ROLE OF ENDOTHELIN-1 IN CARDIAC AND VASCULAR DYSFUNCTION IN STREPTOZOTOCIN-DIABETIC RATS:
EFFECTS OF CHRONIC ENDOTHELIN RECEPTOR BLOCKADE

by

EMI ARIKAWA
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Division of Pharmacology and Toxicology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September, 2002
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Department of Pharmacology and Toxicology
Faculty of Pharmaceutical Sciences
The University of British Columbia
Vancouver, Canada

Date September 24, 2002
ABSTRACT

The present thesis examined the role of endothelin-1 (ET-1), a potent 21-amino-acid vasoconstrictor-mitogen, in diabetic cardiovascular complications. Our previous data showed that diabetic rats exhibited impaired isolated working heart performance, which was ameliorated by chronic treatment with bosentan, a mixed ET\textsubscript{A}/ET\textsubscript{B} receptor blocker. Accordingly, an activated endothelin system may be involved in diabetic cardiomyopathy.

Using streptozotocin (STZ)-induced diabetic rats, a rodent model of Type 1 diabetes, we examined the effects of long-term treatment with ET receptor antagonists on both the cardiac and vascular systems. The primary observations from our studies are that STZ-induced diabetes resulted in cardiac and vascular dysfunction in association with an up-regulation of the expression of the local ET system (i.e. ET-1 and its receptors). Chronic ET receptor blockade with bosentan improved functional cardiac performance and corrected vascular hyper-reactivity to vasoconstrictors in STZ-diabetic rats. These data suggest that exaggerated ET-1 production and/or action may play a role in the development of cardiovascular dysfunction in diabetes.

In the diabetic heart, the cardioprotective effects of bosentan were shown to require the blockade of ET\textsubscript{B} receptors, since selective ET\textsubscript{A} receptor antagonism with ABT-627 did not provide significant improvement in diabetic rat heart function. Moreover, bosentan may improve diabetic cardiac function by blocking the increase in ET-1-evoked coronary vasoconstriction in diabetés. In the vasculature, while chronic bosentan treatment equally ameliorated the hyper-responsiveness to vasoconstrictors in superior mesenteric arteries (SMA) and renal arteries (RA) from diabetic rats, the
underlying mechanisms for this effect appear to be tissue-specific. In SMA, the enhanced vasoconstrictor responses in diabetes seem to be related to an increase in ET\textsubscript{A} receptor-activated thromboxane (Tx) synthesis/release in diabetic vascular endothelium, and bosentan may act by blocking the activation of the ET\textsubscript{A} receptor and/or normalizing the increased expression of the receptor. In RA, however, the beneficial effects of bosentan treatment on the diabetic vasoconstrictor responses are unlikely to be due to an effect on Tx synthesis, but rather, may be exerted through normalization of the vascular responses to TxA\textsubscript{2}. Results presented in this thesis, therefore, provide evidence for a role of ET-1 in cardiovascular dysfunction in diabetes.
1. INTRODUCTION

1.1. Diabetes Mellitus

Definition and classification of diabetes mellitus: the revised diagnostic criteria

Diabetes and its complications

Diabetes and cardiovascular complications

Potential mechanisms underlying diabetic cardiovascular complications: an overview

Animal model of diabetes: streptozotocin-induced diabetes

1.2. Endothelium and the Endothelin System

Endothelium and its role in vascular function

Endothelins: biosynthesis, distribution and clearance

Endothelin receptors
Physiological actions of ET-1 20

Endothelin receptor antagonists 22

1.3. Endothelin—A Possible Link Between Diabetes and Its Cardiovascular Complications 24

Previous data from our laboratory: effects of chronic endothelin receptor blockade in STZ-diabetic rat hearts 25

2. RESEARCH OUTLINE: RATIONALES, OBJECTIVES, AND HYPOTHESES 28

2.1. Overall Hypothesis 30

2.2. Specific Research Objectives and Rationales 30

Studies in the heart 30

Studies in the vasculature 32

3. MATERIALS AND METHODS 35

3.1. Materials 35

3.2. Research Design and Experimental Procedures 36

3.2.1 Care and Treatment of the Animals 36

3.2.2 Experimental Protocols 38

Studies in the heart 38

Study #1 Effects of chronic diabetes and ET receptor blockade with (a mixed ET\(_A\) and ET\(_B\) receptor antagonist) on the tissue level and gene expression of ET-1 in rat hearts 38

Study #2 Effects of chronic ET receptor blockade with bosentan on the tissue level and gene expression of ET\(_A\) and ET\(_B\) receptors in diabetic rat hearts 39
Study #3 Effects of chronic treatment with a selective ET_A receptor blocker on isolated working heart function of diabetic rats

Studies in the vasculature

Study #4 Effects of chronic diabetes and ET receptor blockade with bosentan on the plasma and vascular tissue level of ET-1 in rats

Study #5 Effects of chronic bosentan treatment on the expression of ET_A and ET_B receptors in diabetic rats arteries

Study #6 Effects of chronic bosentan treatment on vascular reactivity in diabetic rats

Study #7 Role of thromboxane A_2 in endothelin-induced hyper-reactivity in diabetic arteries

3.3. Methodology

3.3.1 Isolated Working Heart Function

3.3.2 Isolated Blood Vessel Preparation

3.3.3 Immunohistochemistry

3.3.4 Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

3.3.5 Biochemical Measurements

3.3.6 Systolic Blood Pressure Measurement

3.3.7 Statistical Analyses

4. RESULTS

4.1. General Characteristics

Untreated and bosentan-treated control and STZ-diabetic rats

Untreated and ABT-627-treated control and STZ-diabetic rats
Studies in the Heart

4.2. Study #1
Effects of chronic diabetes and ET receptor blockade with bosentan (a mixed ETA and ETB receptor antagonist) on the heart weight/body weight ratio and on the tissue level and gene expression of ET-1 in rat hearts

4.3. Study #2
Effects of chronic ET receptor blockade with bosentan on the tissue level and gene expression of ETA and ETB receptors in diabetic rat hearts

4.4. Study #3
Effects of chronic treatment with a selective ETA receptor blocker on isolated working heart function of diabetic rats

Studies in the Vasculature

4.5. Study #4
Effects of chronic diabetes and ET receptor blockade with bosentan on the plasma and vascular tissue level and gene expression of ET-1 in rats

4.6. Study #5
Effects of chronic bosentan treatment on the expression of ETA and ETB receptors in diabetic rat arteries

4.7. Study #6
Effects of chronic bosentan treatment on vascular reactivity in diabetic rats

Vascular reactivity in endothelium-intact tissues
Vascular reactivity in endothelium-denuded tissues
Vascular responses to SNP

4.8. Study #7
Role of thromboxane A2 in endothelin-induced hyper-reactivity in diabetic arteries

Plasma thromboxane and prostacyclin metabolite levels
Vascular reactivity--superior mesenteric arteries
5. DISCUSSION

5.1. Overview

5.2. Effects of Diabetes and Chronic Endothelin Receptor Blockade on the Expression of ET-1

5.3. Effects of Diabetes and Chronic Endothelin Receptor Blockade on the Expression of Endothelin Receptors

5.4. Effects of Chronic Endothelin Receptor Blockade on Diabetic Heart Function

5.5. Effects of Chronic Endothelin Receptor Blockade on Diabetic Vascular Function

5.6. Role of Thromboxane A2 in Endothelin-Induced Hyper-Reactivity in Diabetic Arteries

5.7. Concluding Remarks

6. SUMMARY AND CONCLUSIONS

7. REFERENCES
LIST OF SCHEMES

1. Structure of endothelin-1

2. Biosynthesis of endothelin-1

3. Hypothetical mechanisms for the hyper-reactivity of diabetic arteries to vasoconstrictors
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Effect of chronic endothelin receptor blockade on isolated working heart</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>function in control and diabetic rats</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Effect of chronic endothelin receptor blockade on coronary vasoreactivity</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>in control and diabetic rats</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>ET-1-like-immunoreactivity in the left ventricle</td>
<td>61</td>
</tr>
<tr>
<td>4.2</td>
<td>ET-1-like-immunoreactivity in the right ventricle</td>
<td>62</td>
</tr>
<tr>
<td>4.3</td>
<td>ET-1 mRNA levels in ventricles</td>
<td>63</td>
</tr>
<tr>
<td>4.4</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt; receptor-like-immunoreactivity in the left ventricle</td>
<td>66</td>
</tr>
<tr>
<td>4.5</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt; receptor-like-immunoreactivity in the right ventricle</td>
<td>67</td>
</tr>
<tr>
<td>4.6</td>
<td>ET&lt;sub&gt;B&lt;/sub&gt; receptor-like-immunoreactivity in the left ventricle</td>
<td>68</td>
</tr>
<tr>
<td>4.7</td>
<td>ET&lt;sub&gt;B&lt;/sub&gt; receptor-like-immunoreactivity in the right ventricle</td>
<td>69</td>
</tr>
<tr>
<td>4.8</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt; and ET&lt;sub&gt;B&lt;/sub&gt; receptor mRNA levels in ventricles</td>
<td>70</td>
</tr>
<tr>
<td>4.9</td>
<td>Effect of chronic ABT-627 treatment on isolated working heart function in</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>control and diabetic rats</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>ET-1-like-immunoreactivity in superior mesenteric arteries</td>
<td>77</td>
</tr>
<tr>
<td>4.11</td>
<td>ET-1-like-immunoreactivity in renal arteries</td>
<td>78</td>
</tr>
<tr>
<td>4.12</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt; receptor-like-immunoreactivity in superior mesenteric arteries</td>
<td>81</td>
</tr>
<tr>
<td>4.13</td>
<td>ET&lt;sub&gt;A&lt;/sub&gt; receptor-like-immunoreactivity in renal arteries</td>
<td>82</td>
</tr>
<tr>
<td>4.14</td>
<td>ET&lt;sub&gt;B&lt;/sub&gt; receptor-like-immunoreactivity in superior mesenteric arteries</td>
<td>83</td>
</tr>
<tr>
<td>4.15</td>
<td>ET&lt;sub&gt;B&lt;/sub&gt; receptor-like-immunoreactivity in renal arteries</td>
<td>84</td>
</tr>
<tr>
<td>4.16</td>
<td>NE concentration response curve in endothelium-intact arteries from control</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>and diabetic rats</td>
<td></td>
</tr>
<tr>
<td>4.17</td>
<td>ET-1 concentration response curve in endothelium-intact arteries from</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>control and diabetic rats</td>
<td></td>
</tr>
</tbody>
</table>
4.18 NE concentration response curve in endothelium-intact arteries from control and diabetic rats in the presence of L-NAME

4.19 NE concentration response curve in endothelium-denuded arteries from control and diabetic rats

4.20 ET-1 concentration response curve in endothelium-denuded arteries from control and diabetic rats

4.21 NE concentration response curve in endothelium-denuded arteries from control and diabetic rats in the presence of L-NAME

4.22 A. SNP concentration response curve in endothelium-intact arteries from control and diabetic rats
    B. SNP concentration response curve in endothelium-denuded arteries from control and diabetic rats

4.23 A. NE concentration response curve in arteries from control and diabetic rats
    B. ET-1 concentration response curve in arteries from control and diabetic rats

