13  Comparison of blood pressure development and stroke incidence between stroke-resistant and stroke-prone strains of SHR

(a) Blood pressure becomes elevated more rapidly in the stroke-prone substrain, and among males in particular.

(b) Lifespan is conspicuously shorter in the stroke-prone males.

From Yamori (1984) with permission ©Elsevier Science Publishers BV.
fibres of the internal elastic lamina (Coutard & Osborne-Pellegrin, 1991).

1.2.2 Considerations in Using the Wistar Kyoto Rat and the Sprague-Dawley Rat as Normotensive Controls

WKY, SHR and SHR-SP are of common Wistar origin, each derived by selective breeding on the basis of tail-cuff systolic blood pressure. In addition to molecular evidence of genetic heterogeneity in Wistar-Kyoto rats, there is evidence that the normotensive WKY phenotype represents not only the lack of expression of pro-hypertensive traits, but also adaptive attributes which account for the normotensive condition.

The Charles River Sprague-Dawley outbred strain (COBS-CD(SD)) is phenotypically normotensive and less likely than the WKY inbred strain to have congenic abnormalities with respect to the SHR-SP hypertensive strain. It appears that the Charles River Sprague-Dawley has changed over the years. Compared to those distributed in the 1970's, the contemporary animal has a decreased lifespan that is principally attributed to disease and degenerative processes associated with fast growth and high body weight (Nohynek et al, 1993). In contrast to their inbred strains, which are generally priced according to age, the commercial suppliers routinely sell outbred rats at a cost adjusted to body weight, so that more profitable breeding practices would have favoured a high rate of food conversion to growth. For purposes of this research, the most appropriate choice between either phenotypically normotensive control strain would be arbitrary. Compared to WKY rats, the age-related body weight of the Sprague-Dawley rat is even larger in disproportion to SHR-SP, but both presented the problem of whether it was most relevant to age-match or weight-match controls to the SHR-SP. It was decided that the rats would be age-matched, even though by administration of an equivalent drug dose on a milligram per kilogram basis, the absolute dose given to control rats would be greater.
1.3 Therapeutic Potential for Drugs with Anti-thrombotic and Antihypertensive Activity

Hypertension in humans is associated with a number of cardiovascular complications. Notably, myocardial infarction has emerged as the leading complication in treated hypertensives. Platelets are hypersensitive to TXA₂/cyclic endoperoxide agonists, and TP receptors are increased following acute myocardial infarction. Prostacyclin provides an important counterbalancing effect in preventing coronary vasospasm and formation of platelet emboli (Cohen, Shepherd & Vanhoutte, 1983; Willerson et al, 1989; Dorn et al, 1990; Meredith et al, 1993). While aspirin is an inexpensive antiplatelet drug for preventive cardiovascular risk management, TSI-TPA drugs such as ridogrel offer theoretical advantages over aspirin as a long-term preventive strategy (Figure 1.14:a to e).

Even if aspirin was entirely effective in sparing vascular PGHS, it irreversibly attenuates platelet synthesis of endoperoxides. Platelet endoperoxides appear to account substantially as a source of substrate for vascular synthesis of prostacyclin. This has been demonstrated repeatedly by several distinct methodologies. The elegant microcirculation model reported by Chesterman et al (1988), used dual-isotope labelling of arachidonic acid ([³H] arachidonate incorporated into platelets, and [¹⁴C] arachidonate incorporated into human umbilical vein endothelial cells [HUVECs]). They showed that 40 to 80 per cent of vascular PGI₂ is derived from platelet endoperoxides. In fact, pretreatment of HUVECs with aspirin did not greatly impair prostacyclin synthesis in that system. This may suggest that, if endothelial prostacyclin synthesis depends upon the availability of platelet endoperoxides, the low-dose aspirin strategy is likely to compromise vascular PGI₂, regardless of whether vascular PGHS is spared. Similarly, Bordet and colleagues (1990) showed that when platelets were coincubated with endothelial cells, they produced less thromboxane and 1.89-fold more prostacyclin under basal conditions. Following thrombin stimulation, there was an 85 per cent reduction in thromboxane, while prostacyclin generation increased 358 per cent over that
Figure 1.14a: Eicosanoid pathways: Pharmacologically unattenuated situation.

Several arachidonic acid metabolites are potential agonists at vascular and platelet TP receptors. Prostaglandins (principally prostacyclin) and EDRF(NO), have an important role in counteracting TP receptor-mediated vasoconstriction and platelet aggregation. A relative imbalance favouring vasoconstrictor pro-aggregatory mediators over vasodilator/antiaggregatory mediators, may contribute to hypertension and vascular-occlusive complications.
**Figure 1.14h: Eicosanoid pathways: Effects of PGHS inhibitor.**

Inhibiting the prostaglandin \( H \) synthase complex prevents formation of thromboxane \( B_2 \) and its cyclic endoperoxide precursors. This is not necessarily optimal, even if as in the case of aspirin, PGHS inhibition is selective for platelets; platelet and vascular TP receptors remain exposed to non-PGHS-dependent agonists such as isoprostanes; platelet arachidonic acid metabolism is redirected toward lipoxygenase production of \( \text{HpETE} \), which can inhibit vascular prostacyclin synthesis; and if indeed the diffusion of platelet endoperoxide to the vessel wall constitutes a major source of substrate for vascular production of prostacyclin, then the low-dose aspirin strategy, despite its selectivity for platelet cyclooxygenase inhibition, will fail to preserve vascular PG synthesis.
Figure 1.14c: Eicosanoid pathways: Effects of thromboxane synthase inhibition.

More than preserve the vascular capacity to synthesize prostaglandins, they actually enhance it; but while thromboxane synthase inhibition prevents metabolism of cyclic endoperoxide intermediates to thromboxane, the accumulated endoperoxides are themselves potent agonists at platelet and vascular TP receptors.
Figure 1.14d: Eicosanoid pathways: Effects of TP receptor antagonist.

Blocking TP receptors prevents TP mediated vasoconstriction and platelet aggregation by prostanoid and non-prostanoid agonists. Although this approach does not compromise vascular prostaglandin production, neither does it enhance it.
Figure 1.14c: Eicosanoid pathways: Effects of a combination or a dual-acting TSI-TPA. Inhibiting thromboxane synthase in the presence of a TP receptor antagonist offers the theoretical advantage of enhancing diffusible platelet endoperoxides, while preventing platelet aggregation and vasoconstriction by those and other potential TP receptor agonists. The TP receptor antagonism prevents second wave amplification of the platelet aggregatory response and associated vasospasm, and the enhanced antiaggregatory vasodilator prostanoid production that results from concurrent thromboxane synthase inhibition, can function unopposed by TP activation.
generated by an equivalent population of endothelial cells in the absence of platelets. Nowalk & FitzGerald (1989) showed that TSI pretreatment greatly increased the concentration of PGI₂ metabolites emerging from template bleeding time wounds. Yang et al (1996) set up isometric vascular ring tension measurements in a platelet rich bath, and showed that platelet-activating stimuli evoked vascular contractions. In the presence of ridogrel, the same platelet-activating stimuli resulted in relaxation. Badahman & Wilson (1994) showed that in rats pretreated with either a TSI or a TPA, angiotensin II-induced vasoconstriction was reduced, while in rats pretreated with TSI and TPA agents combined, exogenous angiotensin II infusion resulted in a paradoxical net vasodilation. Fitzgerald and coworkers (1988), studied the effects of electrically-induced coronary thrombosis in a canine model. They found that TSI pretreatment alone resulted in PG endoperoxide accumulation, whereas the addition of TPA greatly reduced coronary thrombosis and spasm. The accumulation of cyclic endoperoxides, which can occupy and activate platelet and vascular wall TP receptors, can thus eliminate the benefit of suppressing thromboxane formation, and may explain the unreliability of TSIs to inhibit platelet aggregation (Bertelé et al, 1981; FitzGerald, Reilly & Pederson, 1985).

Thromboxane synthase inhibition results in diversion of endoperoxide metabolism, favouring synthesis of prostaglandins (e.g., PGI₂, PGE₂ and PGD₂) which are capable of stimulating adenylyl cyclase to increase cellular cAMP levels. Combining this action with TP receptor antagonism is important, because of the promiscuity of the TP receptor, which responds to other endogenous agonists, including the endoperoxide intermediates themselves. TSI-TPA drugs have the potential to block proaggregatory vasoconstrictor prostanoid actions at TP receptors, to restore functional integrity to the endothelium, and to enhance the production of antiaggregatory vasodilator prostanoids which activate intracellular cAMP generation. This increased production of prostaglandins may explain how TSI-TPA agents are capable of blunting TP receptor-independent mediators of vasoconstriction, such as angiotensin II, norepinephrine and endothelin (Taddei & Vanhoutte, 1986), and TP receptor-independent mediators of platelet aggregation, such as serotonin, adenine nucleotides, and PAF.
In summary, potential benefits of increased PGI₂ and attenuation of TP activity by TSI-TPA agents such as ridogrel include:

- a theoretically greater anti-aggregatory effect on platelets than is achievable, even with aspirin regimens designed to optimize platelet cyclooxygenase inhibition,
- a natriuretic effect, possibly related to both hemodynamic and tubular effects. These actions are likely to potentiate rather than antagonize antihypertensive therapy,
- an anti-proliferative effect to counteract vascular smooth muscle growth.

Considered along with evidence that TSI-TPA agents confer gastro-mucosal protective (Bunce & Spraggs, 1987; Reeves et al, 1988) and anti-asthmatic benefits (Nagai et al, 1995), these drugs may offer a better therapeutic index, i.e., better efficacy and fewer iatrogenic risks associated with chronic therapy. Large comparative trials are required to assess whether the virtues of chronic preventive TSI-TPA therapy are indeed sufficiently greater than aspirin to outweigh the cost factor. Cost and drug toxicity have an important impact on the feasibility of expanding risk reduction a the larger population, which would include interventions prior to secondary prevention of cardiovascular complications.

1.4 Ridogrel

Ridogrel is one among a growing class of investigational agents which combine TSI and TPA activities in one molecule (Figure 1.15).
Figure 1.15  Chemical structures of ridogrel and examples of other dual-acting TSI-TPA compounds

1.4.1 Structure and Activity

Ridogrel (R68070) is the generic name for (E)-5-[[[(3-pyridinyl)[3-(trifluoromethyl)phenyl]methylene]-amino]oxy]pentanoic acid. A structural feature common to all TSI-TPA agents thus far described in the literature, is the presence of a carboxylate spacially oriented about 0.8 to 1.0 nm. from a basic nitrogen atom of a 3-substituted pyridine or N-substituted imidazole, and also from a benzenesulfonamide structural element. It is postulated that the basic nitrogen forms a complex with the heme-polypeptide, to inhibit synthase activity. The benzenesulfonamide group has affinity for, and hinders activation of TP receptors, in a competitive fashion. In both cases, the carboxylate element is required to promote orientation of the molecule to its targets (Bossche et al, 1992; Soyka et al, 1994; Faull et al, 1995).

1.4.2 Pharmacokinetic / Pharmacodynamic Profile

A pharmacokinetic profile for ridogrel in rats has not been published. In fasted dogs and in humans, more than 90 per cent of an oral dose is rapidly absorbed. Plasma levels peak at 90 to 180 minutes. Its distribution approximates the extracellular fluid volume (0.1 to 0.2 L/Kg). Ridogrel is extensively plasma protein bound. In fasted dogs, the half-life was approximately six hours, and about nine hours in humans.

Thromboxane synthase inhibition specifically hinders hæm-iron catalytic interaction of the enzyme with the C-9 endoperoxide oxygen of PGH₂ (Hecker et al, 1986). The isomerization of PGH₂ to PGI₂ also involves endoperoxide activation, but prostacyclin synthase obtains its specificity through interaction with the endoperoxide oxygen at C-11 (Ullrich et al, 1981). At high (10 μM) concentrations, ridogrel had no effect on prostacyclin synthase (De Clerck et al, 1989; Bossche et al, 1991). In contrast to the cytochrome P450 enzymes having monoxygenase and epoxygenase activity, thromboxane synthase and prostacyclin synthase cannot be reduced by NADPH. Accordingly, Hecker et al (1987) have
proposed that they be more appropriately designated \textit{haem-thiolate isomerases}. Ridogrel was shown to have no effect on monoxygenase reactions involving metabolism of cholesterol (CYP_{11}), pregnenolone (CYP_{XII}), androgen (CYP_{XVIII}), estrogen (CYP_{XXI}), or 11\beta-hydroxylase (CYP_{XII}). Ridogrel had slight effects on regio- and stereo-selective isomerizations of testosterone, but did not inhibit the reduction of testosterone to 5\alpha-dihydrotestosterone (DHT) and 5\alpha-androstane 3\beta,17\beta-diol (Bossche et al, 1992). The activities of cyclooxygenase, platelet adenyl cyclase, platelet cAMP phosphodiesterase, 5-, 12-, and 15-lipoxygenase enzymes were not affected by ridogrel (De Clerck et al, 1989).

A theoretical limitation of ridogrel is its modest (micromolar potency) receptor antagonism, with an IC\textsubscript{50} in the presence of plasma proteins of about 18 \mu M, relative to its potent (nanomolar) thromboxane synthase inhibitory activity, where the IC\textsubscript{50} is as low as 4 nM (Soyka et al, 1994; Bossche et al, 1992).

Ridogrel is a fairly potent uricosuric. Single 400 mg doses of ridogrel in healthy males, decreased serum uric acid levels on average 1.5 mg/dL (Hoet et al, 1990).

Following a seven-day toxicity study, about 60 per cent of rats receiving another dual-acting compound, Z-D1542 at a dose of 500 mg per kilogram (almost 100 times the dose required for complete inhibition of platelet thromboxane synthesis), had an increased average thyroid follicular epithelial cell height. The significance of that finding is uncertain. One possibility is that at high doses, inhibitory activity may extend to the P\textsubscript{450}-dependent isomerase reactions involved in the synthesis of cortisol-binding globulin and thyroid-binding globulins (ICI:Personal Communications).

1.4.3 Summary of \textit{In vitro} and Animal Research Findings

Ridogrel half-maximally inhibits $1 \times 10^4$mol/L U46619-induced platelet aggregation.
at a concentration of $1.2 \times 10^{-6}$ mol/L, and half-maximally inhibits serum thromboxane formation at a concentration of $1 \times 10^{-7}$ mol/L (Hoet et al, 1990). *In vitro*, the absence of vascular EDRF(NO), EDHF and the absence of vascular prostaglandin production derived from platelet cyclic endoperoxides, is likely to result in the accumulation of these potent proaggregatory TP receptor agonists, and leave their actions unopposed. It might be expected that the unequal TSI versus TPA molar potency of ridogrel would result in highly variable measurements of antiaggregatory effects at lower concentrations, as well as a very steep concentration-response emerging at concentrations at which TPA activity becomes evident. In the presence of ridogrel, cAMP and cGMP levels were significantly higher than in the presence of a PGHS inhibitor (indomethacin), a single TSI (CGS13080), or a single TPA (BM13177), following collagen-induced platelet activation in a co-suspension consisting of aspirin-pretreated endothelial cells in platelet-rich plasma (Hoet et al, 1990).

In a canine model of left anterior descending coronary artery occlusion/reperfusion, pretreatment with ridogrel 2.5 mg/kg intravenously did not prevent S-T depression or the occurrence of post-reperfusion ventricular ectopic beats. However, ventricular fibrillation developed in 50 per cent of controls, but in none of the ridogrel treated dogs (De Clerck et al, 1989). Dogs treated with ridogrel 1.25 mg/kg intravenously prior to electrically-induced coronary thrombosis (anodal current of 300µA for 30 minutes), had less extensive thrombosis. Thrombus wet weight was less than 50 per cent of controls, and S-T changes occurred in only 14 per cent of ridogrel treated dogs, compared to 86 per cent of controls. Coronary blood flow was reduced by only 15 per cent versus a 50 per cent reduction in controls (De Clerck et al, 1989).

There is evidence that ridogrel may limit the extension of myocardial infarction into the penumbra. In a study by Golino et al (1993), five groups of 8 New Zealand White rabbits were subjected to 30 minutes of coronary artery occlusion, followed by 5.5 hours of reperfusion. The rabbits were then sacrificed. Infarct size was determined by triphenyltetrazolium chloride staining, and expressed as the percentage of the area at risk of
infarction. Fifteen minutes before reperfusion, the animals were randomized to receive an intravenous bolus of either saline (control), SQ29,548 (TPA), dazoxiben (TSI), ridogrel (TSI-TPA) or the combination of ridogrel+aspirin (PGHS inhibitor). The average infarct size in control animals was 57.7±3.2 per cent of the area at risk, and was not significantly reduced in groups which had received the TPA or ridogrel+aspirin. While infarct size was significantly reduced to 36.7±2.8 per cent of control in the group randomized to TSI, it was reduced to 16.6±3.6 per cent in the group that had received ridogrel. Ridogrel appeared on average to have spared 30% of the myocardium that was otherwise infarcted in controls. It is noteworthy that the TPA alone was of no significant benefit, that aspirin abolished the cardioprotection associated with ridogrel, and that the TSI alone afforded less protection than ridogrel. While TSI-enhanced endoperoxide production may itself be detrimental, a net cardioprotective benefit may result if metabolism of the endoperoxides is redirected toward prostaglandin synthesis. Detrimental effects of endoperoxide accumulation are eliminated when TP receptors are blocked concurrently.

Experiments reported by Janssens et al (1990) indicated that contractions of rabbit femoral or pulmonary arteries are evoked by aggregating blood platelets, and can be prevented with almost equal dose-dependent efficacy by either ridogrel or the 5-HT₂-serotonergic antagonist ketanserin. Serotonin and thromboxane are potent vasoconstrictors released by activated platelets, and appear to co-amplify platelet aggregation and vascular contraction. The antiaggregatory effect of the cyclooxygenase inhibitor suprofen failed to prevent the vascular contractions induced by platelet activation in the absence of ketanserin, whereas ridogrel did. Neither ridogrel nor suprofen inhibited contractions evoked by exogenous administration of serotonin, suggesting that dense-granule release occurs despite achieving a level of cyclooxygenase inhibition sufficient to inhibit platelet aggregation. Alternatively, this observation may further emphasize the important role played by vascular cyclooxygenase-dependent production of modulators which inhibit platelet-induced contraction, and antagonize cyclic flow variations in response to platelet activation. In a subsequent study in which collagen induced a reduction of peripheral blood flow in a feline
model, Loots & De Clerck (1993) demonstrated that the collateral-dependent vascular bed is oversensitive to platelet-derived vasoconstrictors. While 5-HT, rather than arachidonic acid metabolites, appears to be the dominant constrictor agonist, ridogrel does limit the perfusion defect, presumably by curtailing collagen-induced platelet activation in the first place. Indeed, there was a reduced loss of free platelets from circulating blood, as well as attenuated levels of 5-HT and TXB$_2$ in the plasma. These effects were mimicked by TSI alone (dazoxiben), but not by TPA alone (sulotroban). These effects of ridogrel and dazoxiben were prevented by cyclooxygenase inhibition (indomethacin). This seems to reinforce the notion that increased vascular production of vasodilator prostanoids serves to oppose the vasoconstriction induced by platelet-derived monoamine and adenine nucleotide mediators of vasoconstriction, and that attenuation of platelet activation accounts largely for the effectiveness of ridogrel in preserving collateral arterial blood flow after thrombotic obstruction of a larger artery (De Clerck et al, 1990). Yao et al (1990) induced coronary cyclic flow variations by imposing stenosis and endothelial injury in forty-five mongrel dogs, which were divided into four treatment groups to receive either aspirin, a TPA agent (SQ29,548), a TSI agent (dazoxiben), or the dual-acting TSI-TPA agent (ridogrel). Cyclic flow variations were abolished by these treatments in 50%, 71%, 82% and 100% respectively. Epinephrine was infused into dogs in which cyclic flow variations had been abolished, in order to assess the threshold for restoring the flow variations. The dazoxiben and the ridogrel treated groups, were markedly more resistant to higher doses of epinephrine, and flow abnormalities were abolished again with the 5-HT$_2$-antagonist LY 53,857. Both groups became refractory to re-establishing epinephrine-induced flow abnormalities, even at plasma epinephrine concentrations as high as 43.2 ± 9.4 ng/mL. The authors concluded that, compared to PHGS inhibition or TP receptor antagonism, substantially more protection against spontaneous and epinephrine-induced cyclic flow reductions was afforded by a TSI, and even more by ridogrel. Moreover, the combination of ridogrel with a serotonin antagonist appeared to provide complete protection in their canine experimental model.