4.24 NE concentration response curve in control and diabetic rat superior mesenteric arteries in the presence of endothelin receptor blocker

4.25 NE concentration response curve in control and diabetic rat renal arteries in the presence of endothelin receptor blocker

4.26 NE concentration response curve in control and diabetic rat arteries in the presence of dazmegrel

4.27 ET-1 concentration response curve in control and diabetic rat arteries in the presence of dazmegrel

4.28 Concentration response curve to a thromboxane A2 mimetic U46619 in control and diabetic rat arteries

4.29 Ach concentration response curve in control and diabetic rat arteries
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Representative general characteristics of the rats before and at 5 and 10 weeks of bosentan treatment</td>
<td>56</td>
</tr>
<tr>
<td>4.2</td>
<td>General characteristics of the four experimental groups before or after 7 weeks of ABT-627 treatment</td>
<td>57</td>
</tr>
<tr>
<td>4.3</td>
<td>Heart-to-body weight ratio of the rats after 10 weeks of bosentan treatment</td>
<td>60</td>
</tr>
<tr>
<td>4.4</td>
<td>Heart-to-body weight ratio of the rats after 7 weeks of ABT-627 treatment</td>
<td>73</td>
</tr>
<tr>
<td>4.5</td>
<td>Cross-sectional area of the superior mesenteric arteries and renal arteries from the four rat groups</td>
<td>89</td>
</tr>
<tr>
<td>4.6</td>
<td>A. Sensitivities to various agents in the superior mesenteric arteries from the four rat groups</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>B. Sensitivities to various agents in the renal arteries from the four rat groups</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Plasma levels of thromboxane and prostacyclin metabolites in the untreated and bosentan-treated control and diabetic rats</td>
<td>105</td>
</tr>
<tr>
<td>4.8</td>
<td>A. Sensitivities to various agents in the superior mesenteric arteries from the four rat groups</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>B. Sensitivities to various agents in the renal arteries from the four rat groups</td>
<td></td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Free intracellular Ca^{2+}</td>
</tr>
<tr>
<td>+dP/dt</td>
<td>Rate of contraction</td>
</tr>
<tr>
<td>-dP/dt</td>
<td>Rate of relaxation</td>
</tr>
<tr>
<td>2hPG</td>
<td>2-hour plasma glucose</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>6-keto-PGF\textsubscript{1\alpha}</td>
<td>6-keto-prostaglandin F\textsubscript{1\alpha}</td>
</tr>
<tr>
<td>A\textsubscript{260 nm}</td>
<td>Absorbance at 260 nm</td>
</tr>
<tr>
<td>A\textsubscript{280 nm}</td>
<td>Absorbance at 280 nm</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BB rat</td>
<td>BioBreeding rat</td>
</tr>
<tr>
<td>C rat</td>
<td>Control rat</td>
</tr>
<tr>
<td>CA rat</td>
<td>Control ABT-627-treated rat</td>
</tr>
<tr>
<td>CDA</td>
<td>Canadian Diabetes Association</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRC</td>
<td>Concentration response curve</td>
</tr>
<tr>
<td>CT rat</td>
<td>Control bosentan-treated rat</td>
</tr>
<tr>
<td>D rat</td>
<td>Diabetic rat</td>
</tr>
<tr>
<td>DA rat</td>
<td>Diabetic ABT-627-treated rat</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>DT rat</td>
<td>Diabetic bosentan-treated rat</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzymeimmunoassay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ET-1-ir</td>
<td>Endothelin-1-like-immunoreactivity</td>
</tr>
<tr>
<td>ETA-ir</td>
<td>ETA receptor-like-immunoreactivity</td>
</tr>
<tr>
<td>ETB-ir</td>
<td>ETB receptor-like-immunoreactivity</td>
</tr>
<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>Gi</td>
<td>Inhibitory G-protein</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine-nucleotide-regulatory protein</td>
</tr>
<tr>
<td>Gs</td>
<td>Stimulatory G-protein</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Haemoglobin A1c</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N^G-nitro-L-arginine-methyl ester</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAPS Unit</td>
<td>Nucleic Acid Protein Services Unit</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OGGT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGE_2</td>
<td>Prostaglandin E_2</td>
</tr>
<tr>
<td>PGH_2</td>
<td>Prostaglandin endoperoxide H_2</td>
</tr>
<tr>
<td>PGI_2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RA</td>
<td>Renal arteries</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SMA</td>
<td>Superior mesenteric arteries</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TxA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>TxB2</td>
<td>Thromboxane B2</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WKY rat</td>
<td>Wistar Kyoto rat</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS

Publications Related to the Present Thesis:

Manuscripts:


Abstracts:


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DEDICATION

To Mom and Dad,
who are the best parents in the universe, for their love and care

And

To Tomomi,
who is the best sister in the world, for always being there for me
1. INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disease characterized by elevated blood glucose levels. With its increasing prevalence, diabetes is now regarded by some as a public health disorder, meeting the three essential characteristics that define the term: a high disease burden, changing disease incidence implying preventability, and public concern about risk [1]. In 1998/99, approximately 3.5% of Canadians or 0.9 million people aged 12 or older were diagnosed as having diabetes [2]. Given the fact that there are undiagnosed cases of diabetes [3], up to 10% of Canadian adults may presently have diabetes [4]. This number is believed to reach 3 million by 2010 [4].

Diabetes is a serious health concern. Not only is diabetes a major cause of coronary artery disease [5], which is the primary cause of death in Canada, it is also a leading contributing factor in visual impairment and end-stage renal failure in adults in North America [6-9]. Moreover, diabetes is exceedingly costly to both the patient and society. Diabetic patients generally have poorer health and spend more on managing their health than nondiabetic individuals. In the United States, as much as 15% of health care expenditures are spent on the management of diabetes [10].

Definition and classification of diabetes mellitus: the revised diagnostic criteria

Diabetes mellitus is defined as a group of metabolic disorders characterized by the presence of hyperglycemia resulting from defective insulin secretion, insulin action, or both. It is a chronic syndrome affecting carbohydrate, protein and fat metabolism.
Following the 1995 revision of the classification and diagnostic criteria for diabetes by the American Diabetes Association (ADA) [11], the revised scheme was adopted by the World Health Organization (WHO) as well as by the Canadian Diabetes Association (CDA) in 1998 [4, 12]. Under the new system, the classification of diabetes no longer relies on the type of pharmacological treatment used in its management but is primarily based on disease etiologies; the previous terms of “insulin-dependent diabetes mellitus” (IDDM) and “non-insulin-dependent diabetes mellitus” (NIDDM) were eliminated and in their place the terms “type 1” and “type 2” are used instead.

The current classification scheme for diabetes includes four main etiological categories: (1) Type 1 diabetes caused by pancreatic islet β-cell destruction leading to absolute insulin deficiency (~10% of all cases); (2) Type 2 diabetes resulting from insulin resistance with relative insulin deficiency or an insulin secretory defect (~90% of all cases); (3) other specific types of diabetes consisting primarily of specific genetically related forms of diabetes or diabetes associated with other disease or drug use; and (4) gestational diabetes mellitus referring to glucose intolerance with onset during pregnancy [4].

In the new diagnostic criteria, the diagnostic threshold of 11.1 mM used for plasma glucose level 2 hours after a 75-g glucose load in the oral glucose tolerance test (OGTT) has been retained from the previous system; this threshold value was decided based on the finding that at approximately this point the risk of developing microvascular complications such as retinopathy and nephropathy greatly increases [11]. However, it was found that the previous cutpoint used for fasting plasma glucose (FPG) ≥ 7.8 mM did not correlate well with the 2-hour plasma glucose (2hPG) in the
OGTT [11]; as a result, the cutoff level of FPG is now set at 7.0 mM in the newly implemented system [4]. The diagnosis of diabetes can be made by three ways: (1) random plasma glucose value ≥ 11.1 mM in the presence of symptoms of diabetes (i.e. fatigue, polyuria, polydipsia, unexplained weight loss); (2) FPG ≥ 7.0 mM; or (3) 2hPG ≥ 11.1 mM during an OGTT. In all cases, a confirmatory test must be performed on a subsequent day by any of the three methods.

**Diabetes and its complications**

Prior to the Nobel prize winning discovery of the pancreatic hormone insulin in 1921 [13], the outlook for the juvenile form of diabetes was such that this disease rarely lasted long as patients would succumb to fatal consequences due to acute metabolic complications such as diabetic ketoacidosis. With the introduction of insulin therapy, mortality due to acute complications dramatically decreased; however, the prolongation of survival carried with it a high price in terms of morbidity. Ten to 20 years after insulin had become available, the clinical impact of the complications arising from chronic diabetes became much more evident, when a rising number of reports documenting the presence of retinopathy, renal disease as well as heart disease in diabetic patients appeared in the 1940s and 1950s [14].

Regardless of the etiology, long-term diabetes mellitus often leads to a constellation of abnormalities that affect various organ systems and contribute towards the accelerated morbidity and mortality rates found in the diabetic population. The chronic hyperglycemia of this disease is associated with several long-term sequelae, resulting in damage, dysfunction and failure of several organs, including the kidneys,
eyes, nerves, heart and blood vessels. Clinically, those consequences of diabetes may be manifested as end-stage renal failure, blindness, defective nerve conduction, myocardial infarction, angina, congestive heart failure, and impaired wound healing.

**Diabetes and cardiovascular complications**

The findings that diabetic patients are particularly susceptible to cardiac failure and vascular complications have now become an irrevocable fact [15-17]. Up to 80% of mortality in diabetic patients is related to cardiovascular events [18]. The incidence of cardiovascular disease was shown to be increased by two to three fold in the presence of diabetes [18]. Indeed, the American Heart Association has recently stated that “from the point of view of cardiovascular medicine, it may be appropriate to say, diabetes is a cardiovascular disease” [19]. Diseases of large vessels (macroangiopathy) may lead to the development of coronary artery disease, atherosclerosis, peripheral vascular disease, stroke, hypertension and myocardial infarction [20, 21]. Pathological changes in the microvasculature, usually present as structural and functional alterations of the vessel (e.g. basement membrane thickening, increased vascular permeability, microaneurysms) [22], may contribute towards the development of diabetic retinopathy, neuropathy, foot pathology, cardiomyopathy and nephropathy [14, 23]. Cardiac complications in diabetes were previously assumed to mainly develop secondary to coronary atherosclerosis, macroangiopathy and autonomic neuropathy [15]. However, since the study of Rubler et al. [24] reporting the autopsies on four diabetic patients with congestive heart failure in the absence of coronary atherosclerosis 30 years ago, it has become apparent that a specific cardiac muscle disease (diabetic cardiomyopathy)
independent of the above vascular and neural abnormalities also occurs in diabetes [25-29].

Abnormalities in myocardial performance in diabetes concerns both diastolic and systolic function [26, 28, 30-32]. Impairments in diastolic function are one of the earliest signs of left ventricular dysfunction seen in diabetic patients. These include increased left ventricular end diastolic pressure, prolonged isovolumic relaxation times and increased myocardial stiffness, which are indications of defective ventricular relaxation and/or filling [26, 30, 32]. Reductions in systolic function also ensue in diabetic subjects who present with a shorter left ventricular ejection time, a longer pre-ejection period, and a higher ratio of pre-ejection period/left ventricular ejection time accompanied by increased left ventricular end systolic volume and decreased left ventricular ejection fractions [26, 28, 31, 33].

Haemodynamic abnormalities such as changes in blood flow and vascular contractility have been extensively documented in various organs of diabetic patients, including the kidneys, retina, peripheral arteries, and microvessels of peripheral nerves. An increased glomerular filtration rate (GFR) with increased renal plasma flow and an increased kidney size mark the early phase of diabetic nephropathy, while a progressive fall in GFR is seen at a later stage [34, 35]. Blood flow has been shown to be decreased in the retina of diabetic patients [36]. Alterations in either direction (i.e. an increase or a decrease) in reactivity to vasoconstrictors have been noted in the diabetics [37-41]. Impaired vasodilation has also been reported in type 1 and particularly type 2 diabetic patients [42, 43]. Moreover, an impairment of nerve blood
flow and the presence of active epineural arterio-venous shunts have been observed in human diabetic neuropathy [44].

Hyperglycemia is recognized as one of the major factors leading to the generation of chronic diabetic complications [45]. Two landmark multicentre trials in the last decade, the Diabetes Control and Complications Trial (DCCT) [46] and the United Kingdom Prospective Diabetes Study (UKPDS) [47], have unequivocally established that hyperglycemia is the main cause of microvascular complications and that its contribution to cardiovascular events is probably also significant. Over the study period of the DCCT, which averaged 7 years, intensively treated type 1 diabetic patients with better glycemic control were shown to have an approximately 60% reduction in risk in retinopathy, nephropathy and neuropathy when compared with the conventionally treated individuals [46]. Likewise, in the UKPDS, which followed patients with type 2 diabetes for an average of 10 years, an improved blood glucose control was demonstrated to reduce the risk of developing retinopathy and nephropathy and possibly decrease neuropathy. The incidence of the overall microvascular complications was reduced by 25% in patients treated with the intensive therapy (with a median haemoglobin $A_1c$ ($HbA_{1c}$) of 7.0%) when compared with those receiving the conventional therapy (with a median $HbA_{1c}$ of 7.9%). Moreover, it was shown that every percentage point decrease in $HbA_{1c}$ was able to lower the risk of microvascular complications by 35% [47]. Lessons learnt from these studies are that effective glycemic control is important in preventing or reversing the resultant metabolic derangements and complications due to long-term diabetes.
Potential mechanisms underlying diabetic cardiovascular complications: an overview

A number of equally tenable mechanisms, possibly as a consequence of hyperglycemia, have been proposed to mediate the various cardiovascular complications of diabetes. Abnormalities in vascular sensitivity and reactivity to various ligands, alterations in cardiac autonomic function, altered myocardial substrate metabolism, decreased ventricular compliance and hypertrophy have been linked to diabetic cardiomyopathy [16, 48-51]. In addition, abnormalities in various transmembrane and sarcolemmal proteins which control intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(i\)) have also been proposed as important mediators of cardiac dysregulation in diabetes [52, 53]. An increase in oxidative stress has been associated with diabetes as well as its cardiovascular complications [54-57]. Augmented polyol pathway activity and nonenzymatic glycation may play a role as well [45, 58, 59]. Alterations in the activities of guanine-nucleotide-regulatory proteins (G-proteins) have also been implicated in diabetic cardiac and vascular abnormalities [60-68]. As well, the diacylglycerol (DAG)-protein kinase C (PKC) pathway appears to be activated in excess in diabetes and may participate in several of its major complications [69-76]. More recently, the potent endothelium-derived vasoconstrictor peptide endothelin-1 (ET-1) has emerged as a potential factor in the development of diabetic complications [77-86]. The list of the possible mechanisms proposed is long, reflecting the multifactorial nature of the causes underlying diabetic complications. Perhaps each proposed pathway is a different representation of a common underlying pathogenic mechanism; or more likely, perhaps different tissues are susceptible to different mechanisms. The purpose of the present
thesis was to explore the role of one of the above mediators, ET-1, in the development of diabetic cardiovascular complications, with particular reference to streptozotocin-induced diabetes.

**Animal model of diabetes: streptozotocin-induced diabetes**

Animal models of diabetes provide a useful tool in determining the etiologies of the complications caused by diabetes as they feature many physiological and pathophysiological characteristics resembling those found in human diabetes. Genetic type 1 models of diabetes include the spontaneously diabetic BioBreeding (BB) Wistar rat and the NOD mouse. Type 2 models such as insulin resistant fa/fa Zucker rat, fa/fa diabetic Zucker rat, db/db mouse and ob/ob mouse are also widely used. These models allow the identification of the specific genes and environmental triggers that may be involved in the development of human diabetes. However, the use of these genetic animals is associated with problems such as their normoglycemic control littermate also displaying some inborn defects in their metabolic regulatory systems [87, 88].

Diabetes induced in animals by chemicals like streptozotocin (STZ) and alloxan offers a model better controlled for the duration and severity of the diabetic state. Both STZ and alloxan specifically cause β-cell necrosis, leading to the development of a hyperglycemic and hypoinsulinemic state that mimics type 1 diabetes. The degree of diabetes can be controlled by the dose of the compounds. Due to the greater selectivity of the β-cell for STZ and the lower mortality rate seen in STZ-diabetic animals [89, 90], STZ is favored over alloxan as the chief diabetogen.
Streptozotocin (STZ) (2-deoxy-2-(3-methyl-3-nitrosourea) 1-D-glucopyranose) is a broad-spectrum antibiotic extracted from *Streptomyces achromogenes*. Its chemical structure consists of a highly reactive methyl nitrosourea side chain bound to the C2 position of 2-deoxy-D-glucose. While the nitrosourea moiety is responsible for the cytotoxic action of STZ, the glucose moiety is thought to promote the transport of this agent across the cell membrane via a membrane receptor possibly GLUT2, the glucose transporter isoform expressed in the pancreatic β-cells [91]. Three processes are currently believed to be responsible for the β-cytotoxic effects of STZ—methylation of DNA resulting from the formation of carbonium ions (CH$_3^+$) [92], free radical production [93, 94] and nitric oxide (NO) generation [95]. These processes have been proposed to initiate DNA strand breaks resulting in the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) as part of the DNA repair mechanism [92-96]. The PARP utilizes NAD$^+$ as substrate to produce the ADP-ribose units required for the excision-repair process. Exhaustion of NAD$^+$ subsequent to PARP activation leads to the cessation of all NAD$^+$-dependent cellular functions and death of β-cell [92, 96].

Diabetes mellitus induced by STZ in rats exhibits the characteristic symptoms of uncontrolled type 1 diabetes such as polydypsia, polyphagia, decreased weight gain, fasting hyperglycemia, hypoinsulinemia and elevated plasma lipid levels [64, 97-100]. The severity of the diabetic state induced by STZ is dose-dependent [89]; however, the diabetogenic action of STZ in rats appears to be influenced by the route of administration as well as the strain, age and gender of the rats [101]. In our laboratory, the induction of stable diabetes in male Wistar rats is achieved by a single intravenous injection of STZ at a dose between 50-75 mg/kg [99, 102, 103]. In this dose range, the
diabetic rats do not normally require insulin supplement for survival, since the remaining viable pancreatic β-cells release enough insulin to prevent ketoacidosis, as demonstrated by Junod et al. [89].

Rats with chronic STZ-diabetes display many of the cardiovascular aberrations similar to those seen in human diabetic patients [51]. In the rat heart, both systolic and diastolic functions are disturbed by chronic STZ-diabetes. Studies with isolated working heart preparations have demonstrated that the left ventricular function of STZ-diabetic rats is impaired, as shown by a depressed left ventricular pressure development and slower rates of contraction and relaxation in response to increases in left atrial filling pressure in comparison with nondiabetic controls [97, 99, 104, 105]. The impairments in isolated whole heart function are often transferred to isolated tissues and cells of diabetic myocardium; the contraction and relaxation of both isolated papillary muscles and cardiomyocytes from STZ-diabetic rats have been found to be decreased, implicating the involvement of intracellular defects in diabetic cardiac dysfunction [106-108]. Using rodent echocardiography, a recent study following animals longitudinally at different days after induction of diabetes has confirmed that STZ-diabetic rats experienced cardiac functional abnormalities in vivo [109]. Diastolic failure was shown to precede systolic dysfunction in these diabetic rats, similar to events occurring in human diabetes. Significant reductions in diastolic function were observed within 3 days after induction of diabetes by 65 mg/kg i.p. STZ while systolic performance was significantly attenuated only after 35 days [109]. These observations on systolic function are in agreement with our previous reports on isolated working hearts [97, 105]. Furthermore, numerous studies have reported the occurrence of vascular functional
abnormalities in STZ-diabetic rats, as characterized by altered vascular reactivity to vasoconstrictors as well as impaired vasodilation [51, 110-128].

In addition, the fact that STZ-rats are relatively resistant to the development of atherosclerosis makes this diabetic model most suitable for studying aspects of the non-atherogenic form of diabetic cardiomyopathy. Consequently, the STZ-diabetic rat model has been used extensively to elucidate the mechanism/s and mediators of cardiovascular dysfunction in diabetes and in the evaluation of treatment modalities which could prevent or reverse these abnormalities [64, 97-100, 129, 130].
1.2 ENDOTHELIUM AND THE ENDOTHELIN SYSTEM

**Endothelium and its role in vascular function**

The monumental discovery of endothelium-derived relaxing factor by Furchgott and Zawadzki in 1980 [131] made a colossal impact on vascular biology and our understanding of the functional roles of the endothelium. By employing a simple pharmacological technique which assessed the vascular function of isolated vessel preparations, Dr. Furchgott’s group demonstrated the imperative role of the endothelium in mediating the relaxation responses to acetylcholine and proposed the release of a relaxing factor [131], now known to be nitric oxide [132], from the endothelium. Subsequently, the importance of the endothelium in the regulation of the vascular tone was realized and an impetus to investigate the presence of other endothelium-derived vasoactive substances followed.

The endothelium is a layer of cells lining the intimal surface of all blood vessels as well as the cardiac valves and several body cavities. The strategic anatomical location between the circulating blood and vascular smooth muscle allows the endothelium to be involved in normal and pathologic physiology. In addition to serving as a protective covering and permeability barrier to the movement of substances through the blood vessel wall, this monolayer of cells lining the vasculature releases a diverse array of proteins, prostanoids, and other paracrine elements to maintain a delicate balance between vasoconstriction and vasodilation, coagulation and blood fluidity, and inhibition and promotion of vascular growth [133-135].
The endothelium acts as a local modulator of vascular smooth muscle tone and reactivity; it releases both vasodilator (e.g. NO, prostacyclin (PGI$_2$), hyperpolarizing factor) and vasoconstrictor (e.g. ET-1, thromboxane A$_2$ (TxA$_2$), angiotensin II, prostaglandin endoperoxide H$_2$ (PGH$_2$)) mediators in response to a variety of neurohumoral factors as well as to vessel wall mechanical forces [133-135].