There is evidence that ridogrel also enhances tissue perfusion in the microcirculation.
Banic and coworkers (1990) compared surviving tissue areas to the total area of two ventral skin flaps that had been raised 48 hours earlier in three groups of 20 rats. One group served as control, while the others received intraperitoneal injections of either forskolin (cAMP enhancement by direct activation of adenylyl cyclase) or ridogrel. Skin flap viability increased in area from 71% in controls, to 88% in forskolin-treated rats, and 95% in ridogrel-treated rats. Both treatments were associated with a statistically significant (p<0.0001) reduction of skin flap necrosis.

In an ovine model of pregnancy-induced hypertension, ridogrel dose-dependently reduced blood pressure. Neonatal outcomes were improved in the ridogrel group, even in comparison to a positive control group in which blood pressure was normalized by continuous magnesium sulfate infusion. In contrast to 67 per cent mortality in the control groups, neonatal mortality was eliminated and birth weight increased in the ridogrel group. Most of the control ewes that died, were found to have developed haemorrhage in association with consumptive thrombocytopenia (Keith et al, 1994).

1.4.4 Summary of Clinical Experience

Hoet et al (1990a) compared placebo, 400 mg aspirin or 400 mg ridogrel, in a double-blind, randomized, cross-over study of nine healthy nonsmokers. Earlier human studies had determined at two hours post-dosing, a 200 mg dose of ridogrel inhibited only TSI activity, whereas a 400mg dose of ridogrel was required to inhibit U46619- and collagen-induced aggregation. While serum thromboxane formation was completely inhibited by both treatments, ridogrel was clearly more potent than aspirin in prolonging bleeding time. Whereas serum 6-keto-PGF$_{1\alpha}$ and bleeding-time wound intralesional 6-keto-PGF$_{1\alpha}$ were inhibited after aspirin, these parameters were increased after ridogrel. The authors concluded that since aspirin was a more potent inhibitor of aggregation in vitro and less potent at prolonging bleeding time in vivo, local reorientation of cyclic endoperoxide metabolism
toward prostacyclin was likely to account for the stronger *in vivo* inhibition of hemostasis produced by ridogrel relative to aspirin.

Weber et al (1992) extended treatment with ridogrel 300mg b.i.d. for 29 days, in twelve healthy males aged 31-45 years. The regimen was well tolerated. Urinary uric acid excretion rose from the first day and stabilized by the eighth day. Serum uric acid remained at about 50% of pretreatment levels, but returned to pre-medication levels one week following discontinuation of ridogrel. No evidence of tachyphylaxis was observed for the three- to five-fold rightward shift in the concentration-response curves for U46619-evoked *ex vivo* platelet aggregation, over 29 days of treatment. Ex vivo serum thromboxane formation was already inhibited by 99 per cent on the first day of treatment, whereas urinary TXB₂ concentrations did not reach a nadir averaging 71 per cent of baseline, until the seventh day. No reference was made to blood pressure effects. The increases in serum and urinary metabolites of PGI₂ were modest during the course of treatment. This was expected for volunteers in whom cardiovascular disease states associated with platelet activation, were absent.

De Clerck et al (1989b) offer further evidence implicating prostaglandin enhancement in the antiplatelet actions of ridogrel. In five normal adult males, two hours following a single 400 mg dose of ridogrel, bleeding times increased by 380 seconds. This was about double that observed in a separate group of volunteers following a 1 g dose of aspirin. This was despite a similar reduction in *ex vivo* TXB₂ formation, and a greater *ex vivo* antiaggregatory effect attributed to aspirin. One-stage prothrombin times, activated partial thromboplastin times, plasma fibrinogen levels and fibrinolysis in diluted blood, with or without urokinase acceleration, did not differ from baseline in response to ridogrel pretreatment.

In the Ridogrel Versus Aspirin Patency Trial (RAPT), 907 patients with acute myocardial infarction were randomized to receive either ridogrel or aspirin, in addition to
streptokinase thrombolytic infusion. Ridogrel was no more effective than aspirin in enhancing the fibrinolytic efficacy of streptokinase, or enhancing the angiographic flow rates achieved, or at reducing the incidence of major clinical events during hospitalization. However, a post hoc analysis did demonstrate a 32 per cent (p<0.025) reduction in the incidence of new ischemic events (reinfarction, recurrent angina, ischemic stroke). In fact, all six ischemic strokes occurred in the aspirin group, with no excess of hemorrhagic stroke or other serious bleeding complications associated with ridogrel (The RAPT Investigators, 1994).

Hoet et al (1990b) assessed the effects of ridogrel in patients with documented peripheral vascular disease. Plasma levels of β-thromboglobulin (β-TG), platelet factor 4, and serum levels of prostanoid metabolites TXB₂/6-keto-PGF₁α, served as markers of in vivo platelet activation. In randomized double-blind parallel fashion, eleven patients received ridogrel 300 mg b.i.d. for two and a half days, while ten received matching placebo. None of the parameters changed significantly following placebo, whereas ridogrel was associated with a rapid and sustained 50 per cent reduction in plasma β-TG. Urinary 2,3-dinor- and 11-dehydro-TXB₃ fell significantly, and serum 6-keto-PGF₁α was significantly enhanced. Thus in patients with vascular disease, in whom markers of platelet activation are already elevated, ridogrel appears to be effective in modulating platelet hyper-responsiveness.

Only one clinical evaluation, designed to assess the potential for ridogrel to lower blood pressure in patients with essential hypertension, has been reported. In double-blind placebo controlled fashion, Ritter et al (1993) studied the effects following two doses of ridogrel 300 mg orally given 12 hours apart, in 16 hypertensive patients. In contrast to a 92 percent reduction in serum TXB₂, urinary TXB₂ was reduced by only 42 percent in the treatment group. Blood pressure did not change.

In conclusion, the clinical experience with ridogrel has been fairly extensive, but only one small acute study has entertained whether a TSI-TPA agent modulates blood pressure. Ridogrel tends to be well tolerated. Vascular disease destabilizes the interaction between
blood platelets and blood vessels. Mediators released by activated platelets are capable of jeopardizing blood flow to vital tissues, by provoking both vasospasm and thrombosis. While comparable in vitro antiplatelet effects are achieved with PGHS inhibitors, ridogrel appears additionally to enhance vascular prostaglandin synthesis in vivo. Platelet endoperoxide synthesis is exploited, rather than attenuated. Thus, the TSI-TPA interaction in vivo, results in an antiplatelet effect that is qualitatively different from that achieved with aspirin. The findings from animal and clinical investigations imply that in vivo, ridogrel may improve collateral blood flow, reduce vasospasm and perhaps prevent arrhythmias. From a clinical standpoint, the TSI-TPA mechanism may provide greater benefit in heart attack and stroke prevention, than low-dose aspirin.
2. PRESENT INVESTIGATION

2.1 General Introduction

Ridogrel is both an antagonist of TP receptors, and an inhibitor of thromboxane synthase. The experiments that follow, were designed to assess its actions on platelet aggregation and BP using in vitro, ex vivo and in vivo methods. The protocols encompassed both single and repeated dosing. Particular interest was given to the effects of ridogrel on BP, in a genetically hypertensive rat model. Initial experiments were undertaken to assess whether differences in prostanoid regulation could be defined, on the basis of the age and strain, using the rat models proposed for subsequent investigations with ridogrel.

The experimental methods, results and discussion will be dealt with separately for each of the protocols. Overlapping use was made of materials (subjects, equipment and chemical agents) in the various protocols. To avoid redundancy, their descriptions are assembled in Section 3. Likewise, the biochemical techniques used to quantitate prostanoid metabolites (TxB₂, 6-keto-PGF₁α) and PRA, were identical for all protocols. A brief outline of each assay appears in the analytical methods section. The detailed methods are included as appendices.

2.2 Aim of this Study

2.2.1 Hypotheses, Predictions and Approaches

**Hypothesis I.**

*An imbalance of renal prostanoids favouring vasoconstrictor TxA₂ synthesis over vasodilator PGH₂, contributes to the elevated renal vascular resistance characteristic of*
the SHR and SHR-SP.

**Prediction:** At baseline, the daily urinary excretion of TxB\textsubscript{2} will be elevated, and 6-keto-PGF\textsubscript{1\alpha}, as the major opposing prostanoid, will be lower in relative or absolute terms in hypertensive rats, compared to age-matched normotensive strains.

**Approach:** 24-hour urinary collections were undertaken to compare two hypertensive and two normotensive rat strains, employing the least invasive conditions available. Rats were studied in the developing phase (6 weeks), as well as the established phase (16 weeks) of hypertension.

**Hypothesis II.**

*Ridogrel has dual actions that both inhibit the synthesis of proaggregatory thromboxane A\textsubscript{2}/cyclic endoperoxides, and prevent the feedback amplification by thromboxane A\textsubscript{2}/cyclic endoperoxides at platelet TP receptors (the second wave of the blood platelet aggregatory response).*

**Prediction:** It was anticipated that on an equimolar basis, the inhibitory concentration of ridogrel against arachidonic acid-induced whole blood platelet aggregation, and against TxB\textsubscript{2} generation during clotting, would be significantly lower than that of indomethacin.

**Approach:** This prediction was tested using impedance-mode whole blood platelet aggregometry, to produce inhibitory concentration curves for both drugs and controls, following a standardized platelet aggregatory stimulus. The inhibitory concentration of ridogrel on *in vitro* clot generation of TxB\textsubscript{2}, used a radioimmunoassay for serum thromboxane. Both methods employed standard aliquots of whole blood, in the presence of known concentrations of ridogrel, indomethacin and controls.

**Hypothesis III.**

*Ridogrel pretreatment results in redirection of furosemide-stimulated arachidonic acid metabolism toward vasodilatory prostanoid synthesis.*
**Prediction:** A significantly greater amount of 6-keto-PGF$_{1\alpha}$ will be detected in the urine, following an intravenous bolus of furosemide in rats pretreated with ridogrel, compared to controls. A greater BP reduction will follow diuresis.

**Approach:** This hypothesis was tested by pretreating groups of SHR-SP with vehicle, or three doses of ridogrel, prior to receiving a bolus injection of furosemide to stimulate arachidonic acid metabolism. Differences in urinary 6-keto-PGF$_{1\alpha}$ were quantified by radioimmuno-assay, and BP was measured by the indirect tail-cuff method.

**Hypothesis IV.**

*Attainment of effective renal thromboxane inhibition is delayed, and repeated dosing with ridogrel is required to achieve an antihypertensive response.*

**Prediction:** BP will be dose-dependently reduced in SHR-SP, following subchronic dosing with ridogrel.

**Approach:** BP, urinary prostanoids and urinary sodium were measured before and after groups of rats received one of three doses of ridogrel or vehicle, administered at 12-hourly intervals by gavage over a seven day period.

**Hypothesis V.**

*Ridogrel pretreatment modulates the pressor effects of physiologically relevant pressor agents: angiotensin II, norepinephrine, and TxA$_2$.*

**Prediction:** Ridogrel pretreatment will blunt the acute responses to exogenous pressor agents.

**Approach:** Under anaesthesia, direct arterial BP in SHR-SP pretreated with ridogrel or vehicle, was monitored during challenges with incremental doses of the TP receptor agonist U46619, with angiotensin II, and with norepinephrine.
3. MATERIALS

3.1 Subjects

3.1.1 In vitro whole blood studies

Human whole blood was obtained from healthy adult male volunteers who denied ingesting nonsteroidal antiinflammatory drugs for at least two weeks. Rat blood was obtained from breeding stock that were raised with free access to tap water and Prolab R-M-H 3000 chow (Agway Ltd., Syracuse, NY), in a controlled environment providing 12-hour light/dark cycles, temperature (22±2°C) and humidity (55±10%). The animals were cared for in accordance with the principles of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

3.1.2 In vivo rat experiments

All animals used in the experiments were cared for in accordance with the principles of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The protocols were reviewed and approved by the University of Saskatchewan Animal Care Committee (Protocol file numbers: 930057 and 920095). The rats were given free access to distilled water and Prolab R-M-H 3000 chow (Agway Ltd., Syracuse, NY). The manufacturer's list of ingredients for R-M-H 3000 are included in appendix A.

Sprague-Dawley rats were derived from the breeding colony maintained at our animal quarters. They were descendants of a Crl: CD®(SD)BR breeding nucleus purchased in 1995, from Charles River Laboratories (Montréal, QC).
Spontaneously Hypertensive Rats (SHR) were obtained from the breeding colony maintained at our animal quarters. They were descendants of a SHR/NCrI BR breeding nucleus purchased in 1995, from Charles River Laboratories (Montréal, QC).

Wistar Kyoto rats (WKY) were selected from a breeding colony maintained at our animal quarters. They were descendants of a WKY/NCrI BR breeding nucleus purchased in 1995, from Charles River Laboratories (Montréal, QC).

Stroke-prone spontaneously hypertensive rats (SHR-SP) were from a breeding colony maintained at our animal quarters. They were descendants of a SHR-SP/A3N breeding nucleus originally obtained in 1991, from Dr. Carl Hansen (National Institutes of Health, Bethesda, MD).

3.2 Equipment

3.2.1 Tail-cuff systolic blood pressure apparatus

Tail-cuff BP measurements were made using a rat tail occluder cuff and sensor cuff apparatus (Buffington Clinical Systems, Cleveland, OH.), wired for recording on a model 7P1 polygraph (Grass Instruments Co., Quincy, MA). The occluder cuff pressure was calibrated with a mercury manometer. During measurements, rats were placed in Open Broome restrainers (Harvard Apparatus Canada, St. Laurent, QC). The tail-cuff BP measurements were validated against simultaneous direct carotid arterial catheter measurements. Validation experiments are outlined in appendix B.
3.2.2 Direct blood pressure measurement apparatus

Direct carotid arterial BP measurements employed a model P10EZ pressure transducer (Viggo-Spectramed, Oxnard, CA) or a Transpac® pressure transducer (Abbott Critical Care Systems, North Chicago, IL). The analog output of each transducer was calibrated with a mercury manometer, and recorded on a model 7P1 polygraph (Grass Instruments Co., Quincy, MA).

3.2.3 Osmolality equipment

Serum and urine osmolality were measured by the freezing-point depression method, using a model 5004 MicroOsmette® micro-osmometer (Precision Systems Inc., Sudbury, MA).

3.2.4 Autoanalyzer for electrolyte and creatinine assay

Serum and urinary electrolytes and creatinine were measured with a Kodak Ektachem 700® autoanalyzer (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY).

3.2.5 Scintillation counter for measuring beta decay

Beta decay rates in preliminary [³H]inulin and p-[¹⁴C]amino-hippurate clearance studies, and for radioimmunoassay of prostanoids using [³H]thromboxane B₂ and [³H]6-keto-prostaglandin F₁α, were counted on a model LS3801 beta counter (Beckman Instruments Inc., Irvine CA).
3.2.6 Scintillation counter for quantifying gamma emission

[^125I]angiotensin gamma emissions for the radioimmunoassay of plasma renin activity, were counted with an Autogamma® 5000 series gamma counter (United Technologies-Packard Instrument Co. Ltd., Downers Grove, IL).

3.2.7 Whole blood platelet aggregometer

Whole blood platelet aggregation was measured on impedance mode using a two-channel Chrono-log Lumi-Aggregation system (Chronolog Corporation, Havertown, PA). The equipment was linked to an Agg-Link® data acquisition system, with integrated analysis software run on a 386-PC. An illustration is included as appendix C.

3.2.8 Metabolism cages

Timed urine collections, and monitoring of food and water intake, were facilitated by using individual Nalgene® Plastic Metabolic Cages for large rodents (Nalge Co., Rochester, NY). An illustration is included as appendix D.

3.2.9 Inhalational anaesthetic apparatus

Halothane anaesthesia was established using a Fluotec-3® halothane vapourizer (Cyprane Ltd., Keighley, Yorkshire, UK), driven with a 400 KPa/L/minute fresh gas flow consisting of 95% oxygen / 5% carbon dioxide (Carbogen®, Praxair, Mississauga, ON). The circuit was used in a fume hood to exhaust gases. Induction was achieved at 5 per cent halothane, in a flow-through chamber, and anaesthesia was maintained thereafter using an improvised nose-cone (male condom-type disposable urine collection device with rigid plastic collar, Hollister™, Aurora, ON) on an Ayre's-T gas circuit configuration,
delivering between 0.5 to 1.5 per cent halothane as required to minimize hypotension, but extinguish jaw and corneal reflexes.

3.2.10 Gravimetric measurement equipment

Rats, food and water were weighed on a Mettler PC-8000 open platform electronic scale having ± 0.01 g precision (Fisher Scientific Ltd., Ottawa, ON). Urine output was determined gravimetrically using a Mettler College-150 enclosed platform electronic scale having ± 0.001 g precision (Fisher Scientific Ltd., Ottawa, ON). Chemical agents were weighed with a Mettler H54 (± 0.01 mg precision) balance (Fisher Scientific Ltd., Ottawa, ON).

3.2.11 Numbered animal identification equipment

Identification of individual rats was managed by affixing metal ear tags of two types/sources: the monel type (Ealing Science Ltd., St. Laurent, QC) or the stem and washer type (Harvard Apparatus, St. Laurent, QC).

3.3 Chemicals

Chemicals are listed in alphabetical order, followed in parentheses by the sources from which they were obtained:
angiotensin I (Sigma Chemical Co., St. Louis, MO); angiotensin II synthetic human octapeptide (Sigma Chemical Co., St. Louis, MO); arachidonic acid [ChronoPar-390®](Chronolog Corp., Havertown, PA); fiuregrelate (Upjohn Inc., Kalamazoo, MI); furosemide (Sigma Chemical Co., St. Louis, MO); halothane [Fluothane®](Wyeth-Ayerst Inc., Montréal, QC); indomethacin (Merck Frosst Laboratories, Montréal, QC); [3H]-inulin (DuPont Inc., Boston, MA); ketamine hydrochloride [Ketalar®](Parke-Davis, Scarborough, ON); [3H] 6-keto-prostaglandin F1α (DuPont Inc., Boston, MA); [125] iodine
(DuPont Inc., Boston, MA); norepinephrine bitartrate (Sabex Inc., Boucherville, QC); p-
$[^{14}\text{C}]-$amino-hippurate (DuPont Inc., Boston, MA); ridogrel (Janssen-Ortho Inc., North
York, ON); $[^3\text{H}]$ thromboxane B$_2$ (Amersham, Oakville, ON); U46,619 (Cayman
Chemical Co., Ann Arbor, MI); xylazine [Rompun®](Bayvet Div., Chemagro Ltd.,
Etobicoke, ON).
4. ANALYTICAL METHODS

4.1 Radioimmunoassay of thromboxane B₂ in serum and urine

The procedure for radioimmunoassay determination of TxB₂ concentrations in biological samples has been performed and re-evaluated routinely in Dr. Wilson's laboratory. The methods reported by Wilson, McCauley & Tuchek (1984) have since undergone minor modifications, to further improve levels of reproducibility. Extraction and purification steps are undertaken prior to the assay procedure, in order to eliminate cross-reactive substances while maintaining a high rate of analyte recovery. The procedure is detailed in appendix E.

4.2 Radioimmunoassay of urinary 6-keto-prostaglandin F₁α

With the exception of the extraction step, the procedure is similar to that described for radioimmunoassay of urinary TxB₂. The details of the analysis are included as appendix F.

4.3 Radioimmunoassay to determine plasma renin activity

Frozen plasma was thawed in advance. Plasma renin concentration was determined indirectly as a function of the rate of formation of angiotensin I, by the radioimmunoassay method of Stockigt, Collins & Biglieri (1971). The details of this method are included as Appendix E.
4.4 Urine and plasma osmolality

Osmolality of previously frozen (-20°C) 50μL samples were determined by the freezing point depression method, using a model 2000 Micro-osmlette®. The osmometer was calibrated against three commercially prepared standards (100, 500 and 1500 mOsm/kgH₂O) then verified with 300 and 900 mOsm standards.