Endothelium-derived NO is a potent endogenous vasodilator that is derived from the metabolism of L-arginine to L-citrulline by NO synthase [136, 137]. Its physiological importance in modulating homeostatic vascular resistance has been demonstrated by the observation that inhibition of NO synthesis elevates blood pressure [138].

The prostanoids vasodilator PGI$_2$ and vasoconstrictor TxA$_2$ also play a pivotal role in the maintenance of vascular homeostasis. Arachidonic acid is the major precursor of prostanoids and is hydrolyzed from the glycerophospholipids in the plasma membrane via the action of phospholipase A$_2$ [139, 140]. The two cyclooxygenase isoforms, constitutively expressed COX-1 and inducible COX-2, then mediate the oxygenation of arachidonate to yield PGH$_2$ [140]. Conversion of PGH$_2$ occurs via specific synthases to several biologically active end-products including prostaglandins and TxA$_2$ [139]. Prostacyclin synthase and thromboxane synthase, as implied by their names, are responsible for the synthesis of PGI$_2$ and TxA$_2$ respectively [139].

In 1988 Yanagisawa et al. [141] described a novel vasoconstrictive peptide, termed endothelin, which was isolated from cultured porcine aortic endothelial cells. Endothelin-1 (ET-1) belongs to the family of "endothelin peptides" and has been shown to be the most potent vasoconstrictor identified to date in the human.
In addition to their direct effects on the vascular smooth muscle tone, the endothelium-derived vasoactive substances are involved in other roles in maintaining vascular homeostasis. In general, the vasoconstrictors released from the endothelium, such as ET-1, TxA$_2$ and angiotensin II, are prothrombotic and proproliferative. Conversely, endogenous vasodilators such as NO and PGI$_2$ are antithrombotic and antiproliferative. Disturbances in the balance between these vasoactive factors may disrupt vascular homeostasis and play a role in the initiation and progression of vascular diseases.

**Endothelins: biosynthesis, distribution and clearance**

In 1985, Hickey et al. discovered a peptidergic coronary vasoconstrictor produced by cultured endothelial cells. This factor was purified and cloned, and the name “endothelin” was coined in the landmark study of Yanagisawa and colleagues in 1988 [141]. Subsequently, other members of the endothelin (ET) family have been isolated. ETs are comprised of a family of structurally related 21-amino acid peptides consisting of ET-1, ET-2 and ET-3. They are structurally similar to vasoactive intestinal contractor [142], cardiotoxic snake venom sarafotoxins [143] and bibrotoxin [144]. The 21-amino acid ET-1, the predominant endothelin released by vascular endothelial cells, possesses free amino and carboxy termini with four cysteine residues which form two intramolecular disulfide bonds in positions 1-15 and 3-11 (Scheme 1).
Structure of Endothelin-1

The mature endothelin peptides are derived from the preproendothelins (preproET) through the action of two proteases. Each of the preproETs (preproET-1, -2 and -3) has a distinct gene and amino acid sequence with ET-2 and ET-3 differing from ET-1 by two and six amino acids, respectively [145]. Removal of the signal sequence from the 212-amino acid preproET-1 generates proET-1 (195 amino acid) which is then cleaved at dibasic sites by a furin-like enzyme to form the biologically inactive 38-amino acid (39-amino acid in some species) peptide big ET-1. Big ET-1 is converted to the mature ET-1 peptide by hydrolysis of the Trp^{21}-Val^{22} bond by a phosphoramindon-sensitive membrane-bound zinc neutral metalloprotease, endothelin converting enzyme (ECE). ECE, found in a variety of cells including endothelial cells and vascular smooth muscle [146, 147], processes big ET-1 both intracellularly as well as on the cell surface (Scheme 2) [146, 148-150].
SCHEME 2

Biosynthesis of Endothelin-1

Endothelial cells

Endothelin-1 gene

PreproET-1 → Big ET-1

ECE

Big ET-1

ET-1

ECE

Vascular smooth muscle cells

ET_A

ET_B

Various stimuli induce the transcription of preproendothelin-1 (preproET-1) mRNA from the endothelin-1 (ET-1) gene (chromosome 6 in human). PreproET-1 protein is then processed by a furin-like proteases to form big ET-1 that are further cleaved by endothelin converting enzyme (ECE) to mature ET-1. The conversion from big ET-1 to ET-1 can occur intracellularly, or extracellularly on the cell membranes. The majority of ET-1 (75%) are secreted abluminaly (i.e. the smooth muscle side of the endothelial cells); only a small portion gets released into the blood stream. The ET-1 peptide interacts with a pharmacologically selective ET_A and nonselective ET_B receptors.
ET-1 is ubiquitously produced; in addition to vascular endothelial cells which are the predominant source of the peptide in vivo, it is synthesized in vascular smooth muscle (VSM), cardiomyocytes, airway epithelial cells, renal tubular epithelium, glomerular mesangial cells, glia, macrophages, mast cells, fibroblasts, retina and other cells [149, 151-153]. As much as 75% of ET-1 produced by endothelial cells is secreted abluminally toward the vascular smooth muscle side of the cells [154]. The plasma concentration of ET-1 in many species is in the picomolar range, much lower than the pharmacological threshold which is between 0.05-1 nM [155]. As a result, ET-1 is considered as an autocrine/paracrine mediator rather than an endocrine hormone under physiological conditions.

Unlike other peptidic hormones whose regulation can take place at the levels of synthesis, storage and release, the ET-1 level is mainly regulated by synthesis, particularly transcription, due to the fact that many cells producing ET-1 lack storage vesicles or regulatory secretory pathways. Basal control of ET-1 gene transcription is dependent on the activator protein 1 (AP-1) site in the promoter region regulated by the transcription factors c-fos and c-jun [156]. As well, AP-1 regulatory elements respond to PKC activation. PKC may therefore mediate the effects of various stimuli on ET-1 expression through AP-1 transactivation. The levels of preproET-1 mRNA are up-regulated by various chemical and mechanical stimuli including thrombin, catecholamines, angiotensin II, vasopressin, transforming growth factor-β, Ca^{2+} ionophore, insulin, glucose, tumor necrosis factor-α, and haemodynamical shear stress, low-density lipoproteins (LDL) and other factors [141, 149, 150, 157, 158]. A recent study has also demonstrated that the redox-sensitive transcription factor nuclear factor-
kappa B (NF-κB) is involved in induction of ET-1 transcription [159]. Nitric oxide, PGI₂ and atrial natriuretic factor, however, have been shown to decrease ET-1 production in the endothelial cells [160-162], possibly via stimulation of guanylate cyclase and production of cGMP [163].

The plasma half-life of ET-1 is about 4-7 minutes [164]. Plasma ET-1 is primarily cleared by the lungs as demonstrated by a study showing the retention of 80% of bolus-injected ET-1 by this organ during first passage [165]. The clearance of ET-1 is likely to be mediated by the endothelin-B (ET_B) receptor as the selective ET_B receptor blocker BQ788 greatly attenuates the accumulation of intravenously administered radiolabelled ET-1 in lungs and kidneys and slows its clearance from the circulation while the endothelin-A (ET_A) receptor blocker BQ123 does not show such an effect [165]. In renal tissues, neutral endopeptidase (EC 3.4.24.11) is also involved in the control of turnover of ET-1, as shown by the marked increase in urinary endothelin levels by the inhibitors of the endopeptidase [166].

**Endothelin receptors**

Two mammalian receptor subtypes, ET_A and ET_B [167, 168], mediate the actions of ET-1. The two endothelin receptor subtypes, ET_A and ET_B, were originally named to distinguish sites in rat aorta (A) and guinea pig bronchus (B) [169]. The ET_A receptor shows a high specificity for ET-1 and is present on vascular smooth muscle cells [167]. Originally detected in vascular endothelial cells [168], the ET_B receptor, which has an equal affinity for all isopeptides of endothelins, was also demonstrated to exist on vascular smooth muscle cells in humans [170], rats [171], dogs [172] and rabbits [173].
In the heart, both $\text{ET}_A$ and $\text{ET}_B$ receptors are present in atrial and ventricular myocardium, the atrioventricular conducting system and endocardial cells [174], with the $\text{ET}_A$ receptor representing 90% of ET receptors on cardiomyocytes [149, 175]. Other noncardiovascular cells such as airway smooth muscle cells, hepatocytes, brain neurons, osteoblasts, melanocytes, keratinocytes, adipocytes, and various cells in the reproductive tract also express $\text{ET}_A$ receptors. $\text{ET}_B$ receptors are also found in hepatocytes, renal collecting duct epithelial cells, airway smooth muscle cells, osteoblasts, neurons of the central and peripheral nervous system, and various cells of the reproductive tract [149, 176].

Both $\text{ET}_A$ and $\text{ET}_B$ receptors belong to the superfamily of heptahelical transmembrane G-protein-coupled receptors and range from 45 000-50 000 daltons in size. The amino acid structures of the two receptors have a homology of approximately 50%. The diversity of the actions of ET-1 can be explained by the fact that different ET receptor subtypes are coupled to a number of distinct signal transduction cascades through different G-proteins, including $G_s$, $G_q$, $G_i$, and $G_o$, resulting in activation of phospholipase C-β (PLC-β), PLA$_2$, PLD, and PKC, stimulation of Na$^+$/H$^+$ antiporter, modulation of adenylate cyclase activity (inhibition in some tissues but stimulation in others), activation of guanylate cyclase, increase in intracellular calcium, activation of various kinases including S6 kinase and mitogen-activated protein kinases (MAPKs) and induction of immediate early genes (for review, see Ref#177 and #150).
Physiological actions of ET-1

Endothelin-1 exerts a wide range of biological activities, and elicits both short-term effects such as contraction and secretion and long-term effects such as cell growth and migration. ET-1 is the most potent vasoconstrictor identified to date in the human. Subnanomolar concentrations of ET-1 result in dramatic, sustained and dose-dependent systemic vasoconstriction with potency exceeding that of angiotensin II, norepinephrine and leukotriene C4 by several fold [178]. Both ETA and ETB receptor subtypes on VSM cells mediate vasoconstriction [170, 172, 173, 179]. Most endothelial cells express only ETB receptors, activation of which induces production of PGI2 and NO, leading to vasodilatation [164, 180, 181].

The distribution of vascular smooth muscle ETA and ETB receptors and that of endothelial ETB receptors depend on the vascular beds, resulting in regional differences in vascular responses to ET-1. Haemodynamic assessments by Gardiner et al. [182] in conscious unrestrained rats demonstrated that a low bolus dose of ET-1 caused a decrease in renal and mesenteric blood flow but an increase in hindquarter blood flow without affecting the mean arterial pressure or carotid haemodynamics. At a higher dose of ET-1, sustained reductions in renal and mesenteric blood flow were accompanied by transient hyperaemia in the hindquarters. Brief carotid hyperaemia was followed by a marked decrease in blood flow. The initial hyperaemia and the resulting hypotension were counteracted by tachycardia after which hypertension and bradycardia developed as a result of the sustained reductions in blood flow. ET-1-evoked vasoconstriction in the rat renal and mesenteric vasculature is partly mediated by ETB receptors since BQ-3020 (an ETB receptor agonist), and ET-1 in the presence of
a selective ET\textsubscript{A} receptor antagonist (FR139317) have both been demonstrated to induce a vasoconstrictor response in those vascular beds [183].