4.5 Urine and serum sodium and creatinine determinations

Urine and serum electrolytes and creatinine determinations were measured with the Kodak Ektachem 700® autoanalyzer in the clinical laboratories of the Royal University Hospital Department of Pathology.
PLEASE NOTE

Page(s) missing in number only; text follows. Filmed as received.
5. BASELINE COMPARISONS BY AGE AND STRAIN

5.1 Introduction

Increased renal vascular resistance (RVR), and increased renal vasoconstrictor sensitivity to angiotensin II, are characteristic of both the SHR strain and human essential hypertension. They appear to precede the development of hypertension in 6-week old SHRs. Published normative renal prostanoid data from rats that have not been surgically or pharmacologically manipulated, are sparse and inconsistent. The purpose of the present study, was to define baseline characteristics of the rat models, with particular interest in age and strain dependent daily urinary TxB₂ and 6-keto-PGF₁α excretion rates. An independent assessment of four strains at two ages, raised under the same conditions of diet and husbandry, was undertaken for that reason. It was anticipated that in comparison to normotensive rat strains, SHR at 16 weeks of age, would demonstrate basal renal excretion rates of the stable prostacyclin metabolite 6-keto-PGF₁α, that were lower, and/or have elevated basal renal excretion rates of the stable TXA₂ metabolite TxB₂. If so, it would suggest that combined TSI-TPA drug treatment might reverse the imbalance, and delay the onset of hypertension.

5.2 Protocol

Male rats (4 SHR-SP, 4 SHR, 4 WKY and 4 SD) were ear tagged and acclimatized to the laboratory milieu, housed in large (17.5 L) cages from age 4-5 weeks, then distributed individually into four metabolism cages for two days prior to basal 24-hour urine collections. All animals were weighed, and continued to have free access to rat chow and distilled water throughout this experiment. During late evening, between the first and second days of acclimatization, indirect SBP and heart rates were recorded. The tail-cuff procedure involved placing the rat in a pre-warmed plexiglass restrainer (an
electrical heating pad remained warm after being switched off prior to introducing the animal into the restrainer). The occluder cuff was advanced to the proximal end of the tail, and the sensor cuff slid into place just distal to it. The sensor cuff was inflated to a standard 60 mm Hg pressure to stabilize the ventral tail artery directly over the piezo sensor unit within the cuff. The time required to obtain a stable and reproducible pressure measurement varied with each animal. The occluder cuff pressure transducer was calibrated with a mercury manometer and the precision of pen deflections at six points within a zero to 250 mm Hg scale were confirmed on the chart recorder of a model 7P1 Grass® polygraph. The pulse wave from the piezo sensor was fed to a separate channel for simultaneous recording. The sensitivity and gain were adjusted to obtain the best noise-free signal, but no attempt was made to calibrate the tail artery waveform. Obliteration of the uncalibrated tail artery pulse waveform by a cuff inflation pressure of $\leq 230$ mm Hg was verified. At least five cycles were collected on each rat to determine the pressure at which the initial return of tail artery pulse was detected (see appendix B).

In the metabolic cages, the rats were provided with pre-weighed excess rations of RMH3000 rat chow, and a pre-measured excess ration of distilled water. The cages help to separate urine from unconsumed spillage of food, water, stool, hair and dander. Urine was collected over two consecutive timed 24-hour periods from each rat. The urine collecting vials were surrounded by an ice water bath. The food and water remaining at the end of each 24-hour period was subtracted from the starting amounts to quantify water and sodium intake. The sodium content represents 0.44% w/w of the RMH3000 rodent chow. The calculated sodium intake was converted to millimoles, for comparison to 24 hour urinary sodium excretion. The urine samples were centrifuged at $980 \times g \times 10$ min, before the supernatant was transferred to pre-weighed vials and quantified gravimetrically, assuming $1 \text{ g} = 1 \text{ mL}$. Urinary osmolality, sodium, creatinine, TxB$_2$ and 6-keto-PGF$_{1\alpha}$ were assayed according to the methods described in detail in appendices E and F. Tail-cuff SBP was measured again after the second 24-hour urine collection was complete. Grouped by strain, the rats were returned to group cages between re-evaluation periods at ages 11 and 16 weeks. The protocol was subsequently repeated to include four more rats of each strain at 16 weeks.
5.3 Statistical Methods

Interactions of strain and age on urinary TxB₂ and 6-keto-PGF₁α excretion were of primary interest. Two approaches were used for analysis: 1) an analysis of variance using a univariate repeated measures model, to assess age-dependent differences between strains. 2) a one-way analysis of variance, to assess strain differences at each age. Where significant F-tests were demonstrated at p≤0.05, means comparisons were made using the Fisher's Protected Least Significant Differences multiple t-test approach. Data were analyzed using the SuperANOVA® statistical software package for Macintosh (Abacus Concepts Inc., Berkeley, CA). Independent t-tests were used for between strains comparisons. Since the additional four rats of each strain at 16 weeks of age were not littermates, within strain comparisons were made by independent t-tests and Levine's test to assure that no significant differences in means or significant heterogeneity of variances existed between groups.

5.4 Results

The two normotensive strains were similar in most respects at both ages. Likewise, the two spontaneously hypertensive groups were similar. On the basis of age and strain, the greatest differences existed between the stroke-prone spontaneously hypertensive rats and the Wistar Kyoto rats. Results pertaining to differences in growth rate are summarized in Table 5.1. The urinary prostanoid profile is summarized in Table 5.2. The urinary TxB₂ and 6-keto-PGF₁α results shown, are corrected to creatinine and body weight. The corrections had little impact on the proportional relationships between results, in terms of ng of metabolite excreted per 24 hours, before being corrected to creatinine, or prior to compensating for weight. Differences in urine concentration are compared in Table 5.3. Figure 5.1 compares differences in SBP.
Table 5.1: Age-dependent strain differences in body weight, sodium intake and water intake:

<table>
<thead>
<tr>
<th>Body weight (in grams) at 6 weeks:</th>
<th>SHR-SP</th>
<th>WKY</th>
<th>difference between means</th>
<th>95% C.I. of Difference</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>86.4±5.6 g.</td>
<td>126.8±6.5 g.</td>
<td>40.4 g.</td>
<td>19.3</td>
<td>61.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Body weight (in grams) at 16 weeks:</th>
<th>SHR-SP</th>
<th>WKY</th>
<th>difference between means</th>
<th>95% C.I. of Difference</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>313.0±7.2 g.</td>
<td>415.3±2.5 g.</td>
<td>102.3 g.</td>
<td>85.9</td>
<td>118.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sodium intake (mmol/24 hours/100 g body weight) at 16 weeks:</th>
<th>SHR-SP</th>
<th>WKY</th>
<th>difference between means</th>
<th>95% C.I. of Difference</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.25±0.27</td>
<td>2.56±0.21</td>
<td>0.696</td>
<td>-0.04</td>
<td>1.43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Water intake (grams/24 hours/100 g body weight) at 16 weeks:</th>
<th>SHR-SP</th>
<th>WKY</th>
<th>difference between means</th>
<th>95% C.I. of Difference</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.8±1.3 g.</td>
<td>11.4±1.0 g.</td>
<td>0.4 g.</td>
<td>-4.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

SHR-SP rats at age 16-weeks were also about 8% smaller by weight than SHR rats reared under the same conditions.

WKY males at age 16-weeks were approximately 10% smaller than age-matched Sprague-Dawley rats.

Relative to SHR-SP age-matched males at 16-weeks:

WKY rats averaged ~25% greater body weight
S-D rats averaged ~35% greater body weight
Table 5.2  Urinary Prostanoid Profile @ age 6-weeks and 16-weeks

prostanoids in nanograms per 24 hour period volume, corrected to urinary creatinine excretion and body weight.
S: denotes stochastic significance at $p \leq 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>SHR-SP 6-weeks</th>
<th>SHR-SP 16-weeks</th>
<th>WKY 6-weeks</th>
<th>WKY 16-weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>u 6-keto-PGF$_{1\alpha}$</td>
<td>12.1±2.3</td>
<td>24.0±1.8</td>
<td>19.5±3.1</td>
<td>28.3±0.87</td>
</tr>
<tr>
<td>u TxB$_2$</td>
<td>2.68±0.37</td>
<td>3.45±0.31</td>
<td>4.03±0.15</td>
<td>5.95±0.23</td>
</tr>
<tr>
<td>ratio u 6-keto-PGF$_{1\alpha}$ u TxB$_2$</td>
<td>4.51±0.11</td>
<td>7.06±0.46</td>
<td>4.84±0.22</td>
<td>4.76±0.45</td>
</tr>
</tbody>
</table>

Unpaired student t-tests
SHR-SP vs. WKY

@ 6-weeks:

<table>
<thead>
<tr>
<th></th>
<th>diff</th>
<th>95%CI of diff</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>u 6-keto-PGF$_{1\alpha}$</td>
<td>7.4</td>
<td>-1.0</td>
<td>15.8</td>
</tr>
<tr>
<td>u TxB$_2$</td>
<td>1.35</td>
<td>0.09</td>
<td>2.61</td>
</tr>
<tr>
<td>ratio u 6-keto-PGF$_{1\alpha}$ u TxB$_2$</td>
<td>0.33</td>
<td>-0.97</td>
<td>1.63</td>
</tr>
</tbody>
</table>

@ 16-weeks:

<table>
<thead>
<tr>
<th></th>
<th>diff</th>
<th>95%CI of diff</th>
<th>2-tailed p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>u 6-keto-PGF$_{1\alpha}$</td>
<td>4.3</td>
<td>-1.1</td>
<td>10.1</td>
</tr>
<tr>
<td>u TxB$_2$</td>
<td>2.50</td>
<td>1.67</td>
<td>3.33</td>
</tr>
<tr>
<td>ratio u 6-keto-PGF$_{1\alpha}$ u TxB$_2$</td>
<td>2.30</td>
<td>1.24</td>
<td>3.49</td>
</tr>
</tbody>
</table>
### Table 5.3 Strain differences in urine concentration between 16 week old SHR-SP and WKY rats

Using independent t-tests for comparison

<table>
<thead>
<tr>
<th></th>
<th>SHR-SP</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine flow rate (g./24h/100g.BW):</strong></td>
<td>2.49±0.45</td>
<td>4.75±0.28</td>
</tr>
<tr>
<td></td>
<td>diff= 2.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95%CI&lt;sub&gt;diff&lt;/sub&gt;: 0.95 to 3.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value= 0.01</td>
<td></td>
</tr>
<tr>
<td><strong>Urine osmolality (mOsm./Kg.H&lt;sub&gt;2&lt;/sub&gt;O):</strong></td>
<td>2471±125</td>
<td>2373±120</td>
</tr>
<tr>
<td></td>
<td>diff= 97.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95%CI&lt;sub&gt;diff&lt;/sub&gt;: -521 to 327</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value= 0.60</td>
<td></td>
</tr>
<tr>
<td><strong>Serum osmolality (mOsm./Kg.H&lt;sub&gt;2&lt;/sub&gt;O):</strong></td>
<td>273±8</td>
<td>271±9</td>
</tr>
<tr>
<td></td>
<td>diff= 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95%CI&lt;sub&gt;diff&lt;/sub&gt;: -31 to 28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value= 0.93</td>
<td></td>
</tr>
<tr>
<td><strong>Free water clearance:</strong></td>
<td>-0.0465±0.0229</td>
<td>-0.0608±0.0111</td>
</tr>
<tr>
<td></td>
<td>diff= 0.0143</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95%CI&lt;sub&gt;diff&lt;/sub&gt;: -0.0496 to 0.0210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value= 0.32</td>
<td></td>
</tr>
<tr>
<td><strong>Osmotic clearance:</strong></td>
<td>0.0506±0.0239</td>
<td>0.1120±0.0237</td>
</tr>
<tr>
<td></td>
<td>diff= 0.0614</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95%CI&lt;sub&gt;diff&lt;/sub&gt;: 0.0181 to 0.1047</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value= 0.02</td>
<td></td>
</tr>
</tbody>
</table>
@ 6-weeks

**SHR-SP**

n=4  mean ± S.E.M.: 143±3

**WKY**

n=4  mean ± S.E.M.: 122±2

diff= 21 t(5.824;df 5); 95%CI: 12 to 30  p = 0.0021  **

@ 16-weeks

**SHR-SP**

n=8  mean ± S.E.M.: 207.5±14.1

**WKY**

n=8  mean ± S.E.M.: 145.5±7.0

diff= 62 t (3.944;df 10); 95%CI: 27.0 to 97.0  p = 0.0028  **

Figure 5.1  Systolic blood pressure comparisons by age and strain

Independent t-tests are shown with corresponding p-values.

** points to significant differences.
5.5 Discussion

The age-related strain differences in urinary TxB₂ and 6-keto-PGF₁α excretion were contrary to expectation. Strain-by-age 6-keto-PGF₁α excretion rates were not significantly different. Urinary TxB₂ excretion was higher in the normotensive strains. Daily rates of excretion of non-enzymatic hydrolysis products of thromboxane and prostacyclin, are widely accepted to qualitatively reflect their net renal synthesis. However, prostanoid activity is autocrine or paracrine in nature, so the net rate of excretion is insensitive to differences in prostanoid concentrations that may occur at localized sites of action within the kidney. Furthermore, differences in the sensitivity of signal transduction mechanisms might arise from increased receptor density or coupling efficiencies. There is evidence that species differences exist. For example, PGE₂ subserves much the role in rat renal hæmodynamic function, that prostacyclin plays in human kidney. Since PGE₂ was not measured in addition to 6-keto-PGF₁α, the relative balance between opposing prostanoid effects might be underestimated. Another possibility is that modulation of renal thromboxane, is a compensatory response to the development of hypertension.
6. WHOLE BLOOD STUDIES OF THROMBOXANE B₂ 
AND PLATELET AGGREGATION

6.1 Introduction

These studies measure platelet function. Impedence mode platelet aggregometry served as a method to compare the ability of drugs at different concentrations, to inhibit platelet aggregation in whole blood, in response to a standardized stimulus. The ability of coagulating whole blood to produce TxA₂, measured as serum TxB₂, was used to compare the ability of drugs at different concentrations, to limit the TxB₂ generating capacity of identical aliquots of whole blood, when the blood was allowed to coagulate at 37°C. The uninhibited capacity of 1 mL of whole blood to generate TxB₂ varies greatly with the same subject on different days, and between subjects. It does not vary between aliquots of blood from the same sample, if incubated under identical conditions. The blood sampling and dispensing technique for rats is less efficient, and probably accounts for a greater variability between aliquots of the same sample [4.9±0.9%(95CI: 2.2-7.7) for rats, and 2.0±0.8 (95CI:0.1-4.0) for humans]. The inhibitory effects on whole blood platelet aggregation were assessed over a similar range of serial drug dilutions.

6.2 Protocols

6.2.1 Inhibition of whole blood capacity to generate thromboxane B₂ 
during spontaneous clotting

Whole blood was obtained from human volunteers who had not taken aspirin in the past 3 weeks, or other NSAIDs in the past 2 weeks. The phlebotomy site was an antecubital vein, from which blood was drawn into plain glass (red top) Vacutainer® tubes, using a 20 gauge needle on a Vacutainer® system. The blood filled tubes were immediately unstoppered and blood poured
into a polystyrene beaker. The blood from the beaker was drawn into a repeat-pipet, and dispensed as 1.0 mL aliquots into the awaiting 3 mL tubes containing serial dilutions of drug, or controls. The dilution tubes were quickly stoppered, inverted to mix the blood with the drug, and placed into a 37°C water bath to incubate for 45 minutes.

For rat blood ex vivo studies, blood was obtained from up to four rats at a time, following induction of anaesthesia with ketamine 70-90 mg/kg and xylazine 5-10 mg/kg. An 18 gauge short-bevel needle was mounted on a 12 or 20 mL disposable syringe, and 4-5 mL of blood was withdrawn by cardiac puncture. The needle was removed from the syringe before the blood was ejected into a polystyrene beaker. From the beaker, the blood was dispensed in 1.0 mL aliquots using a repeat-pipet, into the awaiting 3 mL tubes containing serial dilutions of drug, or controls. The dilution tubes were quickly stoppered, inverted to mix blood with drug, and placed into a 37°C water bath to incubate for 45 minutes.

At the end of 45 minutes, the clotted blood was centrifuged at 4°C, 980 × g × 20 minutes, and the serum was pipetted into 5 mm × 72 mm glass test tubes, which were sealed and frozen to await assay for immuno-reactive TxB2 concentration.

6.2.2 Inhibition of whole blood platelet aggregation

Blood was collected from healthy volunteers who had not taken aspirin in the past 3 weeks, or other NSAIDs in the past 2 weeks. The blood was drawn from an antecubital vein, into Vacutainer® tubes containing trisodium citrate, and mixed thoroughly. Cuvettes were prepared with 10 μL volume serial dilutions of drug, or controls. Whole blood platelet aggregation was measured on a two-channel Chrono-log Lumi-Aggregation system on impedance mode. Sample preparation involved diluting 450 μL of citrated whole blood with 450 μL of 0.9% saline in a 1mL cuvette. The cuvette contained a disposable siliconized stir bar. Cuvettes containing prepared samples were allowed to equilibrate while stirred at 1000 rpm in the heated sample
blocks to 37°C, and until a stable baseline was attained.

The principle of impedance aggregometry is as follows: each channel has an electrode probe assembly, with two carefully spaced rigid precious metal wires. The probe is immersed in the diluted blood sample, which is stirred at a constant rpm by a teflon-coated stir bar, and maintained at 37°C in the servo-controlled heater block. The probe-cuvette assembly is positioned into the sample chamber. A small electric current passes between the two wires of the electrode. During initial contact with the sample, an equilibrium monolayer of platelets forms on the electrodes. A standard platelet agonist is added to stimulate aggregation; and as the platelet aggregate builds up on the electrodes, it is measured as an increase in electronic impedance (in ohms) over time (in minutes). The data can be evaluated as a function of relative lag-time to response, the slope of the response, or the amplitude of the response.

For each drug-containing sample run in one channel, an untreated control was run in the second. All measurements followed a standard 10 μL stimulus of 0.5 mM (final concentration = 0.005 mM or 5 μM) arachidonic acid emulsified in albumin. The arachidonate was added to both the control and treatment channel in close succession. The moment it was added to each channel, appeared as an artifact on the monitor screen. The increase in impedance was allowed to go to completion, but for comparison purposes, the amplitude of the change was measured at 6.5 minutes from the moment that agonist was added. The response was considered as the aggregatory response in the presence of a known concentration of drug, relative to the maximum response in the control channel. Impedance aggregometry, using the equipment and techniques on hand, provided a moderately sensitive semi-quantitative method. Blood studied under the same controlled conditions, but from different sources, can yield control-response curves to a standard stimulus that differ by 30%.

6.2.3 Statistical methods

For both parameters, thromboxane B₂ generation and platelet aggregation, the objective
was to evaluate the inhibitory potency of ridogrel (a TSI-TPA), and compare its effects to indomethacin (a PGHS inhibitor) and furegrelate (a TSI). Measurements on both parameters were semi-quantitative, evaluating inhibitory concentrations relative to simultaneously run controls. It was demonstrated that twin control responses were identical, and that the responses to identical treatments run simultaneously were also identical. However, the response varies from one control to the next, so it was necessary to run a control simultaneously for each inhibitory drug concentration sample tested. Each control was entered as 100% response, and the response in the treatment channel was determined relative to control (entered as per cent of control). This approach is analogous to that used in agricultural research, based on percentage of kill by a pesticide or herbicide over a range of application rates (Steel & Torrie, 1980). Because there was variation between successive controls (i.e., the variance is binomial in nature), a probit response model, which uses both transformation and weighting, was used for analysis. The computations were run using the probit analysis procedure of a commercial software package (SPSS® for Windows™ Advanced Statistics Release 5, SPSS Inc., Chicago, IL.), run on a 486PC. The procedure provides estimates of intercepts and slopes, and therefore a test of parallelism between the inhibitory concentration curves generated for each drug. Comparing the ratio of concentrations that are equally effective in achieving an inhibitory response that is 50% of control, allows for comparison of their relative potencies.

6.2.4 Results of in vitro whole blood drug concentration-response experiments

Figure 6.1(a)(b)(c) establishes that (a)controls in both channels responded identically following the standard arachidonate 10µL = 0.5 mM stimulus, (b)that in the absence of the stimulus there was no response, and (c)that the inhibitory effect of identical concentrations of drug on arachidonate-stimulated aggregation was likewise identical when run simultaneously.

Figure 6.2 shows the platelet aggregation and spontaneous thromboxane B₂ generation concentration-response curves for indomethacin and ridogrel. With regard to indomethacin, the
Figure 6.1 (a) (b) (c) Whole blood platelet aggregation responses, as they appear in the software graphic display window

(a) The same stimulus (10 µL arachidonic acid 0.5mM) was added to both channels, and resulted an identical platelet aggregation response. This run was a test of the solvent used to dissolve ridogrel (10 µL of 0.4M tataric acid).

(b) The 10 µL arachidonic acid 0.5mM stimulus was added to Channel #2 (STIMULATED CHANNEL), but not to Channel #1 (UNSTIMULATED CHANNEL). A stable baseline was demonstrated in the absence of a stimulus.

(c) Ridogrel (1 x 10^{-7}M) was added to both channels prior to the stimulus, and resulted in overlapping responses.

x-axis scale is 60 sec/division, y-axis scale is 5Ω/division. A typical maximum change in impedance, in response to a 10 µL stimulus of the arachidonic acid 0.5mM reagent, in a whole blood sample from a healthy adult human, is 11 ± 3 Ω.
Figure 6.2  Inhibition of thromboxane B$_2$, and inhibition of platelet aggregation: concentration response curves comparing ridogrel to indomethacin.

- ○ platelet aggregation: indomethacin
- □ TxB$_2$ generation: indomethacin
- ● platelet aggregation: ridogrel
- ■ TxB$_2$ generation: ridogrel
steep portions of the trimmed log-molar concentration-response curves for inhibition of whole blood platelet aggregation, and clot generation of TXB₂ were linear (r²=0.9445 and r²=0.9231 respectively), and were overlapping (Pearson Goodness-of-Fit Chi² = 10.142 df=17 p=0.898). In contrast, concentration-response curves for inhibition of platelet aggregation, and of clot generation of TXB₂, diverged at lower concentrations of ridogrel. With declining concentrations, inhibition of platelet aggregation appeared progressively weaker than inhibition of TXB₂. However, the curves converged at virtually the same concentration for achieving maximal inhibition near 10e-7M. At the lowest ridogrel concentrations tested, inhibition of platelet aggregation was inconsistent. As a result, concentration-dependent inhibition only occurred at concentrations above 5.5e-8M. While iterative curve-fitting described a positive exponential slope with r² = 0.866, some degree of convexity may be artifactual, because more points were clustered at the ineffective end of the concentration-response curve. While furegrelate produced concentration-dependent inhibition of TXB₂ production, parallel to ridogrel, but at 11-fold higher concentration, no concentration-dependent inhibition of platelet aggregation was achieved within the same range of dilutions, despite obtaining consistent and reproducible control responses.

Table 6.1 compares the molar concentrations at which ridogrel and indomethacin inhibited clot generation of TxB₂. Ridogrel had 1850-fold greater inhibitory potency, on an equimolar basis. Table 6.2 compares the molar concentrations at which ridogrel and furegrelate inhibited clot generation of TxB₂. Ridogrel had 11-fold greater inhibitory potency on an equimolar basis.

Table 6.3 compares the molar concentrations at which ridogrel and indomethacin inhibited platelet aggregation. Ridogrel had 75-fold greater inhibitory potency on an equimolar basis. Figure 6.3 demonstrates that in vitro thromboxane inhibitory concentrations of ridogrel did not differ between rat and human whole blood.

6.2.5 Discussion

Concentration-response curves were derived for two parameters:

1) inhibition of thromboxane B₂ generation in whole blood allowed to clot spontaneously
Table 6.1  Results of probit analysis: inhibition of clot thromboxane B<sub>2</sub> generation, comparing the effects of ridogrel to indomethacin

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Molar Concentration at which TXB&lt;sub&gt;2&lt;/sub&gt; Production was Inhibited to 50 per cent of Control</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>ridogrel</td>
<td>4.8E-09</td>
<td>1.0E-10</td>
</tr>
<tr>
<td>indomethacin</td>
<td>8.9E-06</td>
<td>1.6E-07</td>
</tr>
</tbody>
</table>

Estimate of Relative Median Potency: \( \frac{\text{ridogrel IC}_{50}}{\text{indomethacin IC}_{50}} = 5.4E-04 \)

Table 6.2  Results of probit analysis: inhibition of clot thromboxane B<sub>2</sub> generation, comparing the effects of ridogrel to furegrelate

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Molar Concentration at which TXB&lt;sub&gt;2&lt;/sub&gt; Production was Inhibited to 50 per cent of Control</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>ridogrel</td>
<td>7.4E-09</td>
<td>2.0E-09</td>
</tr>
<tr>
<td>furegrelate</td>
<td>8.7E-08</td>
<td>6.7E-08</td>
</tr>
</tbody>
</table>

Estimate of Relative Median Potency: \( \frac{\text{ridogrel IC}_{50}}{\text{furegrelate IC}_{50}} = 8.5E-02 \)

Table 6.3  Results of probit analysis: inhibition of platelet aggregation, comparing the effects of ridogrel to indomethacin

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Molar Concentration at which Platelet Aggregation was Inhibited to 50 per cent of Control</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>ridogrel</td>
<td>8.8E-08</td>
<td>6.7E-08</td>
</tr>
<tr>
<td>indomethacin</td>
<td>6.6E-06</td>
<td>1.9E-07</td>
</tr>
</tbody>
</table>

Estimate of Relative Median Potency: \( \frac{\text{ridogrel IC}_{50}}{\text{indomethacin IC}_{50}} = 1.3E-02 \)
Figure 6.3  Comparing *In vitro* concentration-response curves for ridogrel inhibition of clot thromboxane B₂ generation, between rat and human whole blood
at 37°C over at least 45 minutes

2) inhibition of whole blood platelet aggregation.

The aim of these experiments was to determine the quantitative and qualitative differences between a PGHS-inhibitor (indomethacin), a TSI (furegrelate) and the dual-acting TSI-TPA drug ridogrel.

The finding of greatest interest, is the dissociation of TxB2 formation from platelet aggregation curves. Results from the in vitro whole blood concentration-response experiments, were able to demonstrate dose-dependent reductions in clot TxB2-generating capacity for all three drugs. In two separate attempts, using freshly prepared dilutions, furegrelate did not inhibit platelet aggregation within the same range of dilutions that did inhibit TxB2. The idea of renaming the drug "Frunstregrelate" was entertained. However, the simultaneous control responses to 10 μL arachidonic acid 0.5M-stimulated aggregation in both channels were reproducible within the reference range of 11 ± 3 Ω, and were virtually identical. As well, the solvent (0.9% NaCl, 10 μL alogou), did not alter the control response. This suggests that, although furegrelate was a potent in vitro inhibitor of thromboxane synthesis, it did not reliably inhibit platelet aggregation in a concentration-dependent manner. There is a probable explanation. In vivo, the endoperoxides which accumulate as a result of thromboxane synthase inhibition, are likely to enhance endothelial production of antiaggregatory prostacyclin, but there are few alternate pathways for endoperoxide metabolism in whole blood in vitro. While platelets have some capacity to synthesize antiaggregatory PGD2, it has been shown that platelet aggregation could paradoxically be enhanced by thromboxane synthase inhibition, because it is likely to result in endoperoxide accumulation. It was reported by Mayeux et al (1988), that in washed human platelets, PGH2 had 3-fold higher TP receptor binding affinity than TxB2 (Kd 45 ± 2 nM vs. 163 ± 21 nM), and a longer half-life than TxB2 (5 minutes vs. 30 seconds). Pathological conditions can impair endothelial capacity to generate PGI2, EDRF and other potentially antiaggregatory mediators, so this effect of TSIs is not necessarily unique to in vitro conditions. Ridogrel combines potent TSI activity (IC_{50} 4-7 nM), with relatively modest TP receptor antagonism (IC_{50}
18 μM, in the presence of plasma proteins) (Soyka et al, 1995). This may explain why concentration-dependent platelet inhibition was inconsistent at weaker dilutions, until sufficient receptor-antagonizing concentrations were reached to block endoperoxide-mediated platelet activation. By this reasoning, a dual-acting drug which offered equipotent or greater TP receptor antagonist activity relative to TSI activity, would show no dissociation of antiaggregatory from thromboxane synthase inhibition, and would perhaps represent an even more effective antiplatelet drug than ridogrel.
7. SINGLE DOSE RIDOGREL: DOSE-RESPONSES IN RATS

7.1 Introduction

This group of experiments was done to assess whether acute treatment with ridogrel would result in dose-dependent BP reduction. A prominent component of the increased vascular resistance in SHR-SP rats at 12 weeks of age, is functionally determined and acutely reversible. Anatomical rarification of the resistance vascular bed occurs more slowly, and ultimately predominates as hypertension becomes established. Furosemide, when administered as an intravenous bolus, has been shown to be a potent stimulus for arachidonate release, leading to enhanced eicosanoid synthesis. If TSI-TPA pre-treatment with ridogrel, were to redirect metabolism of augmented concentrations of endoperoxide intermediates toward synthesis of principally vasodilator anti-aggregatory prostanoids, then two mechanisms in particular could mediate an acute fall in BP. First, prostacyclin is known to stimulate adenylyl cyclase activity to increase cAMP, with the potential to modulate vascular sensitivity to pressor hormones and sympathetic tone. This is in addition to its direct vasodilator activity. Moreover, following ridogrel the vasodilator prostanoids would be acting unopposed by mediators of TP receptor activation. A second possible mechanism could derive from a renal vasodilator effect (Badahman & Wilson, 1994); (Cowley, 1994), further contributing to a reduction in BP.

7.2 Protocol

Blood pressure effects were assessed in 4 groups of four 12-week old male SHR-SP rats. Following baseline indirect BP measurements, performed as described in section 5.2, the rats were weighed, and ear-tagged, and randomly assigned to 4 groups of 4 rats/group, each group housed collectively in large cages:
<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Vehicle (methylcellulose 4000cps viscous)</td>
</tr>
<tr>
<td>B</td>
<td>Ridogrel 5 mg/kg susp. by gavage</td>
</tr>
<tr>
<td>C</td>
<td>Ridogrel 25 mg/kg susp. by gavage</td>
</tr>
<tr>
<td>D</td>
<td>Ridogrel 125 mg/kg susp. by gavage</td>
</tr>
</tbody>
</table>

cps: centipoise (unit of viscosity)

Each rat received a single dose by gavage. Each dose was deposited into the stomach as a 500 μL volume, using a 6.5 cm gavage needle passed full length down the oesophagus. At about two hours after dosing, indirect tail-cuff SBP was measured.

The protocol was expanded to assess differences in urinary TxB₂ and 6-keto PGF₁α in control and ridogrel pretreated rats, following furosemide stimulation. Figure 7.1 provides a schematic outline of the protocol. Another sixteen 12-week old male SHR-SP, which were already exposed to frequent handling, including gentle restraint by scruffing, were fitted with numbered ear-tags following baseline measurement of their SBP. Individual rats were placed in commercial metabolic cages, and provided with pre-weighed excess rations of distilled water and RMH-3000 rodent chow containing 0.44% sodium w/w. They were given a 24-hour period to acclimatize to the metabolic cage environment, before recording their weights and commencing a 24-hour baseline urine collection. An ice-water bath surrounded the urine collection receptacles. When the 24 hour urine collection period concluded, the urine was centrifuged at 900 × g for 10 minutes to remove hairs and other suspended contaminants. The supernatant was pipetted into pre-weighed disposable conical urine tubes, then re-weighed to determine the urine output gravimetrically (assumption that 1 g = 1 mL).

Staggered several minutes apart, the rats were gavage-dosed in 4 groups of 4
Figure 7.1  Skeleton protocol for single dose ridogrel experiments
rats, with either vehicle, or ridogrel (5-, 25- or 125 mg/kg) suspended in vehicle. Two hours following dosing, tail-cuff SBP was measured. Each rat then received a left jugular venous injection of furosemide 2mg/kg, according to the technique described by Waynforth & Flecknell (1992). The rats were immediately placed into clean metabolic cages, to collect urine over the next 40 minutes. At 45 minutes, the rats were again removed to measure SBP, then each rat was anaesthetized with combined ketamine 70mg/Kg and xylazine 3mg/kg, before cardiac puncture was performed. A 15 ga. short-bevelled needle on a 10 mL syringe was used to rapidly withdraw about 4 mL of blood. The needle was removed from the syringe before approximately 2 mL of the blood was expelled into an ice cold 3 mL EDTA vacutainer. One mL of blood was deposited into a plain glass vacutainer tube, and incubated in a 37°C water bath. Following blood sampling, rats were euthanized with an intracardiac injection of potassium chloride, while still anaesthetized. The iced EDTA blood samples were centrifuged at 4°C (960 × g × 20 minutes), then the plasma was pipetted into pre-labelled plain 5 mm × 72 mm glass test tubes on ice, and securely stoppered. These samples were stored frozen at -20°C, awaiting assay for PRA (analytical method detailed in Appendix G). After incubating for 45 minutes, the clotted blood samples were taken from the 37°C water bath, and centrifuged at 4°C (960 × g × 20 minutes). Serum was pipetted into pre-labelled plain 5 mm × 72 mm glass test tubes, sealed and frozen at -20°C, awaiting assay for serum TxB₂ concentration. The 40 minute urine volumes were recorded, and the urine was kept frozen at -20°C, awaiting assay for TxB₂ and 6-keto-PGF₁α.

7.3 Statistical methods

In order to check effectiveness of the grouping, the baseline data for each rat was organized into cells after the experiments were completed, according to the treatment group to which they were subsequently assigned. Comparisons of means between treatment group assignments followed ANOVA using the Fisher's Protected

7.4 RESULTS

The results from the single dose studies are presented in Figure 7.2(a)(b), Figure 7.3(a)(b), Figure 7.4(a)(b) and Figure 7.5(a)(b). No grouping assignment differences between cell means were detected post-hoc, for SBP, heart rate, weight, urine output, water intake, or urine TxB₂. Blood for serum TxB₂ and PRA was not drawn at baseline. The mean pre-treatment sodium intake of the group which subsequently became the ridogrel 25 mg/kg treatment group, was significantly greater than two other cells, and nearly significant cf. the pooled baseline:

Pooled baseline 4.34±0.76 n=16 cf. group C n=4

diff 0.92 crit.diff 1.02 p=0.0639.

There were no extreme outliers within the group, and the median was very near the mean. There remains no apparent explanation for their collectively increased food consumption. In retrospect, their higher salt intake does not appear to have biased Group C, in terms of the experimental results.

The SBPs measured approximately two hours post-dosing did not differ significantly between treatment groups, or from the aggregate pre-treatment baseline. The SBPs measured following the 40 minute furosemide-stimulated collection, averaged 16 mm Hg below the pre-treatment baseline. The largest mean difference was for pre-treatment vs. vehicle/furosemide, 23 mm Hg (95%C.I. diff: -23 to 68) p>0.05. Nor did heart rate change significantly with treatment. The mean urine output following furosemide was numerically greater in rats that had been dosed with ridogrel at all doses compared to vehicle, but the increase was not statistically
Figure 7.2  Results from single dose studies with ridogrel:
(a) Pre-treatment body weight
(b) Pre-treatment Na⁺ intake
Figure 7.3 Results from single dose studies with ridogrel:
(a) Group means for systolic blood pressure
(b) Group means for heart rate
Figure 7.4 Results from single dose studies with ridogrel:
(a) Group means for urine thromboxane $B_2$
(b) Group means for urine flow rate
Figure 7.5  Results from single dose studies with ridogrel:
(a)  Group means for *ex vivo* serum thromboxane B₂
(b)  Group means for plasma renin activity
significant, and there was no dose-dependent trend associated with the increase. While post-furosemide urinary TxB2 excretion rates rose in all groups, they did not differ significantly between groups, and neither showed a statistically significant dose-dependent trend.

At 2-3 hours after dosing, which corresponds to the approximate time to reach peak plasma ridogrel concentration after oral dosing in rats (Hoet et al, 1990), ridogrel had not significantly or dose-dependently inhibited furosemide-stimulated urinary TxB2 excretion rates. Meanwhile, all doses of ridogrel resulted in statistically significant inhibition of ex vivo TxB2 production during blood clotting (Table 7.1). In this case, the test for a linear trend between cell mean and cell order was significant p<0.0001, and provided a clear indication that the drug was absorbed and active in the circulation. PRA increased in dose-dependent fashion: Slope= 19.311; r² = 0.5630; Linear F= 31.0732 p= 0.0003 (Table 7.2).

7.5 DISCUSSION

A furosemide intravenous bolus was administered to stimulate arachidonate release. Of the many methods to enhance renal eicosanoid synthesis, furosemide offers certain advantages. First, the diuretic effect allowed collection of timed urine samples in adequate quantity for assay, in non-catheterized animals, over a shorter period surrounding the peak serum concentration of ridogrel. Secondly, the reabsorption rates of renal prostanoid metabolites along the nephron, are minimized at high urine flow rates (Zipser & Smorlesi, 1984), making the excretion rate of urinary TxB2 less dependent on the volume of urine excreted during the collection period. Following acute furosemide-stimulated volume loss, BP did not change significantly with respect to treatment, suggesting that compensatory mechanisms were at play in its maintenance. Activation of the renin-angiotensin-aldosterone system (RAAS), is one
### Table 7.1  Results of acute ridogrel doses on ex vivo serum thromboxane B₂ generation

Fisher's Protected LSD t-tests

<table>
<thead>
<tr>
<th>Means Comparisons</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. Ridogrel 5mg/Kg</td>
<td>&lt; 0.05   *</td>
</tr>
<tr>
<td>Vehicle vs. Ridogrel 25mg/Kg</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Vehicle vs. Ridogrel 125mg/Kg</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Ridogrel 5 vs. Ridogrel 25</td>
<td>&lt; 0.05   *</td>
</tr>
<tr>
<td>Ridogrel 5 vs. Ridogrel 125</td>
<td>&lt; 0.05   *</td>
</tr>
<tr>
<td>Ridogrel 25 vs. Ridogrel 125</td>
<td>NS</td>
</tr>
</tbody>
</table>

### Table 7.2  Results of acute ridogrel doses on plasma renin activity

Fisher's Protected LSD t-tests

<table>
<thead>
<tr>
<th>Means Comparisons</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle vs. Ridogrel 5mg/Kg</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Vehicle vs. Ridogrel 25mg/Kg</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Vehicle vs. Ridogrel 125mg/Kg</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Ridogrel 5 vs. Ridogrel 25</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Ridogrel 5 vs. Ridogrel 125</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Ridogrel 25 vs. Ridogrel 125</td>
<td>&lt; 0.001***</td>
</tr>
</tbody>
</table>
of the major mechanisms to maintain BP in response to acute diuresis, however it is interesting to note that plasma renin activity increased in clear association with ridogrel dose, whereas the urine volume losses were relatively constant across all treatments. The \textit{ex vivo} capacity of clotting blood to generate thromboxane, was inhibited dose-dependently. Following thromboxane synthase inhibition by ridogrel, platelet endoperoxide metabolism may have favoured the synthesis of prostacyclin. In a previous study by Datar, McCauley & Wilson (1987), it was apparent that a sharp rise in PRA occurred, after furosemide injection had evoked an increase in urine 6-keto-PGF$_{1\alpha}$. PRA continued to rise after 6-keto-PGF$_{1\alpha}$ excretion declined, and remained elevated 40 minutes after furosemide (Figure 7.6(a)(b)). It is also possible that ridogrel has a direct action to stimulate renin release, or that indirect interaction with other mechanisms of renin release, such as the adrenergic system, may occur. Studies with other single acting thromboxane synthase inhibitors have shown an increase in renin release, which could be prevented with PGHS inhibitors. Regardless of the mechanism, it is apparent in Figure 7.7, that ridogrel dose is positively tied to PRA, an association which would act counter to, and may have masked the acute antihypertensive response that was hypothesized. Lastly, it has once again been demonstrated that even potent inhibition of \textit{ex vivo} clot generation of thromboxane does not predict a proportionate reduction in the rate of urinary TxB$_2$ excretion.
Figure 7.6 Changes in plasma renin activity following furosemide in rats pre-treated with vehicle or the thromboxane synthase inhibitor furegrelate

(a) It is apparent that PRA rose in succession to the peak 6-keto-PGF$_{1α}$ concentration, and remained elevated beyond the subsequent decline in urinary 6-keto-PGF$_{1α}$ levels. Furosemide 2 mg/kg was administered at Time 0.

(b) In contrast to vehicle-treated rats, PRA was elevated at baseline in the furegrelate pre-treated rats, and rose sharply in response to furosemide.

VEH 1 and VEH 2 were identical injections of 0.03M Na$_2$CO$_3$, and represent a double control where VEH 1 rats subsequently received furegrelate (a repeated measure control), and VEH 2 rats were an independent control. Error bars are SEM.