Apart from acting as a potent systemic vasoconstrictor, ET-1 has marked effects on cardiac vessels and tissues. Low doses of ET-1 have been shown to produce an initial coronary vasodilation in isolated Langendorff hearts [184, 185]. However, the predominant effect of ET-1 in the coronary circulation is a prolonged vasoconstriction [186, 187], which leads to impaired ventricular function [184]. In isolated cardiac tissue preparations such as isolated papillary muscle and paced left atria, ET-1 has been shown to possess positive inotropic and chronotropic effects [153, 186]. In one study, a direct infusion of the selective ET\textsubscript{A} receptor antagonist BQ123 into the human coronary artery resulted in decreases in left ventricular pressure changes, indicating that endogenous ET-1 acts a basal positive inotrope in the human heart [188]. In the kidney, ET-1 reduces renal blood flow and GFR via renal vasoconstriction, thereby reducing urine flow and sodium excretion [153]. ET-1 also exhibits marked mitogenic effects; it stimulates the proliferation of VSM and glomerular mesangial cells [189-191]. As well, ET-1 has potent hypertrophic effects on cardiac myocytes [192]. ET-1 may affect the synthesis of extracellular matrix proteins such as fibronectin and collagen [193, 194], and treatment with endothelin receptor blockers have been shown to attenuate the expression of these proteins in different disease states [195-200].

Endothelins have also other noncardiovascular effects, which affect functions of airway smooth muscle, the kidney, the gastrointestinal tract, endocrine glands and the peripheral and central nervous systems. Moreover, gene knockout experiments have
demonstrated the importance of the endothelin system in the regulation of development [149].

**Endothelin receptor antagonists**

In many disease states, the expression and/or production of ET-1 and its receptors have been reported to be increased [149, 150, 153]. A pathophysiological role for ET-1 has been implicated in a number of diseases including pulmonary hypertension, congestive heart failure, renal failure, myocardial ischemia, hypertension, increased microvascular cell permeability, profound mitogenesis, atherosclerosis and cardiac shock [149, 150, 153, 178, 201-208]. Consequently, there has been a marked interest in developing inhibitors of the endothelin system. Various ET receptor antagonists with different profiles are currently being developed, including bosentan (Tracleer™, Actelion Ltd., Allschwil, Switzerland) and ABT-627 (Abbott Laboratories, Abbott Park, IL, USA).

Bosentan is a nonpeptidic ET receptor antagonist that is selective for both the ETA and ETB receptors [209]. The drug is orally effective and at doses of 100mg/kg/day has been demonstrated to exhibit antihypertensive properties and anti-ischemic effects. We previously used this drug in insulin resistant and hyperinsulinemic fructose hypertensive rats [202]. Bosentan has been demonstrated to competitively antagonize the specific binding of labelled ET-1 with a $K_i$ of 4.7 nM for the ETA receptor and 95 nM for the ETB receptor. It selectively inhibits the binding of ET-1 in isolated rat aorta and blocks contractions elicited by ET-1 and sarafotoxin S6c with a $pA_2$ of 7.2 and 6.0 respectively. The binding of 40 other peptides, prostaglandins, ions and
neurotransmitters is not affected by bosentan [209]. *In vivo*, bosentan has been demonstrated to block the pressor responses to ET-1 (acutely) and exhibit antihypertensive and antihypertrophic effects (chronically). Its profile makes it extremely useful as a pharmacological tool and potential therapeutic agent [209]. Indeed, it was the first ET receptor antagonist approved by FDA for treatment of pulmonary hypertension in November 2001 and is currently available in US and Canada.

ABT-627 is the active enantiomer of the racemate, A-127722, and is a potent and selective ET<sub>A</sub> receptor antagonist [210]. Like bosentan, it is nonpeptidic and orally bioavailable. ABT-627 has been shown to inhibit ET-1 binding to the ET<sub>A</sub> receptor with a K<sub>i</sub> of 0.034 nM. Affinity for the ET<sub>B</sub> receptor is almost 2000-fold less, with a K<sub>i</sub> of 63 nM. It inhibits contraction evoked by ET-1 and sarafotoxin S6c with respective pA<sub>2</sub> values of 9.0 and 6.5. Seven-day oral treatment of 10mg/kg/day ABT-627 was previously demonstrated to effectively attenuate tactile allodynia in STZ-diabetic rats [211].
1.3 ENDOTHELIN—A POSSIBLE LINK BETWEEN DIABETES AND ITS CARDIOVASCULAR COMPLICATIONS

Accumulating evidence has revealed that this endothelium-derived contracting peptide (ET-1) may have an involvement in the pathogenesis and/or reinforcement of diabetic cardiovascular complications [77-86]. Both human and experimental diabetes have been associated with an increase in ET-1 levels [80, 82, 85, 212-218], suggesting that an alteration in ET-1 production in diabetes may be a generalized phenomenon. Elevated glucose levels have been shown to trigger the gene expression and release of ET-1 in cultured endothelial cells [219, 220] as well as diabetic rat glomeruli [215]. An increased urinary excretion of ET-1 has been reported in spontaneously diabetic BB rats [216]. Reports in STZ-diabetes indicate an exaggerated vascular release of ET-1 from diabetic rat mesenteric arteries [213]; an elevated ET-1 mRNA level has also been demonstrated in renal [82] and retinal [85, 218] tissues. Additionally, the levels of ET-1 have been reported to be increased in clinical diabetes, as assessed by urinary excretion and plasma levels [80, 212, 214, 217]. The reactivity of blood vessels to ET-1 has been shown to be altered in diabetes, although much research remains to be done to clarify the nature of the alteration and to elucidate the underlying mechanisms [77-79, 112, 113, 119, 221-224].

Accordingly, an altered ET-1 system could potentially play a role in the development and/or progression of the cardiovascular abnormalities of chronic diabetes. If such a hypothesis were valid, then agents that block the endothelin system, for example, ET receptor antagonists, would be able to minimize end-organ damage in
diabetic cardiovascular system. Indeed, several studies have demonstrated the beneficial effects of ET receptor antagonists in the cardiovascular system in experimental diabetes [85, 199, 200].

**Previous data from our laboratory: effects of chronic endothelin receptor blockade in STZ-diabetic rat hearts**

If ET-1 is a mediator in the development of cardiovascular diseases in diabetes, blockade of its actions should therefore prevent or ameliorate these complications. To test the validity of this hypothesis, we had previously examined the effects of chronic ET receptor blockade (with the ETA and ETB receptor antagonist, bosentan, 100 mg/kg/day po 7-week treatment) on rate of contraction (+dP/dt), rate of relaxation (-dP/dt) and left ventricular developed pressure (LVDP) in isolated working hearts from STZ-diabetic rats (see Figure 1.1) [225]. Data from this study showed that diabetic hearts exhibited a depressed cardiac response, with an inability to respond to increases in left atrial filling pressure. Chronic bosentan treatment was able to partially restore the ability of the diabetic heart to respond to increases in preload when assessed by LVDP and -dP/dt (see Figure 1.1) [225]. We also examined the effects of chronic bosentan treatment on coronary reactivity to ET-1 in diabetic rats (see Figure 1.2, Verma et al., in press [226]). Hearts from STZ-diabetic rats exhibited enhanced coronary pressor responses to ET-1 (at 50 pM and 100 pM), which were normalized by bosentan treatment. Therefore, these findings provide supporting evidence for the participation of ET-1 in the development of cardiovascular dysfunction in diabetes.
FIGURE 1.1
Effect of Chronic Endothelin Receptor Blockade on Isolated Working Heart Function in Control and Diabetic Rats

A. Rate of Relaxation

B. Left Ventricular Developed Pressure

A) Rate of relaxation (-dP/dt) and B) left ventricular developed pressure (LVDP) as a function of increasing left atrial filling pressures in untreated control (C) (n=7), control bosentan-treated (CT) (n=8), diabetic (D) (n=10), and diabetic bosentan-treated (DT) (n=7) rats. Each point is presented as the mean±SEM. *p<0.05, different from C, CT, and DT. #p<0.05, different from D. Adapted from Verma, Arikawa and McNeill, Am J Hypertens 2001; 14:679-687 [225]
FIGURE 1.2

Effect of Chronic Endothelin Receptor Blockade on Coronary Vasoreactivity in Control and Diabetic Rats

A. 50 pM ET-1

Changes in coronary perfusion pressure (CPP) in response to an exposure to A) 50 pM and B) 100 pM ET-1 in untreated control (C) (n=5), control bosentan-treated (CT) (n=5), diabetic (D) (n=6), and diabetic bosentan-treated (DT) (n=6) rats. Each point is presented as the mean±SEM. *p<0.05, different from C, CT and DT. Adapted from Verma et al., Can J Physiol Pharmacol in press [226]
2. RESEARCH OUTLINE: RATIONALES, OBJECTIVES, AND HYPOTHESES

Since the discovery of ET-1 in 1988, considerable evidence has implicated this potent vasoconstrictor-mitogenic peptide in several cardiovascular disease states including hypertension, renal failure, myocardial ischemia, increased microvascular cell permeability, profound mitogenesis, atherosclerosis and cardiac shock [153, 178, 201-208]. An increased ET-1 production and altered vascular reactivity to ET-1 have been demonstrated in experimental and clinical diabetes, suggesting that this peptide may also mediate diabetes-induced cardiovascular dysfunction [84, 113, 214].

If ET-1 is a mediator in the development of cardiovascular diseases in diabetes, blockade of its actions should therefore prevent or ameliorate these complications. Findings from our previous studies support this proposition. We demonstrated that long-term treatment with an ET receptor blocker, bosentan, improved isolated working heart function in STZ-diabetic rats [225]. Furthermore, we found that diabetic rat hearts exhibited enhanced coronary pressor responses to ET-1 which was restored by chronic bosentan treatment to control values (Verma et al., in press [226]). Hence, bosentan may improve diabetic heart function by preventing ET-1 mediated coronary vasoconstriction thereby improving coronary blood flow.

The above observations strongly indicate a role of ET-1 in the development of diabetic cardiovascular complications. Hence, agents that can block the endothelin system, for example, ET receptor antagonists, may be beneficial in minimizing end-organ damage in the cardiovascular system of diabetes. The two primary goals of the present thesis were (1) to investigate the potential role of ET-1 in modulating the
cardiac and vascular dysfunction of STZ-diabetes and (2) to examine the effects of chronic ET receptor blockade on the cardiovascular system in STZ-diabetic rats. The STZ-induced rodent model of Type 1 diabetes is a widely employed model which mimics much of the long-term cardiac and vascular complications of human diabetes. Therefore, it is a suitable model for the current investigation in order to examine the long-term effects of diabetes on the cardiac and vascular function and to determine if ET-1 is involved in those effects.