Figure 7.7  Relative changes in blood pressure, urinary prostanoids and plasma renin activity, by ridogrel dose

Std. error bars. The only significant dose-dependent changes were for serum thromboxane B$_2$ (not shown), and plasma renin activity (PRA).
8. REPEATED DOSE STUDIES COMPARING RIDOGREL TO INDOMETHACIN, KETOPROFEN AND KETOPROFEN CO-ADMINISTERED WITH RIDOGREL

8.1 Introduction

The objectives of these experiments were first, to evaluate the effects of a longer exposure to oral ridogrel treatment on blood pressure, and second, if an antihypertensive effect was observed, to try to determine whether renal prostanoid synthesis changes in parallel with blood pressure.

8.2 Protocol

Male SHR-SP were gavage dosed every twelve hours for seven days. The treatments included vehicle (methylcellulose 4000 cps, 500 µL), one of three doses of ridogrel (1.5 mg/kg, 6.25 mg/kg, or 12.5 mg/kg), one of two non-steroidal antiinflammatory (PGHS-inhibiting) drugs (indomethacin 1.5 mg/kg or ketoprofen 5 mg/kg), and then the combination of ridogrel 12.5 mg/kg and ketoprofen 5 mg/kg. Ridogrel 12.5 mg/kg was also given to age-matched normotensive S-D rats, at the same dosing interval over a seven day period. Four plastic metabolic cages were available for use in these experiments. Accordingly, the experiments were run in treatment groups of four rats at a time. The rats were acclimatized in metabolic cages for a 24-hour period, then the cages were cleaned in preparation for a pretreatment baseline 24-hour urine collection. Tail-cuff BPs were measured and the rats were weighed, before they were dosed with vehicle or drug at zero and twelve hours after commencement of the urine collection period. Preweighed excess rations of R-M-H 3000 rat chow and distilled water were provided each day, and at the end of each day, the food and water remaining was subtracted to calculate consumption. Urine was also collected for each 24 hour period over the seven days of treatment, in conical urine collection tubes surrounded by an ice water bath. The change-
over each day involved a thorough cleaning of the urine collection portion of the cage, weighing the rats, gravimetric quantitation of urine output, of food consumption and of water consumption. The urine samples were centrifuged at 980 x g x 10 min, to remove suspended contaminants. The supernatant was transferred into pre-weighed vials and quantified gravimetrically assuming 1 g = 1 mL. Urine samples were then stored frozen at -20°C, awaiting radioimmunoassay of TxB₂ and 6-keto-PGF₁α, and measurement of urinary creatinine and sodium. At the end of the 24 hour period on Day#7, the rats were weighed, and a final gavage dose of the corresponding drug was administered. Approximately two hours after dosing, a post-treatment series of SBP determinations were made and averaged, prior to anaesthetizing the rat with combined ketamine 70 mg/kg and xylazine 8 mg/kg, to permit blood collection by cardiac puncture. Blood samples for PRA and serum TxB₂ were prepared and stored as described in section 7.2 (P. 92). The rats were euthanized under anaesthesia by intracardiac injection of KCl.

8.3 Statistical methods

A one-way analysis of variance for baseline SBP was carried out, and followed by post-hoc Student-Newman-Keuls all-pairs comparisons, to determine whether pre-treatment SBP differences existed between any of the groups of SHR-SP. Paired Student's t-tests were used to compare baseline to Day#7 SBPs within groups. Between-groups comparison of changes in SBP were made by one-way analysis of variance, and where there were significant F-tests, post-hoc means comparisons were made using the Fisher's Protected Least Significant Differences multiple t-test procedure. The same analysis was used for between-groups comparison of plasma renin activity. Multivariate analysis of variance was used to evaluate changes in the urinary creatinine-corrected excretion rates of TxB₂ and 6-keto-PGF₁α, urine sodium excretion, sodium and water intake, and changes in body
weight. The statistics were performed using the SPSS® statistical software release 5 (SPSS Inc, Chicago IL), run on a 486PC, and using the SuperANOVA® statistical software package (Abacus Concepts Inc, Berkely, CA), run on a Macintosh LE computer.

8.4 Results

Figure 8.1 and Table 8.1 summarize SBP measurements in each group of rats before and after twice-daily dosing over seven days. The Sprague-Dawley rats had systolic blood pressures significantly lower than the 12-week old SHR-SP males. There were no significant pre-treatment SBP differences between any of the groups of SHR-SP rats. Following seven days of twelve-hourly dosing, the vehicle-treated group demonstrated a progression to higher systolic pressures. In contrast, SHR-SP rats that were treated with ridogrel, had systolic pressures at the end of Day#7 that were lower than baseline, and the mean reduction from baseline increased with dose (Figure 8.2). At all doses of ridogrel, the change from pre-treatment to Day#7 was significant compared to vehicle treated rats. The blood pressure reduction from their respective baselines, did not approach a 0.05 level of statistical significance at the two lower doses of ridogrel (1.5 mg/kg and 6.25 mg/kg) however, the mean systolic pressure for the group of SHR-SP rats treated with ridogrel 12.5 mg/kg, was significantly lower than baseline, and was markedly lower than the vehicle treated group (95% confidence interval for the difference: -71.2 to -31.8, unpaired t=5.519, df=21, two-tailed p<0.0001). Figure 8.3 shows the SBP measurements for individual rats before and after repeated dosing with vehicle or ridogrel 12.5 mg/kg, and demonstrates the variability in the antihypertensive response to ridogrel, even at the highest dose tested. SBPs among S-D rats likewise treated with ridogrel 12.5 mg every twelve hours for seven days, showed no change. Ridogrel does not evoke a hypotensive response.

Over the treatment period, the urinary TxB2 excretion rates were reduced from
Figure 8.1  Group comparisons of indirect systolic blood pressures before and after seven days of treatment
Table 8.1  Comparison of baseline with post-treatment indirect systolic blood pressure measurements between groups

| GROUP       | CONTROL vehicle q12h | Ridogrel 1.5mg/Kg q12h | Ridogrel 6.25mg/Kg q12h | Ridogrel 12.5mg/Kg q12h | Indometacin 1.5mg/Kg q12h | Keto-profen 5mg/Kg q12h | Ket.5mg+Rid.12.5mg/Kg q12h | S-D rats
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>MEAN</td>
<td>184</td>
<td>224</td>
<td>208</td>
<td>192</td>
<td>179</td>
<td>156</td>
<td>200</td>
<td>173</td>
</tr>
<tr>
<td>n=</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SEM</td>
<td>8.6</td>
<td>6.3</td>
<td>11.3</td>
<td>4.9</td>
<td>11.7</td>
<td>7.8</td>
<td>6.1</td>
<td>6.7</td>
</tr>
<tr>
<td>Md</td>
<td>175</td>
<td>225</td>
<td>209</td>
<td>195</td>
<td>178</td>
<td>152</td>
<td>202</td>
<td>163</td>
</tr>
<tr>
<td>95:L</td>
<td>165</td>
<td>210</td>
<td>172</td>
<td>177</td>
<td>142</td>
<td>131</td>
<td>187</td>
<td>159</td>
</tr>
<tr>
<td>95:U</td>
<td>203</td>
<td>238</td>
<td>244</td>
<td>208</td>
<td>216</td>
<td>181</td>
<td>213</td>
<td>187</td>
</tr>
<tr>
<td>min</td>
<td>152</td>
<td>180</td>
<td>180</td>
<td>179</td>
<td>154</td>
<td>131</td>
<td>153</td>
<td>137</td>
</tr>
<tr>
<td>max</td>
<td>229</td>
<td>260</td>
<td>234</td>
<td>202</td>
<td>207</td>
<td>181</td>
<td>234</td>
<td>217</td>
</tr>
</tbody>
</table>

**DIFFERENCES**

| Mean_{diff} | 39.6                | -15.3                 | -23.3                   | -27.1                   | -3.0                      | 26.1                      | 23.0                         | 9.8              |
| 95:L_{diff} | 17.3                | -36.5                 | -57.8                   | -45.7                   | -58.3                     | -14.9                     | -34.2                        | -7.2             |
| 95:U_{diff} | 61.9                | 6.0                   | 11.3                    | -8.6                    | 52.3                      | 67.1                      | 80.2                         | 26.7             |
| p=          | 0.0015              | 0.1069                | 0.1503                  | 0.0056                  | 0.8987                    | 0.1703                    | 0.3633                        | 0.2099           |
Figure 8.2  Comparison of blood pressure changes (Day#7 SBP minus Day#0 SBP) across treatment groups, normalized to the within group baselines

Error bars = 95% CI
Figure 8.3  Blood pressure changes for individual rats in the vehicle and ridogrel 12.5 mg/kg treated groups

Bars represent baseline measurements, ▲ represent follow-up SBP measured on Day#7.
Upper panel  Vehicle-treated rats [n = 11].
Lower panel  Ridogrel 12.5 mg/kg treated rats [n = 12].
baseline in all except the vehicle group. Ketoprofen 5 mg/kg every 12-hours was less effective than indomethacin or ridogrel, but the combination of ketoprofen 5 mg/kg with ridogrel 12.5 mg/kg produced the most profound reduction in urinary TxB₂. Treatment differences in \textit{ex vivo} serum TxB₂ inhibition are compared in Figure 8.4. Compared to vehicle treated rats, all treatments significantly inhibited clot generation of TxB₂. \textit{Ex vivo} thromboxane inhibition by ridogrel significantly increased with dose (Table 8.2). Ridogrel treatment was associated with significant dose-related increases in PRA (Table 8.3 and Figure 8.6). Figure 8.7 shows the change in 6-keto-PGF₁α urinary excretion rates. In ridogrel treated rats, the change in 6-keto-PGF₁α urinary excretion varied less, and was above the 50th percentile of vehicle treated rats, but no statistically significant increase above baseline occurred over the treatment period.

Table 8.4 reveals no significant difference in sodium balance averaged over the seven day treatment period, whereas Figure 8.8 suggests that the reduction in blood pressure comparing Day#7 to baseline, was associated with sodium retention. Given the steepness of the renal function curve, it is probably less than would be expected if only a reduction in renal perfusion pressure had been imposed, e.g., by suprarenal constriction of the aorta (Nagaoka et al, 1981); (Roman & Zou, 1993); (Beierwaltes, Arendshorst & Klemmer, 1982).

4.2.4.5 Discussion

Repeated dosing experiments, examined whether extended combined TP receptor antagonism and inhibition of thromboxane synthase activity by ridogrel, would have an antihypertensive effect during the steep phase of hypertension development in untreated 12 week old male SHR-SP rats. Ridogrel not only prevented the progression of hypertension that occured in vehicle-treated SHR-SP males, but was associated with a dose-related reduction. At 12.5 mg/kg every 12-hours, the BP reduction was significant compared to both the vehicle and baseline. As was shown in Figure 8.3 (page 109), all of the vehicle-
Figure 8.4  Treatment group comparison of *ex vivo* clot generation of thromboxane B$_2$ at Day#7

Error bars span the 95% CI
Table 8.2  Ridogrel dose-dependent inhibition of \textit{ex vivo} colt generation of thromboxane \(B_2\)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>linear: between columns</td>
<td>1</td>
<td>42803.0</td>
<td>42803.0</td>
</tr>
<tr>
<td>non-linear: between columns</td>
<td>4</td>
<td>33014.0</td>
<td>8253.5</td>
</tr>
<tr>
<td>residual: within columns</td>
<td>23</td>
<td>8570.2</td>
<td>372.6</td>
</tr>
<tr>
<td>totals:</td>
<td>28</td>
<td>84387.0</td>
<td></td>
</tr>
</tbody>
</table>

slope=-11.564, \(r^2=0.5072\), \(F=114.8698\), \(p<0.0001\)

Table 8.3  Ridogrel dose-dependent enhancement of plasma renin activity

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>linear: between columns</td>
<td>1</td>
<td>449.82</td>
<td>449.82</td>
</tr>
<tr>
<td>non-linear: between columns</td>
<td>3</td>
<td>73.26</td>
<td>24.42</td>
</tr>
<tr>
<td>residual: within columns</td>
<td>16</td>
<td>423.99</td>
<td>30.29</td>
</tr>
<tr>
<td>totals:</td>
<td>18</td>
<td>947.07</td>
<td></td>
</tr>
</tbody>
</table>

slope 3.570, \(r^2=0.4750\), \(F=14.8529\), \(p<0.0018\)
Figure 8.6  Treatment group comparison of *ex vivo* plasma angiotensin I generation rate (plasma renin activity)

Error bars span the 95% CI
SHR-SP males 12th to 13th wk

Vehicle gavaged q12h X 1-wk

Ridogrel 12.5mg/Kg q12h X 1-wk

24-hr observation period

Figure 8.7 Superimposed boxplots showing the change in 24 hour urinary 6-keto-prostaglandin F₁α excretion rates over the 7 day treatment period, comparing vehicle and ridogrel-treated rats

Corrected to urinary creatinine. The lighter boxplots represent the daily average change from baseline of 4 vehicle-treated rats, and the darker superimposed boxplots represent that of rats gavaged every 12-hours with ridogrel 12.5 mg/kg.
Table 8.4  Comparison of sodium intake, sodium excretion, and cumulative sodium balance in SHR-SP rats pretreated with ridogrel 12.5 mg/kg v. control

<table>
<thead>
<tr>
<th>Sodium Intake (mmol. per 24 hours):</th>
<th>TREATMENT GROUP</th>
<th>MEAN</th>
<th>SE OF MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (Vehicle)</td>
<td>3.286</td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>Ridogrel 12.5mg.</td>
<td>3.320</td>
<td>0.072</td>
<td></td>
</tr>
</tbody>
</table>

Mean difference = 0.034  
C.I. diff: -0.223–0.290  
p = 0.79

Equality of variances: F = 1.84  p = 0.17

<table>
<thead>
<tr>
<th>Urinary Sodium Excretion (mmol. per 24 hours):</th>
<th>TREATMENT GROUP</th>
<th>MEAN</th>
<th>SE OF MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (Vehicle)</td>
<td>2.547</td>
<td>0.103</td>
<td></td>
</tr>
<tr>
<td>Ridogrel 12.5mg.</td>
<td>2.448</td>
<td>0.065</td>
<td></td>
</tr>
</tbody>
</table>

Mean difference = 0.099  
C.I. diff: -0.348–0.150  
p = 0.42

Equality of variances: F = 2.30  p = 0.09

<table>
<thead>
<tr>
<th>Cumulative Sodium Balance (mmol. over 7 days):</th>
<th>TREATMENT GROUP</th>
<th>MEAN</th>
<th>SE OF MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (Vehicle)</td>
<td>20.115</td>
<td>0.414</td>
<td></td>
</tr>
<tr>
<td>Ridogrel 12.5mg.</td>
<td>19.292</td>
<td>0.357</td>
<td></td>
</tr>
</tbody>
</table>

Mean difference = 0.823  
C.I. diff: -1.954–0.308  
p = 0.15

Equality of variances: F = 1.23  p = 0.37

(a) Rodent chow was the only source of sodium, 0.44% w/w of food consumed.
(b) Means are daily means across the 7 day treatment period.
(c) A running total of daily sodium intake minus daily sodium output, 7 days x 4 rats/group.

Same rats as in Figure 8.8, but these tables represent the average of 7 consecutive days, whereas the figure shows only baseline v Day#7 data.
Figure 8.8  The ratio of urinary sodium excretion over sodium intake, in relation to the change in blood pressure, comparing baseline v Day#7, in one set of vehicle and one set of ridogrel 12.5 mg/kg treated rats

Arrows indicate temporal direction of change from Day#0 to Day#7. Sodium excretion decreased along with blood pressure in the ridogrel group, and increased along with the increase in blood pressure in the vehicle-treated group.
treated rats progressed to higher pressures after one week, whereas the majority of ridogrel-treated rats (mostly those with higher baseline pressures) regressed to lower than baseline pressures. Even the rats which showed minimal change from baseline were remarkable, considering that rats treated with vehicle alone invariably developed increased BP over the same period.

Thromboxane synthase inhibition alone cannot account for the blood pressure modulating effects of ridogrel. Indeed, indomethacin, and even more so the combination of ketoprofen and ridogrel, produced comparable or greater reductions in both urinary excretion rates and \textit{ex vivo} clotting capacity to generate TxB$_2$, but those treatments were not associated with BP reduction. Since cyclooxygenase inhibition attenuated the antihypertensive action of ridogrel, it suggests that redirection of endoperoxide intermediates toward synthesis of principally vasodilator prostanoids was important, and may act unopposed in tandem with TP receptor antagonism. Increased adenylyl cyclase activity leading to elevated intracellular cAMP, is the dominant signal transduction mechanism enhanced by the vasodilator prostaglandins. Enhancement of cAMP is likely to elevate the threshold for vascular smooth muscle contraction, even in response to vasoconstrictors such as norepinephrine, angiotensin II, aginine vasopressin and serotonin. Tesfamariam & Ogletree (1995) have demonstrated that one- and two-week treatment with the TP antagonist ifetroban did not affect blood pressure, but normalized endothelium-dependent vasorelaxation in spontaneously hypertensive rats. It is possible, that if the investigators had included a TSI to enhance prostaglandin synthesis, a BP reduction would have occurred. Badahman & Wilson (1994) have demonstrated that, while the thromboxane synthase inhibitor furegrelate alone prevented angiotensin II-induced renal vascular vasoconstriction, the combination of furegrelate with the TP receptor antagonist SQ29,548 resulted in a reduction in vascular resistance to well below baseline. In effect, angiotensin II became a vasodilator as opposed to merely a less potent vasoconstrictor, as was the extent of effects with either TSI or TPA alone. PGHS inhibition with ketoprofen prevented the antihypertensive effect of ridogrel, thus unopposed vasodilator prostanoid
enhancement appears requisite to the BP modulating effect of extended treatment. A leftward shift in the renal function curve was not evident. The question remains whether an equivalent reduction in renal perfusion pressure by a distinct mechanism, such as by servo-controlled suprarenal aortic stenosis, would have resulted in less sodium excretion than with ridogrel.
9. RESPONSES TO PRESSOR AGENTS IN ANÆSTHETIZED RATS

9.1 Introduction

It was previously demonstrated that combined TSI with TPA pretreatment resulted in a renal vasodilator response to angiotensin II. Presently, extended pretreatment with ridogrel 12.5 mg/kg reduced blood pressure in 12-week old male SHR-SP rats. It seemed plausible that ridogrel might indeed modulate the pressor effects of physiologically relevant pressor agents. Several endogenous vasopressors, in addition to having direct vasopressor activity, also stimulate the release of arachidonate from hormone-responsive pools (Figure 9.1). Ridogrel pretreatment potently inhibits thromboxane synthase activity, so that more platelet-derived endoperoxides may be available for vascular synthesis of prostacyclin. In addition, TP receptor antagonism would prevent opposing vasoconstrictor effects of the endoperoxides themselves, and any alternative or preformed TP receptor agonists (such as isoprostanes) that may be liberated with arachidonic acid. The objective was to assess whether ridogrel pretreatment modulates vascular resistance to such an extent, that a net reduction in BP might be detected at the level of the systemic circulation.