In the heart, we examined if chronic diabetes altered the expression of ET-1 and its receptors and if the cardioprotective effects of the mixed ET<sub>A</sub> and ET<sub>B</sub> receptor blocker bosentan in diabetes were related to its effects on the expression of those genes. In addition, we determined if chronic treatment with a selective ET<sub>A</sub> receptor antagonist (ABT-627, Abbott Laboratories, Illinois) could improve heart function in diabetic rats. In the vascular studies, we examined if vasoreactivity was altered in arteries from long-term diabetic rats and if chronic treatment with bosentan could normalize the vascular defects found in diabetes. We also attempted to elucidate whether diabetes-induced vascular dysfunction involved an alteration in the expression of ET-1 and its receptors as well as an interaction between ET-1 and TxA<sub>2</sub>.
2.1 OVERALL HYPOTHESIS

ET-1 may play a role in the development of the cardiovascular complications in Type 1 diabetes. Elevated glucose levels in diabetes may result in an increase in ET-1 levels in the heart and vasculature. Moreover, the expression of ET receptors may be altered in diabetes in a manner which reinforces the unfavorable effects of ET-1. Hence, we hypothesized that an activated endothelin system may lead to cardiac failure as well as vascular dysfunction in the diabetic states, and that chronic endothelin receptor blockade may therefore normalize cardiac and vascular functions in diabetes by blocking ET-1 actions.

2.2 SPECIFIC RESEARCH OBJECTIVES AND RATIONALES

STUDIES IN THE HEART

Study #1. To determine the effects of chronic diabetes and ET receptor blockade with bosentan (a mixed ET\textsubscript{A} and ET\textsubscript{B} receptor antagonist) on the tissue level and gene expression of ET-1 in rat hearts

Chronic endothelin receptor blockade with bosentan has been shown to improve cardiac function in diabetic rat hearts [225], suggesting that an activated endothelin system, such as an up-regulation of ET-1 expression, may be involved in diabetic cardiomyopathy. Moreover, high glucose levels have been demonstrated to increase the production of ET-1 via a PKC dependent pathway [219, 220, 227]. Previous studies have also demonstrated that ET-1 may stimulate its own production possibly via the ET\textsubscript{B} receptor cells [228-230]. Furthermore, treatment with a mixed ET receptor blocker was shown to attenuate the elevated renal ET-1 levels in diabetes [82]. Hence, ET receptor...
blockers may exert their beneficial effects by preventing the autoinduction of ET-1. We believed that increased ET-1 levels in diabetes might be responsible for the development of diabetic cardiac dysfunction. Hence, hearts from STZ-diabetic rats would exhibit an increased peptide and/or mRNA level of ET-1. As well, bosentan might correct cardiac function in diabetes by normalizing the expression of ET-1.

**Study #2. To examine the effects of chronic ET receptor blockade with bosentan on the tissue level and gene expression of ET\textsubscript{A} and ET\textsubscript{B} receptors in diabetic rat hearts**

Alterations in ET-1 action in diabetes may be due to changes in the expression of ET receptors. Deng et al. [85] have demonstrated an up-regulation of ET receptor (both ET\textsubscript{A} and ET\textsubscript{B}) mRNA in the presence of elevated levels of ET-1 in retina isolated from long-term diabetic rats. Similar effects have been noted in rats with congestive heart failure wherein increased ET-1 levels are associated with parallel and paradoxical increases in ET-1 binding sites [231]. In addition, an increase in cardiac ET-1 binding sites, with no change in affinity of ET-1, has been observed in 2, 4, and 6-week STZ-diabetic rats [86]. However, whether such an increase in the expression of ET-1 receptors occurs in our diabetic rat cardiac tissues is unknown. Hence, we examined the expression of ET\textsubscript{A} and ET\textsubscript{B} in both untreated and bosentan-treated diabetic rat cardiac tissues. Alterations in ET receptor level might be important in the pathogenesis of cardiac complications of diabetes. Bosentan might correct cardiac function in diabetes by normalizing the levels of ET receptors.
**Study #3. To study the effects of chronic treatment with a selective ET\textsubscript{A} receptor blocker on isolated working heart function of diabetic rats**

The non-specific nature of the dual ET\textsubscript{A}/ET\textsubscript{B} receptor blocker bosentan prevents us from determining which specific receptor subtype(s) may be responsible for mediating the pathogenic role of ET-1. Moreover, results from Study #2 in which there was an increased expression of ET\textsubscript{A} receptors in both the right and left ventricles of diabetic rats suggest that ET\textsubscript{A} receptors may have an important role in the development of diabetic cardiac failure. Hence, the purpose of the present study was to determine if selective blockade of ET\textsubscript{A} receptors would provide cardioprotective effects in diabetes. *We believed that ET-1 might mediate negative changes to the heart via ET\textsubscript{A} receptors in diabetes and that chronic treatment with a selective ET\textsubscript{A} receptor blocker ABT-627 would protect the diabetic hearts against impairments in cardiac function.*

**STUDIES IN THE VASCULATURE**

**Study #4. To examine the effects of chronic diabetes and ET receptor blockade with bosentan on the plasma and vascular tissue level and gene expression of ET-1 in rats**

Elevated glucose levels have been shown to trigger the gene expression and release of ET-1 [219, 220, 227], possibly through the activation of PKC [220]. Hence, the hyperglycemic states of STZ-diabetes should result in an increase in plasma and vascular ET-1 levels. *An increased ET-1 level in diabetes might be responsible for the development of diabetic vascular abnormalities. Hence, arteries from STZ-diabetic rats would exhibit an increased level of ET-1.*
Study #5. To determine the effects of chronic bosentan treatment on the expression of ET<sub>A</sub> and ET<sub>B</sub> receptors in diabetic rat arteries

Alterations in ET-1 vascular action in diabetes (see Study #6) may be due to changes in the expression of ET receptors. We hypothesized that altered levels of ET receptors might be important in the pathogenesis of vascular complications of diabetes. Thus, we believed that bosentan would ameliorate vascular reactivity in diabetes (see Study #6) by normalizing the levels of ET receptors.

Study #6. To determine the effects of chronic bosentan treatment on vascular reactivity in diabetic rats

Vascular responses to various vasoactive agonists including ET-1 have been reported to be altered in diabetes. Studies have suggested that an activated endothelin system may be responsible for diabetic vascular complications [85, 199]. The alterations in vascular reactivity found in diabetes might be mediated by ET-1 which have been found to be increased in diabetes. Thus, chronic blockade of ET-1 action with bosentan should prevent or ameliorate vascular dysfunctions in diabetes.

Study #7. To determine the role of thromboxane A<sub>2</sub> in endothelin-induced hyper-reactivity in diabetic arteries

Results from Study #6 showed that the removal of the endothelium abolished the enhanced vascular responses to NE and ET-1 observed in diabetic arteries. Furthermore, we have previously demonstrated that the hyper-responsiveness to NE in
superior mesenteric arteries from diabetic rats was corrected by cyclooxygenase inhibition with indomethacin [116]. This implied that an altered synthesis of endothelium-derived vasoconstrictor eicosanoids such as TxA$_2$ [116, 232] could be a factor in the enhanced NE responses of the diabetic vessels. Indeed, an increase in TxA$_2$ release from diabetic aorta has been previously noted [232]. Moreover, ET-1 is a potent inducer of TxA$_2$ synthesis/release [233-235]. On the other hand, there is evidence suggesting that TxA$_2$ may stimulate the release of ET-1 [236]. Therefore, an interaction between the endothelin and thromboxane systems might result in an increase in ET-1 and TxA$_2$ levels in diabetes, which then in turn mediated/potentiated the endothelin-induced increase in vascular reactivity to vasoconstrictors in diabetes. Hence, acute blockade of ET receptors or inhibition of thromboxane synthesis should ameliorate the vascular functional defects in diabetic arteries.
3. MATERIALS AND METHODS

3.1 MATERIALS

Acetonitrile, acetylcholine (Ach) chloride, BQ123, 3,3'-diaminobenzidine tetrahydrochloride with cobalt enhancer (Sigma Fast™ DAB with Metal Enhancer), human/porcine endothelin-1 (ET-1), gum arabic, indomethacin, disodium EDTA (ethylenediaminetetraacetic acid), methyl formate, N⁶-nitro-L-arginine-methyl ester (L-NAME), norepinephrine (NE) hydrochloride (arterenol hydrochloride), REDTaq™ DNA polymerase, sodium nitroprusside (SNP), streptozotocin (STZ), U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α) were obtained from Sigma, St. Louis, MO. Halothane was purchased from Halocarbon Laboratories, North Augusta, SC. Sodium pentobarbital (Somnotol®) was from MTC Pharmaceuticals (Cambridge, ON). Bosentan and ABT-627 were generous gifts from Actelion, Allschwil, Switzerland and Abbott Laboratories, Illinois, respectively. Dazmegrel (UK-38485) was generously provided by Pfizer, Ltd, Sandwich, Kent, UK. Rabbit polyclonal anti-ET-1 antiserum and radioimmunoassay (RIA) kits for ET-1 were bought from Peninsula Laboratories Inc., Belmont, CA. Vectastain Elite kits were purchased from Vector Laboratories, Inc., Burlingame, CA. Sheep polyclonal anti-ETₐ receptor antibodies were obtained from Research Diagnostics, Inc., Flanders, NJ. Sheep polyclonal anti-ETₐ receptor antibodies were from Maine Biotechnology Services, Inc., Portland, ME. RNA STAT-60™ reagent system was purchased from Tel-Test, Inc., Friendswood, Texas. SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase System, Oligo(dT)₁₂-₁₈ primers, dNTP mix were purchased from Canadian Life Technologies Inc., Burlington, ON. RNase inhibitor was obtained from Roche Diagnostics Canada, Laval, Quebec. ET-1,
ET$_A$ receptor, ET$_B$ receptor and $\beta$-actin primers for the PCR assay were synthesized by Biotechnology Laboratory, Nucleic Acid Protein Services (NAPS) Unit, University of British Columbia, Vancouver, BC. Amprep 500mg C2 mini-columns, thromboxane B$_2$ (TxB$_2$) enzymeimmunoassay (EIA) kits and 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) EIA kits were obtained from Amersham International, Little Chalfont, Buckinghamshire, UK. Hexane was from Fisher Scientific, Nepean, ON. Enzymatic colorimetric assay kits for glucose and cholesterol were purchased from Boehringer Mannheim, Laval, Quebec. Enzymatic colorimetric assay kits for triglyceride were obtained from Boehringer Mannheim and Sigma, St. Louis, MO. Radioimmunoassay kits for insulin were from Linco Research Inc., St. Charles, MO. Unless otherwise stated, all other chemicals were reagent grade and obtained from either Sigma, St. Louis, MO, BDH Inc., Toronto, ON, or Fisher Scientific, Nepean, ON.

3.2 RESEARCH DESIGN AND EXPERIMENTAL PROCEDURES

3.2.1 Care and treatment of the animals

Male Wistar rats of approximately 200-250 g were obtained either from the Animal Care Unit, University of British Columbia, Vancouver, BC or from Charles River Laboratories Inc., Montreal, Quebec. They were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. The rats were housed on a 12-hour light (7:00-19:00)-dark cycle and received rat chow (LabDiet 5001, PMI® Nutrition International, Inc., Brentwood, MO) and water ad libitum. Rats were randomly assigned to two groups. One group received a single caudal vein injection of STZ (Sigma, St. Louis, MO) (dissolved in saline (0.9% sodium chloride) at 60mg/mL) at
a dose of 60 mg/kg (under halothane anesthesia) and served as the diabetic group. The other group was injected with saline and served as the age- and weight-matched control group. The rats injected with STZ were checked for hyperglycemia (set at ≥15 mM in our laboratory) at 72 h using Glucostix (Miles Canada Inc., Etobicoke, ON) reagent strips read by a Glucometer II (Ames, Miles Laboratories, Elkhart, Indiana).

**Oral bosentan treatment (for all studies except Study #3)**

In all studies except Study #3, the effect of chronic bosentan treatment on the cardiovascular system in control and diabetic rats was investigated. The control and diabetic rats were further divided into untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT). One week after STZ injection, oral bosentan treatment (100 mg/kg/day by daily oral gavage; bosentan suspended in 1% gum arabic) was administered to the CT and DT groups for 10 weeks while the C and D rats received the vehicle (1% gum arabic (Sigma, St. Louis, MO)). The therapeutic efficacy of this dose has been previously demonstrated by studies reporting a blood pressure lowering effect at this dose [202]. The treatment was chosen to last for 10 weeks (i.e. 11 weeks of diabetes) because, from our past experience as well as reports from other laboratories, both cardiac and vascular abnormalities of long-term diabetes are expected to be seen in STZ-diabetic rats at this time point [97, 116, 120, 123, 237]. Five-hour fasted blood samples were collected from the tail vein at basal, week 5 and week 10 of the bosentan treatment for determination of plasma glucose, triglyceride and insulin levels. Systolic blood pressure was measured by the tail-cuff method as described in Section 3.3.6 at basal, week 5 and week 10.
Oral ABT-627 treatment (for Study #3)

In Study #3, the effect of chronic selective blockade of ET$_A$ receptors on the diabetic rat heart function was examined. Rats were also divided into four groups: untreated control (C), control ABT-627-treated (CA), diabetic (D) and diabetic ABT-627-treated (DA). One week after induction of diabetes, ABT-627, an oral selective ET$_A$ receptor blocker synthesized by Abbott Laboratories, Illinois, was administered to the CA and DA groups in the drinking water at a dose of 5 mg/kg/day for 7 weeks. Five-hour fasted blood samples were collected from the tail vein at basal and week 7 of the ABT-627 treatment for biochemical measurements. Systolic blood pressure was measured at basal and week 7.

3.2.2 Experimental protocols

STUDIES IN THE HEART

Study #1

Effects of chronic diabetes and ET receptor blockade with bosentan (a mixed ET$_A$ and ET$_B$ receptor antagonist) on the tissue level and gene expression of ET-1 in rat hearts

To evaluate the long-term effects of diabetes and bosentan treatment on cardiac ET-1 expression, tissue peptide ET-1 levels were assessed by immunohistochemistry while ET-1 mRNA expression was examined by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. After 10 weeks of bosentan treatment, rats from all four experimental groups were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). For each animal, the chest
was opened, and the heart was quickly excised and rinsed in saline. For immunohistochemistry, ventricles were isolated and immersed in 10% formalin (for 8 hours). Slide sections of paraffin-embedded tissues were prepared by Wax-it, Histology Services, University of British Columbia, Vancouver, BC and subsequently processed for immunohistochemical analysis of ET-1-like-immunoreactivity (ET-1-ir). For the RT-PCR assay, ventricles were frozen in liquid nitrogen. Frozen ventricles were stored at –70°C until the RT-PCR assay was performed.

Study #2

**Effects of chronic ET receptor blockade with bosentan on the tissue level and gene expression of ET\textsubscript{A} and ET\textsubscript{B} receptors in diabetic rat hearts**

The effects of chronic bosentan treatment on cardiac expression of ET receptors in control and diabetic rats were studied. Same heart tissue samples of all four rat groups from Study #1 were used for immunohistochemical analyses of ET\textsubscript{A} receptor-ir (ET\textsubscript{A}-ir) and ET\textsubscript{B} receptor-ir (ET\textsubscript{B}-ir) as well as RT-PCR assessment of ET\textsubscript{A} and ET\textsubscript{B} receptors.

Study #3

**Effects of chronic treatment with a selective ET\textsubscript{A} receptor blocker on isolated working heart function of diabetic rats**

This study was undertaken to determine if selective blockade of ET\textsubscript{A} receptors would provide cardioprotective effects in diabetes. Control and diabetic rats were either treated with the oral selective ET\textsubscript{A} receptor antagonist ABT-627 (5 mg/kg/day) or not
treated for 7 weeks as described above. At the end of ABT-627 treatment, hearts were isolated from all four rat groups, and myocardial performance was assessed by isolated working heart function procedures.

**STUDIES IN THE VASCULATURE**

**Study #4**

Effects of chronic diabetes and ET receptor blockade with bosentan on the plasma and vascular tissue level of ET-1 in rats

The effects of chronic diabetes and bosentan treatment on plasma and vascular tissue ET-1 levels were examined in this study. Following 10 weeks of bosentan treatment, rats from all four experimental groups were anaesthetized with sodium pentobarbital (60 mg/kg), and blood samples for the determination of plasma ET-1 level were obtained via cardiac puncture. Plasma was separated and stored at -70°C until assayed. Superior mesenteric arteries and renal arteries were isolated from all rats and immersed in 10% formalin (for 8 hours). Slide sections of paraffin-embedded tissues were prepared by Wax-it, Histology Services, University of British Columbia, Vancouver, BC and subsequently processed for immunohistochemical analysis of ET-1-ir. Superior mesenteric arteries and renal arteries were selected in order to examine the role of ET-1 in arteries from different branches of the vascular tree as regional variations in the responses to ET-1 have previously been observed [182]. These two arteries are frequently used in vascular studies largely because of the ease in their isolation and handling. Furthermore, while superior mesenteric arteries and renal arteries are still considered to be large vessels, they do have a smaller diameter and therefore make a
greater contribution to peripheral vascular resistance than do aortae. Mesenteric arteries are generally accepted to be more representative for "resistance" vessels. The mesenteric circulation of the rat receives approximately one-fifth of the cardiac output [238] and, thus, alterations in this vasculature would significantly affect the normal regulation of blood flow to the organs. Renal arteries were chosen since they provide blood supply to the kidneys; alterations in these arteries would potentially affect normal renal function and may have a role in the development of diabetic nephropathy, which is another major complication related to diabetic angiopathy where ET-1 has also been implicated.

Study #5

Effects of chronic bosentan treatment on the expression of $\text{ET}_{A}$ and $\text{ET}_{B}$ receptors in diabetic rat arteries

The effects of chronic bosentan treatment on vascular tissue levels of ET receptors in control and diabetic rats were assessed. The same tissue samples of superior mesenteric arteries and renal arteries of all four rat groups from Study #4 were used for immunohistochemical analyses of $\text{ET}_{A}$-ir and $\text{ET}_{B}$-ir.

Study #6

Effects of chronic bosentan treatment on vascular reactivity in diabetic rats

At the end of 10 weeks of bosentan treatment, vascular reactivity studies were performed in superior mesenteric arteries and renal arteries isolated from the four experimental rat groups. Isolated arteries were cut into two rings with the endothelium
gently removed from one ring and the other left intact. Rings were suspended in individual tissue baths. Following an initial equilibration period (60 minutes), the tissues were exposed to agonists and isometric vascular responses recorded according to the following protocol: (i) a cumulative concentration response curve (CRC) to norepinephrine (NE) ($10^{-9}$-$10^{-4}$ M) in the absence and presence of the nitric oxide (NO) synthase inhibitor N$^G$-nitro-L-arginine-methyl ester (L-NAME) ($10^{-4}$ M incubated for 30 minutes prior to CRC), (ii) a cumulative CRC to acetylcholine (Ach) ($10^{-9}$-$10^{-4}$ M) in rings precontracted with $10^{-6}$ M NE, (iii) a cumulative CRC to sodium nitroprusside ($10^{-10}$-$10^{-5}$ M) in rings precontracted with $10^{-6}$ M NE and (iv) a cumulative CRC to ET-1 ($10^{-10}$-$3\times10^{-8}$ M).

**Study #7**

**Role of thromboxane A$_2$ in endothelin-induced hyper-reactivity in diabetic arteries**

Data from Study #6 showed that contractile responses to NE and ET-1 were enhanced in diabetic arteries. This hyper-reactivity was ameliorated by chronic bosentan treatment, suggesting a role for ET-1 in this vascular defect. Furthermore, the exaggerated vasoconstrictor responses in diabetic rats were demonstrated to be endothelium-dependent and could be blocked by cyclooxygenase inhibition, implicating the involvement of endothelium-derived vasoconstrictor eicosanoids such as TxA$_2$ in this effect. Therefore, experiments were conducted (1) to investigate if hyper-reactivity in diabetic arteries was mediated by direct vascular actions of ET-1 through its receptors, (2) to determine if TxA$_2$ is involved in this hyper-responsiveness to vasoconstrictors in diabetes, and (3) to examine if the beneficial effects of long-term
bosentan treatment seen in diabetic arteries were related to its blocking actions of the direct effects of ET-1 and/or its effects on the interaction between the endothelin and TxA₂ systems. Thus, contractile responses to vasoconstrictors were assessed following *in vitro* incubation of (1) bosentan, (2) a selective ETₐ receptor blocker, BQ123 (Sigma, St. Louis, MO), and (3) a thromboxane synthase inhibitor, dazmegrel (Pfizer, Sandwich, Kent, UK). As well, to evaluate the vascular reactivity to TxA₂ in diabetic arteries, vascular responses to U46619 (Sigma, St. Louis, MO), a TxA₂ analogue, were examined.

Following 10 weeks of bosentan treatment, blood samples were obtained via cardiac puncture for determination of plasma thromboxane and prostacyclin levels. Vascular reactivity studies were performed in superior mesenteric arteries and renal arteries isolated from the four experimental rat groups. Isolated arteries were cut into two rings. Rings were suspended in individual tissue baths. Following an initial equilibration period (60 minutes), the tissues were exposed to agonists and isometric vascular responses recorded according to the following order: (i) a cumulative CRC to Ach (10⁻⁹-10⁻⁴ M) following precontraction with 10⁻⁶ M NE, (ii) a cumulative CRC to NE alone (10⁻⁹-10⁻⁴ M), (iii) a CRC to NE constructed in the presence of either BQ123 (1 µM incubated for 30 minutes prior to CRC) or bosentan (1 µM for 30 minutes), (iv) a CRC to NE in the presence of dazmegrel (1 µM for 30 minutes), (v) a CRC to U46619 (10⁻¹²-10⁻⁶ M), (vi) a CRC to ET-1 (10⁻¹⁰-3x10⁻⁸ M) constructed either in the absence or in the presence of dazmegrel (1 µM for 30 minutes).
3.3 METHODOLOGY

3.3.1 Isolated working heart function

Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). The chest was opened, and the heart was excised and mounted on the working heart apparatus. Perfusion was first initiated in the retrograde manner through the aorta. Following cannulation of the left pulmonary vein, cardiac work was initiated by switching from the retrograde mode to the working heart mode such that buffer entered the left ventricle via the left atrium and exited through the aorta. The aortic outflow was subjected to a constant afterload of 6 R.U. (resistance units). The heart was paced at a constant rate of 300 beats/min. A 20 gauge needle, inserted through the apex of the heart into the left ventricle was used to measure left ventricular pressure via a Stratham P23AA pressure transducer. The perfusion fluid used was Chenoweth-Koelle solution [composition (mM): NaCl 120, KCl 5.6, CaCl$_2$ 2.18, MgCl$_2$ 2.1, glucose 10.0 and NaHCO$_3$ 19.2], aerated with 95% O$_2$ and 5% CO$_2$ and maintained at 37°C. Left ventricular performance was assessed in terms of rate of contraction (+dP/dt), rate of relaxation (-dP/dt) and left ventricular developed pressure (LVDP) in response to increasing left atrial filling pressures (preload) as described previously [99].