9.2 Protocols

9.2.1 Maximum subpressor dose-response to infused angiotensin II.

As a preliminary project, an assessment was made to establish that SHR-SP rats were more sensitive to angiotensin II in terms of both the dose required to elicit a systemic pressor response, and the corresponding change in renal vascular resistance at a maximum

119
Figure 9.1 In addition to their direct actions, several vasoactive hormones stimulate arachidonic acid turnover.
subpressor dose. Eleven SHR-SP and six WKY rats were anesthetized with combined ketamine 80 mg/kg, xylazine 8 mg/kg, and atropine 0.04 mg/kg intramuscularly and instrumented with a left carotid arterial line (PE-50 O.D.: 0.965 mm, I.D.: 0.58 mm). The left internal jugular vein was cannulated with two PE-10 (O.D.: 0.61 mm, I.D.: 0.28 mm)(Intramedic®, Clay Adams Div. of Becton Dickinson & Co., Parsippany, NJ). The three lines were passed subcutaneously to exit at the nape, and enclosed in a plastic guard. The urinary bladder was cannulated with a double-flanged 15 ga tube via a small abdominal incision. The proximal flange was retained within the bladder by a purse-string, and the second flange was stabilized by sutures between the muscle and skin so that the distal tip protruded beyond the skin closure. The rats recovered over 48 hours, then they were placed in a plexiglass restrainer and the carotid line attached to a calibrated pressure transducer (P10EZ, Viggo-Spectramed, Oxnard, CA). The analog input from the transducer was fed to a model 7P polygraph recording system(Grass Inst. Co., Quincy, MA). One of the venous lines was attached to a 10 mL syringe via a 30 ga needle to deliver $[^{14}\text{C}]$PAH 0.0118 μCi/min with a syringe pump (Model 901, Harvard Apparatus Co., Dover, MA). The other venous line was used to deliver a loading dose of $[^{14}\text{C}]$PAH 0.4 μCi before attaching it via a 30 ga needle to a 0.9% NaCl solution, infused at 0.018 mL/min by a second syringe pump (Model 975, Harvard Apparatus Co., Boston, MA). When the baseline arterial pressure stabilized, a timed urine collection was commenced. The point at which at least 50 μL of urine was obtained, served as the midpoint of the collection period. At that midpoint, a 250 μL blood sample was drawn from the carotid line, then the line was flushed with 250 μL of saline. At an equal time after the midpoint, the urine collection was concluded. The venous line that had been infusing saline was switched to saline containing angiotensin II, and an infusion was commenced at 1 ng/kg/min, then increased by increments of ≥2 ng/kg/min, every five minutes until a ≥ 5 mm Hg increase in mean arterial pressure was observed. At that point, the infusion rate was tapered back by approximately 1 ng/kg/min, until the pressor response was extinguished (the syringe pump infusion rate increments did not allow for a uniformly additive dose change). Finally, the infusion rate was returned again to the presumed
pressor dose, to confirm the pressor effect. The maximum subpressor dose was thus recorded for each rat, and that dose was maintained during a second $[^{14}\text{C}]$PAH clearance period. The blood drawn at the midpoints of the two clearances was centrifuged, and equal aliquots of plasma and urine were dispensed into separate scintillation vials (Fisher Scientific Ltd., Ottawa, ON) to which 10 mL of scintillant (Amersham Canada Ltd., Oakville, ON) was added. Beta decay was counted for each sample, and effective renal plasma flow was calculated according to the formula:

\[
\text{ERPF} = \frac{\text{urine PAH (cpm)}}{\text{plasma PAH (cpm)}} \times \frac{\text{urine vol (\mu L)}}{\text{time period (min.)}} \times \frac{100 \text{g}}{\text{rat wght (g)}}
\]

An estimate of renal vascular resistance was calculated according to the formula:

\[
\text{Estim. RVR} = \frac{\text{MAP (mm Hg)}}{\text{ERPF}}
\]

units: mm Hg/mL/min/100g.

9.2.2 Effect of ridogrel on pressor responses to a graded dose infusion of angiotensin II

In order to compare rats pretreated with ridogrel to those pre-treated with vehicle, this experimental protocol examined pressor responses to a graded dose infusion of angiotensin II. Male 12-week old SHR-SP were dosed by gavage with either vehicle ($n=3$, methylcellulose viscous 4000 cps), or ridogrel 12.5 mg/kg every 12 hours for two days = four doses ($n=4$ rats). Approximately two hours after the last dose, the rats were anaesthetized with the long-acting injectable combination $\alpha$-chloralose 80 mg/kg and ethyl carbamate 400 mg/kg i.p. The left carotid artery and the left jugular vein were cannulated as described in the previous protocol. The carotid line was attached to the pressure transducer, and measurements were made from polygraph chart recordings. The venous lines were attached to a mini syringe pump (model 975, Harvard Apparatus, Boston, MA).
A stable 15-minute baseline arterial pressure was demonstrated during 0.26 mL/minute infusion of sodium carbonate 2 mM solution, prior to initiating infusion of angiotensin II. A rate-controlled infusion of angiotensin II was titrated incrementally between 5, 10, 20, 40, and 80 ng/kg/min, with up to 20-minute vehicle intermissions between each dose, to allow arterial pressure to return to baseline.

9.2.3 Ridogrel effects on pressor responses to bolus injections of the TP receptor agonist U46,619, norepinephrine and angiotensin II, in anaesthetized rats

Ridogrel is a potent TSI, but a relatively modest TP receptor antagonist. While 12.5 mg/kg every 12-hours is four times the per kilogram ridogrel dose required in humans to produce a 70 per cent inhibition in ex vivo U46,619-induced platelet aggregation, its absorption, bioavailability and clearance may differ for juvenile male SHR-SP during repeated dosing. This dose-response study was performed, in part, to establish that effective TP receptor antagonism was achieved after 7 days of twice-daily dosing with ridogrel 12.5 mg/kg. In addition, the use of this preparation was extended to assess the relative pressor responses to a range of bolus injected doses of norepinephrine and angiotensin II.

Eight rats (SHR-SP males at 11-weeks of age) were assigned to treatment: n=4 per group to receive either ridogrel 12.5 mg/kg in 0.5 mL of a methylcellulose viscous vehicle by gavage every 12-hours, or gavaged with vehicle alone on the same dosing schedule. On day zero, the rats were weighed, and preliminary SBPs and heart rates were measured by the indirect tail-cuff method. On day seven, approximately two hours after the last dose of ridogrel, weight, SBP and heart rate were repeated using the same methods. Subsequently, each rat was anaesthetized with halothane. The left carotid artery was cannulated with PE50 tubing, and the left jugular vein cannulated with PE10 tubing. The carotid line was attached via a pressure transducer (Transpac®️, Abbott Critical Care
Systems, North Chicago, IL), to the polygraph chart recorder, and calibrated to mercury for direct carotid arterial blood pressure monitoring. Once a stable pressure wave was demonstrated, U46,619 0.5 μg (in 100 μL volume of 20% K-H buffer) was injected as a bolus through the jugular venous line. Pressure was allowed to return to baseline before 2.5 μg of U46,619 was bolus injected. This procedure was repeated with U46,619 at doses of 5.0, 10, and 20 μg, then norepinephrine at doses of 0.1, 1.0 and 10μg (in 100 μL volume of normal saline), then angiotensin II at doses of 10, 20, 40, 80 and 160 ng (in 100 μL volume of normal saline). Finally, the three drugs were administered at very high doses: angiotensin II 400 ng, norepinephrine 40 μg and U46,619 40 μg. When the measurements concluded, the halothane concentration was increased, and the rats were euthanized by an intravenous injection of KCl.

9.3 Statistical methods

9.3.1 Maximum suppressor dose-response to infused angiotensin II.

To assess strain differences in the sensitivity to angiotensin II, the maximum suppressor dose found for each rat was entered as the dependent variable in an analysis of variance, with strain as the independent variable. A one-way ANOVA was also used to assess strain-dependent changes in MAP and estimated RVR at baseline, and at the maximum suppressor dose of angiotensin II. Paired t-tests were used to compare within-strain changes in MAP and estimated RVR, between baseline and at the maximum suppressor dose of angiotensin II.

9.3.2 Effect of ridogrel on pressor responses to a graded dose infusion of angiotensin II

A two-way (treatment * dose) analysis of variance for repeated measures model was used, with change in arterial pressure at each dose planned as the basis for post-hoc
means comparisons by Scheffé's S test. From the results, the half-width of the 95% confidence interval was used in Stein's calculation, to estimate the sample size required to have detected a 15 mm Hg difference.

9.3.3 Ridogrel effects on pressor responses to bolus injections of the TP receptor agonist U46,619, norepinephrine and angiotensin II, in anaesthetized rats.

Pretreatment measurements of weight, systolic pressure and heart rate between treatment and control groups, were compared using unpaired t-tests. Differences between preliminary (Day Zero) and final (Day Seven) measurements were made by paired t-tests. Areas under the acute systolic pressor-response curves were compared, by excising the tracing from the chart recorder paper, and entering the weights of the paper for each drug \times dose \times rat in an analysis of variance for repeated measures. Treatment (ridogrel or vehicle) and nested dose for each drug were the independent variables. Since one of the control rats was lost due to surgical misadventure, it resulted in an unbalanced number of observations within cells. Mean square estimates were used to compute quasi F tests by Satterthwaite's procedure, and orthogonal contrasts were used for treatment \times dose-within-agonist single degree of freedom comparisons. Within-treatment means of paper weights, representing the area under the peak systolic pressor-response curves at each dose of corresponding agonists, were subjected to independent t-tests, from which 95% confidence error bars were constructed.

9.4 Results

9.4.1 Maximum subpressor dose-responses to infused angiotensin II

The results are summarized in Table 9.1. At baseline, the MAP differed
Table 9.1  Results of strain-dependent maximum subpressor angiotensin II dose-finding experiments

<table>
<thead>
<tr>
<th></th>
<th>SHR-SP</th>
<th></th>
<th>WKY</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>@ 16-weeks:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditions</td>
<td>Basal</td>
<td>Angiotensin II</td>
<td>Basal</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Ang II Dose</td>
<td>0</td>
<td>8.2 ± 1.4</td>
<td>0</td>
<td>10.0 ± 2.3</td>
</tr>
<tr>
<td>(ng/Kg/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>135 ± 4</td>
<td>137 ± 5</td>
<td>67 ± 3</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>C[14C]PAH (mL/min)</td>
<td>3.28 ± 0.21</td>
<td>1.49 ± 0.07</td>
<td>8.78 ± 0.50</td>
<td>5.16 ± 0.25</td>
</tr>
<tr>
<td>RVR estimate MAP/C[14C]PAH</td>
<td>41.5 ± 5.2</td>
<td>92.7 ± 11.1</td>
<td>7.7 ± 1.1</td>
<td>13.7 ± 1.5</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>11</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>
significantly between SHR-SP and WKY rats: difference = 68 mm Hg, 95% CI$_{\text{diff}}$ = 57 to 79, $p<0.0001$. What was defined as the maximum subpressor dose of angiotensin II, was not statistically different between strains:

\[
\text{SHR-SP} = 8.2 \pm 1.4 \text{ ng/kg/min};
\]
\[
\text{WKY} = 10.0 \pm 2.3 \text{ ng/kg/min}.
\]

and the within-strain changes from baseline MAP in response to these doses, were not significant.

Strain differences in the baseline surrogate estimates of RVR were highly significant: difference = 33.8 mm Hg/mL/min/100 g, 95% CI$_{\text{diff}}$ = 21.9 to 45.6, $p<0.0001$; as were strain differences in estimated RVR responses to the maximum subpressor doses of angiotensin II: difference = 79.9 mm Hg/mL/min/100 g, 95% CI$_{\text{diff}}$ = 54.0 to 103.9, $p<0.0001$. Strain differences in the magnitude of within-strain change from baseline, differed markedly as well: difference = 43.2 mm Hg/mL/min/100 g, 95% CI$_{\text{diff}}$ = 24.7 to 61.7, $p<0.0004$.

9.4.2 Effect of ridogrel on pressor responses to a graded dose infusion of angiotensin II

The results are summarized in Table 9.2. Although not statistically significant, the mean pressor responses to angiotensin II at doses between 10.0 to 40.0 ng/kg/min were lower in rats pretreated with ridogrel (Figure 9.2).

9.4.3 Ridogrel effects on pressor responses to bolus injections of the TP receptor agonist U46,619, norepinephrine and angiotensin II, in anaesthetized rats.

Pretreatment weight, indirect systolic blood pressure, and heart rate were virtually identical for rats assigned to either group. At treatment Day#7, weight had increased by:
Table 9.2  Mean arterial pressure dose-response to angiotensin II infusions in SHR-SP pretreated with ridogrel 12.5 mg/kg vs. control SHR-SP given vehicle alone

<table>
<thead>
<tr>
<th>ANG II inf. dose</th>
<th>TREATMENT</th>
<th>Between group differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>log dose</td>
<td>ng/kg/min</td>
<td>vehicle, n=2</td>
</tr>
<tr>
<td>1.00</td>
<td>10.0</td>
<td>9.4 ± 6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.31</td>
<td>20.6</td>
<td>13.7 ± 13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.60</td>
<td>40.0</td>
<td>26.7 ± 16.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9.2  Dose-response curves showing the effect of angiotensin II on mean arterial pressure in SHR-SP pretreated with ridogrel v vehicle

Rats were pretreated with ridogrel 12.5 mg/kg (n=3), or methylcellulose vehicle (n=2), by gavage every 12 hours × 4 doses. Two hours after the last dose, rats were anaesthetized (α-chloralose + ethylcarbonate) and cannulated for carotid pressure monitoring during jugular venous infusion of angiotensin II.
control = 14 ± 3 g; ridogrel = 11 ± 3 g, the between-groups difference was not significant. Heart rates had not changed significantly: control = 9 ± 5; ridogrel = 4 ± 6, and were not different between groups at treatment Day#7. Indirect systolic blood pressure changes were in opposite directions: control = 13; ridogrel = -11; difference = 23 mm Hg, 95%CI_{diff} = -68 to 20, p=0.2261.

Comparisons of areas under the systolic pressor-response curves are presented in Figure 9.3. The responses to the lowest doses and to the highest doses administered were not different, so that for all three agonists the curves converged at both ends within the range of doses used, with statistically significant separation at intermediate doses.

9.5 Discussion regarding responses to pressor agents in anaesthetized rats

The results comparing the maximum subpressor dose of angiotensin II in SHR-SP to WKY rats, suggest that the renal vasculature was sensitive to the vasoconstrictor effects of angiotensin II, at doses that did not increase the mean arterial pressure. Compensatory mechanisms may resist acute angiotensin II-induced changes in mean arterial pressure, but physiological parameters such as peripheral vascular resistance and cardiac output were not measured in these experiments. However, the results do support that, in addition to SHR-SP rats having a higher baseline renal vascular resistance, their renal vasculature is also more sensitive, given that they responded with greater reductions in renal blood flow at any given dose of angiotensin II in comparison to WKY rats. The difference in maximum subpressor doses defined for each strain differed by 1.8 ng/kg/min in this underpowered experiment. Sample size calculations based on these results predict that an additional eight to ten rats per group would have been required to detect a minimum difference of 1.8 ng/kg/min. Inadequate sample size was also evident in the experiments comparing mean arterial pressure responses to infused angiotensin II in SHR-SP rats treated with either ridogrel or vehicle. Stein's calculation estimated a sample size
Figure 9.3 Areas under the systolic pressor-response curves, comparing the responses to three agonists in ridogrel-pretreated vs vehicle-treated SHR-SP

Rats were pretreated with ridogrel 12.5 mg/kg (n=4), or methylcellulose vehicle (n=3), by gavage every 12 hours x 7 days. Two hours after the last dose, under halothane anaesthesia, rats were cannulated for carotid arterial pressure monitoring during jugular venous bolus injections of the agonists. Error bars span 95% CIs.
of eight rats per group would have been required to test the hypothesis that ridogrel blunted the pressor response to angiotensin II. Moreover, differences may have increased if ridogrel pretreatment had been extended for more than two days. Variability in between-subject baseline blood pressures following anaesthesia was of concern, as was the question of whether tachyphylaxis occurred over the course of an infusion; so rather than pursue these experiments, the approach was revised to use bolus doses and include other pressor agents. The switch to halothane was made to permit better control over the depth of anesthesia as gauged by the minimum concentration of halothane required to extinguish the corneal and jaw reflexes. While the dose-responses to U46,619 were lower in ridogrel pretreated rats, the dose-response curve were not as flattened or rightwardly shifted as anticipated. The competitive TP receptor antagonism afforded following subchronic dosing with ridogrel might still be rather modest at 12.5 mg/kg. The paper-cutting method used to compare areas under the peak systolic pressor-response curve was admittedly crude (Figure 9.4); and since heart rate was not included as a covariate during anesthesia, if an increase in heart rate contributed to the pressor effect it might have also exaggerated it as a result of a greater ink saturation of the chart paper within the area under the curve. Taken together, the results support that ridogrel pretreatment increases the threshold for agonist-induced vascular contractions. The effect is not restricted to the modulation of pressor responses to agonists that directly stimulate TP receptors, although TP receptor mediated effects may contribute indirectly to the net pressor effects of agonists such as norepinephrine and angiotensin II.
This portion of tracing excised and weighed:

Figure 9.4  Example of the approach to measuring the area under the systolic pressor-response to a bolus injection of norepinephrine 10μg/kg

A scalpel blade was used to cut along the outside (systolic) edge of the tracing, from the beginning of the pressor response to where the systolic pressure returned to baseline. The paper was excised along a straight line connecting the preinjection diastolic pressure to the re-established baseline, and weighed on a precision balance.
10. SUMMARY AND CONCLUSIONS

In summary, intricate mechanisms have evolved so that under physiologic conditions, blood is maintained in a fluid state. Haemostasis involves coordinate thrombotic and vasospastic mechanisms, which are capable of responding explosively when a vessel wall is breached. The endothelium is placed strategically between the blood and the vessel wall. The functional integrity of the endothelium has a principal role in the homeostatic regulation of platelet-vessel wall interactions, and the mediation of both vascular tone and haemostasis. Arachidonic acid is the dominant substrate for cyclic endoperoxide synthesis by the PGHS complex. Cyclic endoperoxides in turn, serve as the common substrate for the synthesis of both TxA$_2$ and PGI$_2$. Platelets are highly capable of synthesizing TxA$_2$, but not PGI$_2$. Conversely, the vessel wall is efficient at synthesizing PGI$_2$, but produces little TxA$_2$. Importantly, in the SHR, vascular PGHS is less active than in platelets, and the diffusion of platelet-derived endoperoxides appears to be a major source of substrate for the vascular synthesis of PGI$_2$. Despite a striking overlap of the biochemical pathways by which they are synthesized, TxA$_2$ and PGI$_2$ have virtually opposite biological effects on platelet aggregation and vascular tone.

Ridogrel is an investigational drug with dual activities as a potent inhibitor of thromboxane synthase, and a modest antagonist at TP receptors. In contrast to aspirin, or to drugs which exhibit either TSI or TPA activity alone, the dual action of ridogrel exploits a biochemical interaction between platelets and the vessel wall. The TSI action preserves the synthesis of diffusible platelet endoperoxides, while the TPA action prevents the feedback amplification of platelet aggregation and vasospasm, which would otherwise result from TP receptor activation by the endoperoxides themselves. More endoperoxide is made available to the vessel wall for PGI$_2$ synthesis. PGI$_2$ is coupled to increased intracellular cAMP generation, and is both a potent inhibitor of platelet aggregation, and a vasodilator. As a result, ridogrel may have relatively more therapeutic potential for reducing the risk of vascular catastrophies that are associated with essential hypertension,
such as myocardial infarction and stroke.

If a relative imbalance of prostanoid actions, favouring vasoconstrictor TxA₂ synthesis over vasodilator PGI₂, contributes to the elevated renal vascular resistance characteristic of the SHR and stroke-prone substrain, it is not reflected by an increased rate of urinary TxB₂ excretion, or by a reduced rate of urinary 6-keto-PGF₁α excretion. Radioimmunoassays of these metabolites in 24 hour urine samples showed a lower concentration of TxB₂ in the hypertensive rats, relative to the normotensive strains. If a reduction in renal TxA₂ synthesis is a compensatory response to the development of hypertension, it would be a compensatory mechanism that is augmented by ridogrel.

In vitro, a discrepancy was apparent between the high molar potency of ridogrel to inhibit thromboxane synthase, relative to its modest antagonism at TP receptors. A linear inhibitory concentration-response could be demonstrated for TxB₂ generation during clotting of whole blood, but arachidonic acid-stimulated whole blood platelet aggregation was not inhibited at the lower corresponding concentrations. Effective inhibition of platelet aggregation likely requires higher concentrations. At higher concentrations, sufficient TP receptor antagonism is achieved, and able to compete with the agonist effects of an accumulating concentration of cyclic endoperoxide intermediates that develops in consequence to thromboxane synthase inhibition. This limitation may be less relevant at the vascular interface in vivo, since the vascular wall may effectively metabolize platelet endoperoxides to prostaglandins and prostacyclins. Some prostaglandins and the prostacyclins have potent antiaggregatory actions.

The hypothesis that ridogrel pretreatment results in redirection of arachidonic acid metabolism toward vasodilatory prostanoid synthesis, was indirectly supported. Following extended ridogrel treatment, BP was dose-dependently reduced in SHR-SP. This antihypertensive effect was not demonstrated when the PGHS inhibitor ketoprofen was administered concurrently. Furthermore, ridogrel pretreatment modulated the pressor
effects of angiotensin II and norepinephrine, even though the degree of TP receptor antagonism to U46,619 appeared to be rather modest. This appears to support and extend previous research, which has demonstrated that TSI, TPA, or combined TSI-TPA agents modulate contractile responses in various regional vascular beds to angiotensin II (Badahman & Wilson, 1994), serotonin (DeClerck et al, 1990), endothelins (Taddei & Vanhoutte, 1986), 20-HETE (Escalante et al, 1986), somatostatin (Shirahase et al, 1993) and even the hyperresponsive myogenic reduction in arteriolar diameter associated with hypertension (Huang, Sun & Koller, 1993).

The antihypertensive efficacy of ridogrel is not immediate in its onset, it is not associated with a hypotensive effect, and it does not appear to be associated with a marked diuretic effect. As in the single dose studies contained in this thesis, the study in humans diagnosed as having mild to moderate primary hypertension, reported by Ritter et al (1993), did not detect an antihypertensive response after two doses of ridogrel. This appears to be the only other investigation with ridogrel, to look for a BP modulating effect in a hypertensive model.

In these studies, an antihypertensive response to ridogrel was demonstrated using BP measurements made seven days apart. While a single dose does not lower BP, repeated dosing does. Obviously, BP reduction begins at an intermediate point in time. The longer the delay in BP reduction, the steeper the decline would have to be. Given the uncertainty of ridogrel's effect on renal sodium excretion, it is important to determine whether a sufficient leftward shift in renal function develops. A leftward shift would be requisite to maintaining the BP reduction over an extended duration of treatment. Telemetric blood pressure measurement is an expensive prospect, but it would have permitted constant monitoring over a chronic treatment period, in unrestrained animals. Additionally, bona fide renal function studies and haemodynamic studies would have been required to compare ridogrel treatment against control, and more precisely characterize the renal response to ridogrel.
11. FUTURE DIRECTIONS

There is now impetus to pursue the investigation of the antihypertensive effect of TSI-TPA drugs, using the more sophisticated BP telemetric methodology. The ability to measure cardiac output along with BP, would aid in understanding the haemodynamic effects of ridogrel.

Further studies could be refined to assess the spectrum of the pressor-response modulating effect of ridogrel. The data acquisition equipment used for this thesis, has since been upgraded to a digital system, which will permit much higher data resolution. The anaesthesia can be improved by using a 70% nitrous oxide/30% oxygen gas flow, so that as little as 0.5% isoflurane will achieve a stable depth of anaesthesia, with far less cardiovascular depression than occurred with halothane alone. With this refined method, it is practical to explore whether ridogrel pretreatment will blunt the pressor dose-response to other agonists in a reproducible manner. It would be of interest to include attenuation of the pressor effects of endothelin-1, angiotensin II, 5-hydroxytryptamine, norepinephrine, adrenalin, N-omega-nitro-L-arginine (NOLA), arginine vasopressin, somatostatin, and interleukin-2.

It is also relevant to explore whether these are class effects, reproducible with other TSI-TPA drugs, for example (Z)D-1542 and DT-TX30SE. Further efforts are warranted to ascertain whether enhanced synthesis of vasodilator prostanoids, and their actions through signal transduction mechanisms coupled to increased intracellular cAMP generation, account for a broad-spectrum antipressor effect. It should be confirmed whether PGHS inhibition eliminates the antipressor effect, and in the presence of PGHS inhibition, whether dibutyryl cAMP or fenoldopam would restore the effect. In combination with a PDE4 (cAMP-specific) phosphodiesterase inhibitor such as rolipram, R80122 or 3-isobutyl-1-methylxanthine (IBMX), a greatly augmented antipressor effect of TSI-TPA pretreatment might result, relative to the effect of the phosphodiesterase inhibitor alone.
12. REFERENCES


Brass LF, Shaller CC, Belmonte EJ. Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein dependent mechanism distinct from that of thrombin. *J. Clin. Invest.* 1987;79:1269-1275.


Irvine RF. How is the level of free arachidonic acid controlled in mammalian cells? *Biochem. J.* 1982;204:3-16.


13. APPENDICES
Appendix A:

Nutritional monograph for Prolab R-M-H 3000 rodent chow, Agway Ltd., NY.
### Nutrient Content

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>22.5%</td>
</tr>
<tr>
<td>Fat</td>
<td>5.5%</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1.5%</td>
</tr>
<tr>
<td>Fibre</td>
<td>4.5%</td>
</tr>
<tr>
<td>Ash</td>
<td>5.5%</td>
</tr>
<tr>
<td>Gross energy</td>
<td>4.4 Kcal/g</td>
</tr>
<tr>
<td>Digestible energy</td>
<td>3.7 Kcal/g</td>
</tr>
<tr>
<td>Metabolizable energy</td>
<td>3.5 Kcal/g</td>
</tr>
</tbody>
</table>

### Mineral Content

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>0.97%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.85%</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.95%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.44%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.21%</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.15%</td>
</tr>
<tr>
<td>Iron</td>
<td>289 mg/kg</td>
</tr>
<tr>
<td>Copper</td>
<td>23.0 mg/kg</td>
</tr>
<tr>
<td>Fluorine</td>
<td>35.0 mg/kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>61.7 mg/kg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.18 mg/kg</td>
</tr>
<tr>
<td>Zinc</td>
<td>84.0 mg/kg</td>
</tr>
<tr>
<td>Iodine</td>
<td>1.1 mg/kg</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.96 mg/kg</td>
</tr>
</tbody>
</table>

### Amino Acid Content (%)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.22</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.63</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.34</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.44</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.10</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.35</td>
</tr>
<tr>
<td>Histadine</td>
<td>0.51</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.10</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.83</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.41</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.42</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.13</td>
</tr>
<tr>
<td>Proline</td>
<td>1.34</td>
</tr>
<tr>
<td>Serine</td>
<td>1.06</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.91</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.27</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.83</td>
</tr>
<tr>
<td>Valine</td>
<td>1.15</td>
</tr>
</tbody>
</table>

### Vitamin Content

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>IU/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>20,229.0</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>1,045.0</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>55.62</td>
</tr>
<tr>
<td>Thiamine</td>
<td>11.50 mg/kg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>10.38 mg/kg</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>21.92 mg/kg</td>
</tr>
<tr>
<td>Niacin</td>
<td>72.45 mg/kg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>8.84 mg/kg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.67 mg/kg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.40 mg/kg</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.06 mg/kg</td>
</tr>
<tr>
<td>Choline</td>
<td>1,542.0 mg/kg</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.97 mg/kg</td>
</tr>
</tbody>
</table>
Appendix B:

Validation of indirect tail-cuff systolic blood pressure technique by comparison with direct carotid arterial catheter systolic blood pressure measurements.
Appendix B:
Validation of the indirect tail-cuff systolic blood pressure measurement method:

Introduction

Measurements were undertaken to assess the level of concurrence between direct arterial blood pressure measurement, and the indirect tail-cuff methodology used for this thesis work.

Methods for indirect blood pressure measurement have been employed for years (Byrom & Wilson, 1938; Williams, Harrison & Grollman, 1939). Direct measurement is invasive, and despite various swivel devices which permit a reduced level of restraint, the occlusion of a major artery and the presence of an intravascular foreign body were likely to have a confounding impact on platelet activation and prostanoid synthesis (Popp & Brennan, 1981; Bünag, 1991; Waynforth & Flecknell, 1992). Telemetry blood pressure monitoring systems, offer a compromise between obtrusive and invasive methods, but were prohibitively expensive in this instance. Various types of sensors have been used for indirect measurement. All sensors detect the re-emergence of blood flow, distal to a tourniquet calibrated to manipulate the barrier pressure surrounding a proximal segment of an appendage (usually the caudal artery). Several variables have been noted to affect the accuracy of indirect tail-cuff measurements. Examples include the matching of tail circumference to the width of the occluding cuff, the anatomically defined cephalo-caudal pressure gradient in the tail, confounding vasoconstriction of the vessel below 38.1±0.6°C, or vasoconstriction in response to sound (added to or subtracted from the steady background noise) in the laboratory (Yen et al, 1978).

Arterial blood flow to the rat tail subserves a thermoregulatory role. Blood flow is increased to dissipate heat, and reduced to conserve body heat (Rand, Burton & Ing, 1965). The differential control of vascular tone in this specialized region of the circulation must be taken into account when measuring systemic blood pressure. The pressure in the tail-artery may be more or less responsive to vasoactive agents than the core circulation (Buñag, Mueting & Riley, 1975). Sympathetic "fright" responses cause vasoconstriction in the tail-artery of rats, particularly those unconditioned to restraint, light, noise and the tactile disturbance of repeated cuff inflations (Borg, 1977). These are exaggerated, and positively correlated with age, in the innately hyperkinetic SHR-SP strain (Chiueh & Kopin, 1978; Yen et al, 1978; Lundin & Norlander, 1986; Boone & McMillen, 1994). It is relevant to note that blood pressures measured by different methods (radiotelemetry, exteriorized catheter or tail-cuff), when recorded simultaneously under the same conditions of restraint, were similar at all time points. In contrast, when these methods are considered independently, the baseline blood pressures monitored in unrestrained rats by radiotelemetry were distinctly lower, compared to measurements made from direct exteriorized (tethered) catheters, which in turn tended to be lower than measurements made by indirect tail-cuff (box-restrained) methods in matched groups of
rats (Bazil et al., 1993; Balakrishnan & McNeill, 1996). It appears that differences in the degree of restraint associated with each method, correspond to differences in the level of psychogenic stress. The unrestrained conditions under which rats implanted with radiotelemetric devices can be monitored, is presumably least intrusive. Restraint and the rat's perception of tail-cuff inflation are unavoidable by the indirect method. Consequently, baseline SBP estimations made in rats by the indirect method will be elevated, commensurate with a more alert state. However, for comparing similarly acclimatized control groups of rats, there is no reason to expect that the level of stimulation experienced by treatment groups of rats would differ, unless an anxiolytic (or conversely, an increased sensitivity to stress) was attributable to the treatment itself. Enhanced irritability has been shown for rats treated with streptozotocin or high dietary fructose; conversely, sympathoinhibitory or sedating antihypertensive agents such as β-blockers, central α2 agonists such as clonidine and the centrally-acting I1-imidazoline receptor agonists have been shown to attenuate blood pressure and heart rate responses to psychological stress in humans (Julius, 1995; Kulka, Tryba & Zenz, 1995) and rats (Blanc, Grichois & Elghozi, 1991; Meehan, Tomatzy & Miczek, 1995). An exploration of whether ridogrel has any such psychoneuroendocrine effects has not been addressed elsewhere, and was not within the scope of this thesis.

Several validation experiments have shown a high level of concurrence between indirect tail-cuff and direct blood pressure measurement under different conditions of warming, restraint, disease models, drugs and for different technologies applied to detect the pulse (Pfeffer, Pfeffer & Frohlich, 1971; Buñag, Mueting & Riley, 1975; Buñag & Butterfield, 1982; Wen et al, 1988). Clearly, it remained appropriate to validate the indirect measurements with the equipment and experimental conditions used in this research.

Materials

Four male SHR-SP were selected at 12 weeks of age from our breeding colony maintained from SHR-SP/A3N stock originating from NIH, Bethesda, MD. PE50 and PE10 polyethylene tubing was used to fashion arterial and venous cannulae (Intramedic®Clay Adams Div. Becton Dickinson & Co., Parsippany, NJ). which were gas sterilized (ethylene oxide) in gas permeable pouches (Tower DualPeel®Pharmaseal Div., Baxter Healthcare Corp, Valencia, CA). The piezo-electric pulse sensor and 15mm. occluder cuff apparatus (Buffington Clinical Systems, Cleveland, OH), and the pressure transducers (TransPac®Abbott Laboratories, North Chicago, IL) were calibrated to mercury (Baumanometer®W.A. Baum Co., NY,NY) for input to separate channels of a data acquisition system (MacLab 4e® via a MacLab QUAD Bridge®, ADInstruments, Castle Hill, NSW, Austr.) running a multi-channel recording software package (CHART®ADInstruments, Castle Hill, NSW, Austr.) on a Power Macintosh 7200/120 PC/C computer (Apple Computer Inc, Cupertino, CA). Occluder cuff inflation/deflation
pressure cycles were controlled by an automated cuff pump (Model 20NW, iitc Inc., Landing, NJ) to provide a high degree of consistency in the rate of inflation, the peak and trough cut-off pressures, the delay prior to deflation and a uniform linear deflation rate. The temperature inside the restrainer was monitored with a model A0A212 Tele-Thermometer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH), and the rats' core temperature was monitored via rectal probe to a model 44TA Tele-Thermometer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH).

Drugs
angiotensin II (Sigma), chymotripsin (Sigma), gentamicin sulfate (Novopharm, Toronto, ON), heparin sodium (Leo Laboratories, Pickering, ON), ketamine hydrochloride (Ketaset™, Ayerst, Montreal, QC), midazolam (Versed®, Hoffman-La Roche, Mississauga, ON), norepinephrine bitartrate (Sax at Inc., Boucherville, QC), penicillin G sodium (Novopharm Ltd., Toronto, ON), polyvinyl-pyrrolidone 360,000MW (Sigma), U46619 (Cayman Chemicals, Ann Arbor, MI), xylazine (Rompun™, Bayvet Div., Chemagro Ltd, Etobicoke, ON).

Methods
Surgical anaesthesia was achieved following an intramuscular injection of combined ketamine 90 mg/kg and xylazine 7.5 mg/kg. Each of four male 12 week old SHR-SP were instrumented with a PE50 left carotid arterial line and a PE10 left internal jugular venous line. The lines were filled with PVP solution containing heparin and chymotripsin to prolong patency, and were capped. The lines were exited at the nape, and enclosed in a plastic protector. The 1 cm ventral midline incision was closed with 6-0 polybutester monofilament. Prophylactic antibiotic coverage was provided with penicillin G 250,000 iu and gentamicin 2 mg ip administered intraoperatively. Analgesia was provided with buprenorphine 0.01 mg/kg q12h × 36 hrs. The rats recovered uneventfully over six to eight days, at which time they were returned to the adjustable plexiglass restrainer to which they were accustomed, and the lines were exposed. The carotid line was bled, irrigated with heparinized saline, and attached to a pressure transducer. The occluder cuff was placed over the tail, proximal to the sensor cuff. An adjustable heating pad under the restrainer was used to maintain the restrainer temperature around 26-28°C, which kept the animals' rectal temperature at 38.1±0.6°C. The carotid line and occluder cuff pressure transducers were calibrated to mercury. Mild sedation greatly facilitates tail-cuff measurements. Midazolam 0.7 mg/kg was administered slowly via the intravenous line. The midazolam did not cause any noticeable reduction in direct carotid blood pressure, while it did adequately reduce the frequency of tail movements. Obliteration of the uncalibrated tail artery pulse waveform by a peak occluder cuff inflation pressure of ≤268 mm Hg was verified (Figures B1 to B3). The cuff pump duty cycle was manually triggered to initiate a steep inflation to 268 mm Hg, a 2 second delay, a linear deflation rate of 5 mm Hg/sec, and to terminate compression completely at 80 mm Hg. At least five
Figure B1  Two cycles of occluder cuff inflations, showing that the return of tail artery pulsation was detected by the sensor at a cuff pressure almost identical to the direct carotid artery systolic blood pressure measurement.
Figure B2  Concurrence between direct and indirect measurements is maintained during the depressor response to hydralazine
Concurrence between direct and indirect measurements is maintained during the pressor response to angiotensin II
cycles were collected on each rat at baseline, to compare the simultaneous carotid artery systolic pressure with the pressure at which the initial return of tail artery pulse was detected. Subsequently, the same procedure was used to establish whether the magnitude of pressor responses were different in either artery, following intravenous bolus injection of norepinephrine 10 μg/kg or angiotensin II 80 ng/kg, and following the depressor response evoked by hydralazine 0.4 mg/kg, and finally a bolus injection of U46619 10 μg/kg was given. Following intravenous injection of each vasoactive agent, at least four measurements were collected approximately 0.5 minutes apart. The time between injections of the different agents corresponded to that required for return to the previous baseline, or at least to stabilize at a new baseline pressure.

Analytical methods

Several validation experiments have published statistical comparisons of two or more methods that simultaneously measured the same parameter, based upon correlational or regression analyses (Maistrello & Matscher, 1969; Bünag, 1973; Pfeffer, Pfeffer & Frohlich, 1971). Within the context of its research application, an obvious problem exists in deciding where the high level of stochastic significance due to coincidence ends, and the level of concurrence between methods begins to assume scientific importance (Altman & Bland, 1983). The assumption of homogeneity of within-subject and pooled variances for both methods was confirmed. Separate paired two-tailed t-tests were used for comparison of simultaneous direct and indirect systolic blood pressure measurements for baseline, pressor and depressor responses. A $z_{\text{diff}} \leq 1.0$ mm Hg was considered in advance to be desirable, and $z_{\text{diff}} \leq 2.5$ sufficient to establish an acceptable level of concurrence between measurements by the two methods. If the homogeneity of variances assumption could be met, and a minimum 10 sets of paired baseline measurements (≥five per rat) could be pooled, then where a mean difference of zero resided within a $95\% \text{CI}_{\text{diff}}$ half-width ≤2.0, it would represent an acceptable level of concurrence between methods, to establish this indirect tail-cuff method as a valid cross-sectional estimate of peak carotid systolic blood pressure in 12 week old male SHR-SP. Since the measurement technique is likely to influence the parameter it is used to measure, it is necessary that experiments include SHAM-manipulated control group comparisons, and to assume that the treatments were without influence on the level of arousal elicited by the measurement procedure.

Results

Tables B1 to B5 display the extent to which the level of occluder-cuff pressure at which tail-artery pulsation was detected, varied from the simultaneous systolic pressure measured directly in the carotid artery. No changes in carotid arterial wave amplitude occurred to suggest kinking, thrombus or vessel wall encroachment on the catheter lumen. The magnitude of peak systolic pressor responses that were measured directly in the
Table B1  Concurrence between indirect and direct systolic blood pressure measurements, under control conditions

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Paired Observations</th>
<th>METHOD</th>
<th>Group Mean</th>
<th>S.E.M</th>
<th>Indirect / Direct Ratio [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (CONTROL)</td>
<td>30</td>
<td>INDIRECT</td>
<td>171.8</td>
<td>26.5</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIRECT</td>
<td>172.1</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>$\bar{x}_{\text{diff}}$</td>
<td>S.E.M._diff</td>
<td>95%CI $\frac{1}{2}$-width</td>
<td>t-value</td>
<td>df</td>
<td>2-tail significance</td>
</tr>
<tr>
<td>0.217</td>
<td>0.438</td>
<td>0.897</td>
<td>0.49</td>
<td>29</td>
<td>0.625</td>
</tr>
</tbody>
</table>

Indirect v Direct Systolic
Baseline Conscious Restrained

12-wk old male SHR-SP

![Graph showing correlation between indirect and direct systolic blood pressure measurements with Rsq = 0.993]
Table B2 Concurrency between indirect and direct systolic blood pressure measurements, following norepinephrine

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Paired Observations</th>
<th>METHOD</th>
<th>Group Mean</th>
<th>S.E.M</th>
<th>Indirect / Direct Ratio [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine 10µg/Kg.</td>
<td>21</td>
<td>INDIRECT</td>
<td>201.5</td>
<td>32.0</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIRECT</td>
<td>204.0</td>
<td>32.5</td>
<td></td>
</tr>
<tr>
<td>( \bar{x}_{\text{diff}} )</td>
<td>S.E.M_{\text{diff}}</td>
<td>95% CI 1/2-width</td>
<td>t-value</td>
<td>df</td>
<td>2-tail significance</td>
</tr>
<tr>
<td>2.476</td>
<td>0.931</td>
<td>1.941</td>
<td>2.66</td>
<td>20</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Indirect v Direct Systolic

Norepinephrine Response

12-wk old SHR-SP

![Graph showing systolic blood pressure measurements](image)

\( R^2 = 0.9828 \)
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Paired Observations</th>
<th>METHOD</th>
<th>Group Mean</th>
<th>S.E.M</th>
<th>Indirect / Direct Ratio [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II 10ng/Kg.</td>
<td>23</td>
<td>INDIRECT</td>
<td>221.1</td>
<td>33.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIRECT</td>
<td>221.2</td>
<td>33.9</td>
<td></td>
</tr>
<tr>
<td>( \bar{x}_{\text{arr}} )</td>
<td>( \text{S.E.M.}_{\text{arr}} )</td>
<td>95%CI ½-width</td>
<td>t-value</td>
<td>df</td>
<td>2-tail significance</td>
</tr>
<tr>
<td>0.078</td>
<td>0.846</td>
<td>1.755</td>
<td>0.09</td>
<td>22</td>
<td>0.927</td>
</tr>
</tbody>
</table>

Indirect v Direct Systolic

Angiotensin II Response

12-wk old male SHR-SP

![Graph showing the relationship between indirect and direct systolic blood pressure measurements, following angiotensin II](image)

\[ \text{Rsq} = 0.9857 \]
Table B4  Concurrence between indirect and direct systolic blood pressure measurements, following hydralazine

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Paired Observations</th>
<th>Method</th>
<th>Group Mean</th>
<th>S.E.M</th>
<th>Indirect / Direct Ratio [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydralazine 0.4mg/Kg.</td>
<td>12</td>
<td>INDIRECT</td>
<td>137.8</td>
<td>7.5</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIRECT</td>
<td>138.1</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>δ₃₅ []; S.E.M.₃₅;</td>
<td>95%CI ½-width t-value df</td>
<td>0.300</td>
<td>0.787</td>
<td>1.732</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Indirect v Direct Systolic

Hydralazine Response

12-wk old male SHR-SP

\[ \text{Rsq} = 0.9896 \]
Table B5  Concurrence between indirect and direct systolic blood pressure measurements, pooling all paired data

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Paired Observations</th>
<th>METHOD</th>
<th>Group Mean</th>
<th>S.E.M</th>
<th>Indirect / Direct Ratio [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooling all of the paired data</td>
<td>86</td>
<td>INDIRECT</td>
<td>187.5</td>
<td>40.7</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIRECT</td>
<td>188.2</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.E.M.dif</td>
<td>95%CI ½-width</td>
<td>t-value</td>
<td>df</td>
</tr>
<tr>
<td></td>
<td>0.743</td>
<td>0.381</td>
<td>0.757</td>
<td>1.95</td>
<td>85</td>
</tr>
</tbody>
</table>

Indirect v Direct Systolic
Paired Data for All Treatments Pooled
12-wk old male SHR-SP [n = 4]

![Graph showing concurrence between indirect and direct systolic blood pressure measurements]
carotid artery following intravenous bolus injection of angiotensin II 80 ng/kg, averaged 49 mm Hg, compared to the 32 mm Hg pressor response following intravenous bolus injection of norepinephrine 10 µg/kg. However, unlike angiotensin II, norepinephrine caused vasospasm and loss of tail artery pulsation, which necessitated waiting for the return of the tail artery pulse before simultaneous direct and indirect measurements could be made; meanwhile the pressor effect had decayed considerably. In contrast, comparisons between the two methods could begin at the height of the angiotensin II pressor response. The magnitude of pressor responses measured directly in the carotid artery, following intravenous bolus injection of U46619 10 µg/kg averaged 78 mm Hg, and induced a sustained loss of the tail artery pulse, so that simultaneous measurements could not be made at all. The onset of the depressor response evoked by hydralazine 0.4 mg/kg was gradual, falling to as low as 96 mm Hg systolic.

Conclusions:

The results serve to establish an acceptable level of concurrence, to conclude that the indirect tail-cuff method is a valid cross-sectional estimate of peak carotid artery systolic blood pressure, in 12 week old male SHR-SP rats, within the experimental conditions established for this thesis.
Appendix C:

Illustration of the Chrono-Log® Lumi-Aggregation system used for the *in vitro* whole blood platelet aggregation studies.
Appendix D:

Illustration of the Nalgene® metabolic cages, including modifications.
Nalgene® Plastic Metabolism Cages

Cover
Large, vented. Keeps animal securely in cage. Can be removed for easy access to animal.

Upper Chamber
Made of transparent polycarbonate to admit total room light. Surface is smooth, garse-proof, noiseless. Cleans easily in warm soapy water.

Feeder Chamber

Feeder Drawer

Collection Funnel and Separating Cone
Unique funnel and cone design assures immediate and complete separation of feces and urine. Prevents urine from entering feces tube and eliminates urine washover.

Feces Collection Tube
Fecal pellets roll down sides of funnel and are collected in feces collection tube.

Urine Ring

Urine Collection Tube
Graduated in cubic centimeters for convenient measurement.

Cage Volume
Total cage volume of 328 cubic inches surpasses ILAR recommendations; accommodates rats weighing up to 300 grams.

Water Bottle
Calibrated in milliliters.

Spillage Drain
Prevents water from entering collection funnel.

Support Grid
Surface area is 63 sq. in., surpassing ILAR recommendations for grid surfaces.

Stand
Stainless steel. Supports cage, gives easy access to all parts.

Hopper is a modification to prevent carriage of food into the cage.

Urine flows along the inside surfaces of the collection funnel and is diverted by the urine ring directly into the urine collection tube.
Appendix E:

Radioimmunoassay of thromboxane B$_2$ in serum and urine.
Radioimmunoassay of Thromboxane B₂ in Serum and Urine

The extraction method differs slightly for urine versus serum. A previously assayed sample of urine is routinely carried over as a quality control (QC), to monitor interassay variation. The QC and urine samples are pipetted in 5 mL aliquots to 50 mL centrifuge tubes, and spiked with [³H]TxB₂ (25 μL = 1500 dpm). When the sample volume is less than 5 mL, it is brought to volume with purified water. The original urine volume and collection period are referenced to each sample, and the dilution factors are accounted for throughout the procedure. Serum samples of 200 μL are initially spiked with [³H]TxB₂ (25 μL = 1500 dpm), and vortexed with 200 μL of ethanol in a 10 mm × 75 mm polystyrene test tube, then centrifuged at 2230 × g × 20 min. The supernatant is poured into 50 mL centrifuge tubes and diluted with 5 mL of purified water. The [³H]TxB₂ tracer amount is added for calculation of fractional recovery by comparison to the total count (TC) controls (comprised of 500 mL of the tris gel pH 7.4 working buffer, similarly spiked but not subjected to the extraction and purification steps, but instead, pipetted directly into scintillation counting vials). The tris buffer, per litre of water, contains 6.08 g tris acetate, 9.00 g sodium chloride, 1.00 g polyvinylpyrrolidone (MW = 40,000), 3.4 mL of 0.5M magnesium sulfate, 1.6 mL of 0.1M calcium chloride, and 2.0 mL of 10N hydrochloric acid. Only urine samples (including the QC) are brought into the pH range 6.8 to 7.4, with 1M potassium hydroxide or 1M hydrochloric acid. To remove neutral lipids, all samples (serum and urine) are washed with 15 mL hexane for 10 min, then centrifuged at 1300 × g × 5 min, and the hexane discarded. The aqueous layer is brought to pH 3.3 to 3.8 with concentrated formic acid, and immediately extracted with 30 mL chloroform, shaken for 10 min, and centrifuged at 1300 × g × 5 min, before discarding the aqueous layer. The organic fractions are evaporated to dryness in 50 mL conical tubes, maintained at 37°C in a water bath, under a jet of anhydrous nitrogen gas.

Subsequent to extraction, serial column liquid chromatography is used for further purification of samples. The solvent systems are all comprised of chloroform:heptane: methanol:acetic acid, which vary in their capacity to elute TxB₂, by increasing the relative v/v proportion of methanol. Sephadex LH-20 columns (130 mm × 10 mm) are pre-conditioned with solvent (100:100:30:2). The sample residues are taken up in 400 μL of solvent (100:100:10:2) by vortexing for 30 s, and each applied to a separate column. The sample tubes are further rinsed with 200 μL of the same solvent, and also added to the column. The columns are eluted with the following solvent systems in sequence: 10 mL of (100:100:10:2), 10 mL of (100:100:20:2), 20 mL of (100:100:25:2). 40 mL conical tubes are used to collect the later fraction known to contain the analyte. The purified fraction is evaporated under nitrogen at 37°C. At that point, the residues can be stored at -20°C for up to three days prior to assay. The radioimmunoassays are performed in duplicate, in 10 mm × 75 mm polystyrene tubes. Following their purification, the evaporated samples are solubilized by vortexing with 500 μL of tris buffer for one minute.
Tubes containing standard concentrations of TxB₂, are prepared in duplicate: 6.25, 12.5, 25, 50, 100, 200 and 400 pg. The assay is set up according to Table E1. Each tube is mixed by vortexing, and incubated at 37°C for 3 hours. During incubation, spike recoveries are measured by adding 150 µL of the tris-buffered sample to vials containing 9 mL of scintillation fluid, and are counted alongside the TC vials for 10 minutes/vial. At the end of the incubation, the assay tubes are chilled in ice water for 10 min, then 1 mL of dextran⁷⁰-coated charcoal (1:10 w/w) is added to all tubes, and mixed by vortexing. All tubes are centrifuged at 4°C, 2230 x g x 20 min. The supernatant is transferred to counting vials containing 9 mL of scintillant, and each is counted for 10 minutes. Note that the supernatant from the TC tubes is added to the two vials containing 50 µL of [³H]TxB₂. Software on the scintillation counter computer is used to fit a spline-interpolated curve to the Scatchard plot of the standards. This yields the standard curve, from which the sample concentrations are extrapolated. The final TxB₂ concentrations are adjusted for per cent recoveries, and sample dilution factors. Serum TxB₂ concentrations are reported as ng/mL, whereas urine concentrations can be calculated in terms of concentration (ng/vol) or rate (ng/sample period). The interassay variability is 8%-13%. Recoveries are between 60% and 80%. The standard curves vary less than 5% from the predicted curve.

<table>
<thead>
<tr>
<th>TUBE</th>
<th>tris (µL/tube)</th>
<th>Std. or sample</th>
<th>[³H]TxB₂ (5000 dpm / 50 µL)</th>
<th>anti-TxB₂ Ab (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>TC</td>
<td>150</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>NSB</td>
<td>NSB</td>
<td>150</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>blank</td>
<td>blank</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Standards</td>
<td>Standards</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>QC</td>
<td>QC</td>
<td>50</td>
<td>0</td>
<td>50; 100</td>
</tr>
<tr>
<td>urine</td>
<td></td>
<td>50</td>
<td>0</td>
<td>50; 100</td>
</tr>
<tr>
<td>samples</td>
<td></td>
<td>75</td>
<td>50</td>
<td>25; 50</td>
</tr>
</tbody>
</table>

* 50 µL = 5000 dpm [³H]TxB₂ for total counts are pipetted directly into counting vials during the extraction step.
Appendix F:

Radioimmunoassay of urinary 6-keto-PGF$_{1\alpha}$
Radioimmunoassay of Urinary 6-keto-prostaglandin F$_{1\alpha}$

A previously assayed urine sample is included as a quality control (QC), to monitor interassay variation. Sample urines are added to counting vials in 5 mL aliquots, or brought up to volume with purified water. 1 mL of tris buffer is added to a 12 mm × 75 mm tube. The QC, urine samples, and the tube of tris buffer are each spiked with 50 µL = 4000 dpm [3H]6-keto-PGF$_{1\alpha}$. The test tube of spiked buffer is mixed, and 300 µL of the contents pipetted into two counting vials to measure total counts (TC). Pre-packed octadecyl (C18) columns are pre-conditioned by flushing with 20 mL of methanol and 20 mL of purified water. Samples are brought to pH 3.0-3.2 with 1N HCl, and applied to the columns, followed by 3 rinses (total 20-30 mL) of purified water. The columns are eluted with 20 mL of 15% v/v methanol, then 20 mL of hexane, and the eluents discarded. Methyl formate 10-15 mL is added to the columns, and the fraction is collected in 40 mL conical tubes, each containing 3 drops of methanol. The methyl formate fractions are evaporated to dryness under nitrogen at 37°C. Sephadex LH-20 columns measuring 10 mm × 130 mm, are conditioned with 20 mL of chloroform:heptane:methanol:acetic acid (100:100:30:2). The extracted sample residues are solubilized by vortexing with 47 µL of methanol for 60 s, 453 µL of solvent (100:100:0:2) for 30 s, then 500 µL (100:100:20:2) for 30 s. Samples are applied to the column, and the conical tube rinsed with another 200 µL of the (100:100:20:2) solvent, which is also added to the column. The columns are eluted with the following chloroform:heptane:methanol:acetic acid solvent systems in sequence: 10 mL of (100:100:10:2), 9 mL of (100:100:20:2), 20 mL of (100:100:25:2). The latter fraction is collected in 40 mL conical tubes, and evaporated to dryness under nitrogen at 37°C. The residues can be stored at -20°C for up to three days prior to assay.

For the assay, the sample residues are solubilized in 1 mL of tris buffer, by vortexing for 1 minute. Duplicate assays are set up in 10 mm × 75 mm polystyrene tubes, according to Table F1. Assay tubes are pipetted into vials with 9 mL of scintillant, and counted for 10 min each, along with the TC vials. Following incubation, 1 mL of dextran-coated charcoal (1:10 w/w) is added to each assay tube, mixed, then centrifuged at 4°C, 2230 × g × 20 min. Supernatants are transferred to counting vials containing 9 mL of scintillant, and each vial is counted for 10 minutes. Sample 6-keto-PGF$_{1\alpha}$ concentrations are extrapolated from the standard curve that is developed from a spline-fitted Scatchard plot. The concentrations are corrected to account for recovery and volume factors. The interassay variability is 8%-15%. Recoveries range between 50% to 75%. Standard curves vary less than 5% from the predicted curve.
### Table F1  Set-up for duplicate 6-keto-PGF$_{1a}$ RIA

<table>
<thead>
<tr>
<th>TUBE</th>
<th>tris (µL/tube)</th>
<th>Std. or sample</th>
<th>$[^3]$H]TxB$_2$ (5000 dpm / 50 µL)</th>
<th>anti-TxB$_2$ Ab (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
<td></td>
</tr>
<tr>
<td>TC TC</td>
<td>400</td>
<td>400</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>NSB NSB</td>
<td>400</td>
<td>400</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>blank blank</td>
<td>200</td>
<td>200</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Standards</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>QC QC</td>
<td>100</td>
<td>0</td>
<td>100; 200</td>
<td>50</td>
</tr>
<tr>
<td>urine samples</td>
<td>100</td>
<td>0</td>
<td>100; 200</td>
<td>50</td>
</tr>
</tbody>
</table>

* 50 µL = 5000 dpm $[^3]$H6-keto-PGF$_{1a}$ for total counts are pipetted directly into counting vials during the extraction step.
Appendix G:

Radioimmunoassay technique used for determining the rate of angiotensin I generation in plasma (plasma renin activity).
Radioimmunoassay for Determining the Rate of Angiotensin I Generation (Plasma Renin Activity)

This assay method does not measure renin directly, instead, renin is allowed to react with endogenous plasma angiotensinogen to generate angiotensin I, and the angiotensin I is then quantified by radioimmunoassay. Since excess substrate is present in the plasma, the amount of angiotensin I generated, is directly proportional to the renin activity. Plasma is incubated at 37°C under conditions designed to inhibit angiotensin I degradation. The angiotensin I concentration is determined before and after incubation. A standard amount of [125I]-labelled angiotensin I and a standard amount of anti-angiotensin I antibody, are incubated at 4°C in the presence of either known quantities of unlabelled angiotensin I, or plasma containing endogenous angiotensin I to be measured. The competition of the labelled angiotensin I for antibody binding sites with plasma angiotensin I, is compared to that of known quantities of angiotensin I. Bound antibody is separated from free angiotensin I with dextran70-coated charcoal, which adsorbs the unbound fraction.

Equipment
Disposable 12 mm x 75 mm polystyrene tubes
Disposable 12 mm x 75 mm culture tubes
Disposable micropipettes (10 µL and 20 µL)
Eppendorf 200 µL pipettor
gamma emission scintillation counter
refrigerated centrifuge

Reagents
1. EDTA (Na2) 11.2% (or use 50 µL/mL of 0.3M to yield 15 mM)
2. 8-hydroxyquinoline 4.9% (use 10 µL/mL to yield 3.4 mM)
3. BAL 5% (use 10 µL/mL to yield 5 mM), or using BAL from Calbiochem, 50 µL
BAL solubilized in peanut oil and benzyl benzoate (9:1)
4. 8-hydroxyquinoline - BAL mixture 1:1
5. tris acetate buffer (0.1M) pH 7.4 is prepared fresh (6.05 g of tris base in 500 mL of purified water is pH adjusted with approximately 2.4 mL of glacial acetic acid)
6. tris acetate buffer with lysozyme is prepared fresh (1 mg of crystallized 2x lysozyme/mL)
7. barbital buffer pH 7.4 (3.825 g of NaCl, 0.7357 g of Na barbital, and 0.4857 g of Na acetate trihydrate, is dissolved in 450 mL of purified water. The pH is adjusted with approximately 25 mL of 0.1N ammonium chloride)
8. dextran-coated charcoal stock solution (2.5 g of charcoal is suspended in 0.25 g of dextran70 dissolved in 100 mL of barbital buffer)
9. dextran-coated charcoal working solution (dilute stock 1:4 with barbital buffer)
10. [125I]angiotensin I (dilute with tris acetate buffer with lysozyme, so that 0.2 mL will give approximately 10,000cpm)
11. angiotensin I standards (The stock standard is 1 mg/mL. 5 mL of purified water is
added to 5 mg of angiotensin I, and frozen. )

12. Intermediate standard solution of angiotensin I 50 μg/mL, is made by diluting 0.5 mL of stock solution with 10 mL of purified water. The intermediate solution can be thawed and refrozen several times.

13. Working standard solution of angiotensin I 100 ng/mL, is made by diluting 10 μL of the intermediate solution with 5 mL of tris acetate buffer with lysozyme on the day of the assay.

14. Anti-angiotensin I antisera is maintained as a frozen stock solution. 0.1 mL is diluted in 100 mL of tris lysozyme buffer, and stored frozen. On the day of the assay, it is further diluted 1:6 with tris lysozyme buffer, in quantity sufficient to provide 0.2 mL/tube to be assayed. The dilution is adjusted as required to result in approximately 50% binding.

**Sample preparation**

1. Blood is collected into ice cold EDTA vacutainer tubes, kept on ice until centrifuged at 4°C to remove the plasma for assay. The plasma may be stored frozen.

2. 5 mL of plasma is dispensed into "Tube A", and 25 μL EDTA + 10 μL BAL - 8-hydroxyquinoline added. The tube is mixed well, and 0.25 mL is transferred to a second "Tube B". "Tube A" is incubated in a 37°C water bath for 3 hours. "Tube B" is kept in an ice water bath. At the end of the 3 hour incubation, "Tube A" is placed in an ice water bath.

**Procedure**

1. Preparation of angiotensin I standard concentrations:
   a. add 0.6 mL of working standard to 9.4 mL of tris lysozyme buffer, and mix.
   b. add 0.4 mL of working standard to 9.6 mL of tris lysozyme buffer, and mix.
   c. add 2 mL of (b) to 2 mL of tris lysozyme buffer, and mix.
   d. add 2 mL of (c) to 2 mL of tris lysozyme buffer, and mix.
   e. add 2 mL of (d) to 2 mL of tris lysozyme buffer, and mix.
   f. add 2 mL of (e) to 2 mL of tris lysozyme buffer, and mix.

2. Polystyrene 10mm × 75 mm tubes are labelled in duplicate, and reagents are added according to **Table G1**.

3. All tubes are mixed by vortex, and incubated in a refrigerator for 20-24 hours.

4. The working solution of dextran70-coated charcoal is stirred briskly on a magnetic stir plate, while 1 mL is pipetted into each of the tubes (except TC).

5. The tubes are mixed with a vortex mixer, then centrifuged at 2230 × g × 20 min.

6. The supernatant is transferred into similarly labelled 10mm × 75 mm glass tubes.

7. The glass tubes are sealed with parafilm, and all tubes (including TC) are counted using a gamma emission scintillation counter for 5 minutes.

8. A standard curve is constructed using computerized software, and the angiotensin I concentration generated in the samples are extrapolated from it.
<table>
<thead>
<tr>
<th>TUBE LABEL</th>
<th>BUFFER (mL)</th>
<th>Standard or Sample</th>
<th>AngI-free plasma (µL)</th>
<th>$[^{131}I]$Ang I (mL)</th>
<th>Antisera (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>NSB</td>
<td>0.7</td>
<td>0</td>
<td>20</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>125</td>
<td>0</td>
<td>0.5 mL (f)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>250</td>
<td>0</td>
<td>0.5 mL (e)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0.5 mL (d)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0.5 mL (c)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0.5 mL (b)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>3000</td>
<td>0</td>
<td>0.5 mL (a)</td>
<td>20</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>CA10</td>
<td>0.5</td>
<td>10 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>CB10</td>
<td>0.5</td>
<td>10 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>CA20</td>
<td>0.5</td>
<td>20 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>CB20</td>
<td>0.5</td>
<td>20 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>XA10</td>
<td>0.5</td>
<td>10 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>XB10</td>
<td>0.5</td>
<td>10 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>XA20</td>
<td>0.5</td>
<td>20 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>XB20</td>
<td>0.5</td>
<td>20 µL</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>