3.3.2 Isolated blood vessel preparation

Rats were anaesthetized with an intraperitoneal injection of pentobarbital. The abdomen was opened and the superior mesenteric and renal arteries were isolated. Arteries were isolated from each rat, cleaned of adherent connective tissue and cut into two rings (2-3 mm in length). Some rings were denuded of the endothelium by gently
rolling a stainless steel rod on the intimal surface of the vessel while the endothelium was preserved for the other ring. Successful removal of the endothelium was assessed by the loss of the relaxation responses to $10^{-5}$ M Ach. The tissues were suspended on wire hooks in isolated tissue baths containing modified Krebs-Ringer bicarbonate solution with the following composition (in mM): NaCl (118), KCl (4.7), CaCl$_2$ (2.5), KH$_2$PO$_4$ (1.2), MgSO$_4$ (1.2), NaHCO$_3$ (25), dextrose (11.1) and disodium EDTA (0.026), maintained at 37°C, and oxygenated with 95% O$_2$ and 5% CO$_2$. Each ring was subjected to a resting tension of 1.0 g (superior mesenteric arteries) and 0.75 g (renal arteries) determined to allow for maximum force generation from pilot data. Following an initial equilibration period (60 minutes), isometric vascular responses to various vasoactive agents were recorded and cumulative concentration response curves constructed according to the protocols described above in section 3.2.2. For each concentration, a plateau was obtained before the subsequent dose was added. After each concentration response curve (CRC), buffer was replaced several times to wash the tissues until the resting tension of each tissue was reached. At the end of the experiment, the tissues were removed, blotted dry, and the cross-sectional area of each vascular ring was calculated \[\text{cross-sectional area (mm}^2\) = \frac{\text{weight (mg)}}{\text{length (mm)} \times \text{density (mg/mm}^3\)}\]. The density of the arteries was assumed to be 1.05 mg/mm$^3$ [239]. The absolute tension generated was corrected for cross-sectional area and expressed as g/mm$^2$. Agonist pD$_2$ values (-log ED$_{50}$) were calculated by nonlinear regression analysis of the individual CRC's and used as an index of sensitivity [239].
3.3.3 Immunohistochemistry

**ET-1**

Immunohistochemical analysis of ET-1-like-immunoreactivity (ET-1-ir) was performed in ventricular and vascular tissues from the four experimental groups. Ventricles, superior mesenteric arteries, renal arteries were immersed in 10% formalin (for 8 hours) and embedded in paraffin by Wax-it, Histology Services, University of British Columbia, Vancouver, BC. Slide sections of paraffin-embedded tissue were dewaxed in xylene, preincubated with 0.6% hydrogen peroxide in methanol for 1½ hour (to block endogenous peroxidase activity) and blocked with 5% normal goat serum for 1 hour (room temperature). The slides were subsequently incubated with a rabbit polyclonal anti-ET-1 antiserum (Peninsula Laboratories Inc., Belmont, CA, USA) at a 1:1000 dilution for 48 hours at 4°C. The slides were then washed in phosphate buffered saline (PBS) for 10 minutes, and immunostained with an avidin-biotin-peroxidase system (Vectastain Elite kit, Vector Laboratories, Inc., Burlingame, CA) using diaminobenzidine (with cobalt enhancer, Sigma, St. Louis, MO) as the chromagen. Slides incubated with non-immune normal rabbit serum (vs. anti-ET-1 antiserum) served as the negative control. A digital imaging system was used for assessment of ET-1-ir. Slides were viewed through a Nikon Diaphot TMD inverted microscope. A video camera connected to an IBM compatible computer with Northern Eclipse Software (Empix Imaging Inc., Mississauga, ON) converted the data to a digital image, each with a gray value ranging from 0 to 255. For each tissue, two (for renal arteries) or four (for ventricles and superior mesenteric arteries) different images were taken. Areas with an equal size were selected for each image. A threshold above which pixels were counted
was established. Any areas darker than the threshold would be recognized as “objects”. Percent object area (= total object area/selected area X 100%) from each image was obtained. Values from the images were averaged for each rat tissue.

**ET receptors**

Tissue ET receptor protein expression levels in the ventricles and arteries were assessed by immunohistochemical analyses of ET\(_A\) receptor-ir (ET\(_A\)-ir) and ET\(_B\) receptor-ir (ET\(_B\)-ir) using similar methods employed for ET-1-ir as described above with a few modifications. Briefly, endogenous peroxidases were blocked by 1.5-hour incubation in 0.6% H\(_2\)O\(_2\) methanol, and the slides were blocked with 5% normal rabbit serum for 1 hour at room temperature. The slides were then subsequently incubated with either sheep polyclonal anti-ET\(_A\) receptor antibodies (at a 1:180 dilution, Research Diagnostics, Inc., Flanders, NJ) or sheep polyclonal anti-ET\(_B\) receptor antibodies (at a 1:250 dilution, Maine Biotechnology Services, Inc., Portland, ME) at 4°C for 45 hours. Non-immune sheep serum was used as control for verifying the specificity of staining. The slides were then washed with PBS and immunostained with an avidin-biotin-peroxidase system from Vector Laboratories, Inc. The intensity of staining was assessed using the digital imaging system and Northern Eclipse software as described above.
3.3.4 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

**ET-1**

Tissue total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [240] with RNA STAT-60™ reagent system (Tel-Test, Inc., Friendswood, Texas) according to the manufacturer's instructions. The concentration of extracted RNA was measured by determining the absorbance at 260 nm ($A_{260\text{ nm}}$). The purity and integrity of RNA were assessed by determining the ratio $A_{260\text{ nm}}/A_{280\text{ nm}}$ and by gel electrophoresis on 1% agarose gel stained with ethidium bromide. First strand cDNA synthesis was performed using the SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase System (Life Technologies Inc., Burlington, ON). Total RNA was added to oligo(dT) primers (Life Technologies Inc., Burlington, ON), denatured at 70 °C for 10 minutes. Reverse transcription was carried out by the addition of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and dNTPs (Life Technologies Inc., Burlington, ON) in the presence of 40 U RNase inhibitor (Roche Diagnostics Canada, Laval, Quebec) and 10 mM dithiothreitol (DTT) at 42°C for 50 minutes in a total reaction volume of 20 µL. The reaction was terminated by a 15-minute incubation at 70°C. The resulting RT products were stored at −20°C until used. The amplification was carried out using the following cDNA sequences. ET-1 sense primer (5'-GCT CCT GCT CCT CCT TGA TG -3') and ET-1 antisense primer (5'- CTC GCT CTA TGT AAG TCA TGG -3') with a predicted product size of 499 bp were used [85, 241]. The housekeeping gene β-actin sense primer (5'-TGG TGG GTA TGG GTC AGA AGG-3') and antisense primer (5'- ATC CTG TCA GCG ATG CCT GGG-3') with a predicted product size of 813 bp [85, 242] was used as an internal control for coamplification with ET-1. Reactions
were carried out in a volume of 50 μL using RT products from 150 ng RNA and 5 U REDTaq™ DNA polymerase (Sigma, St. Louis, MO). The PCR amplification was carried out as follows: the initial cycle using 3 minutes at 94°C (denaturation), 1 minute at 60°C (annealing) and 3 minutes at 72°C (extension); subsequent cycles of PCR using 45 seconds at 94°C (denaturation), 45 seconds at 60°C (annealing) and 1 minute at 72°C (extension). The PCR amplification was completed with a final extension step (7 minutes at 72°C). Conditions for the PCR assay were optimized prior to the actual experiment by analyzing PCR products with variable amounts of template, primer concentration and cycle numbers to establish the linearity of the PCR amplification. Since β-actin is highly expressed, fewer cycles are required for its amplification. The primers for β-actin were added into the reaction tube after 7 cycles (i.e. during the denaturation step of the 8th cycle). Hence, 31 cycles and 24 cycles of amplification were performed for ET-1 and β-actin, respectively. The PCR products were electrophoresed on 1.5% agarose gels (75 V for 2 hours), stained with ethidium bromide, visualized by ultraviolet transilluminator, and photographed. The photographs were scanned, and the PCR products were analyzed by densitometry using a computer with Quantity One® software (PDI Inc., Huntington Station, NY). The densitometric values of the amplified products of ET-1 were normalized against those of the housekeeping gene β-actin. Sequencing of the PCR products were carried out by Biotechnology Laboratory, Nucleic Acid Protein Services (NAPS) Unit, University of British Columbia, Vancouver, BC to ensure the accuracy of the PCR amplification.
ET Receptors

RT-PCR was performed for ET receptors as described above with a few modifications. The amplification was carried out using the following primers: for $ET_A$ receptor sense primer (5'-TTC GTC ATG GTA CCC TTC GA-3') and antisense primer (5'-GAT ACT CGT TCC ATT CAT GG-3') with a predicted size of 546 bp, and for $ET_B$ receptor sense primer (5'-TTC ACC TCA GCA GGA TTC TG-3') and antisense primer (5'-AGG TGT GGA AAG TTA GAA C-3') with a predicted size of 475 bp, were used [241]. $ET_A$, $ET_B$ and $\beta$-actin were coamplified in the same reaction tube. Thirty-one cycles and 24 cycles of amplification were chosen for ET receptors and $\beta$-actin, respectively, after the linearity of the PCR amplification had been established. The densitometric values of the amplified products of $ET_A$ and $ET_B$ receptors were normalized against those of the housekeeping gene $\beta$-actin. Sequencing of the PCR products were done by Biotechnology Laboratory, NAPS Unit, University of British Columbia, Vancouver, BC to ensure the accuracy of the PCR amplification.

3.3.5 Biochemical measurements

Blood samples were collected from the tail vein, except at termination when samples were obtained by cardiac puncture. Samples were centrifuged in a desktop microcentrifuge (14000 rpm (19283 x g) for tail vein samples and 4500 rpm (3645 x g) for cardiac samples at 4°C for 25 minutes, Beckman Allegra 21R Centrifuge, Beckman Instruments, Inc., Spinco Division, Palo Alto, CA) to separate plasma. Plasma was aliquoted and stored at or below −20°C until assayed.
**Measurement of plasma ET-1**

A quantity of 2 mL of plasma was acidified with 0.5 mL HCl (2M) and centrifuged at 8000 x g for 10 minutes. Extraction of ET-1 from plasma was performed using Amprep 500mg C2 mini-columns (Amersham International, Little Chalfont, Buckinghamshire, UK). The supernatant was passed through the column, washed initially with 5 mL of water (containing 0.1% trifluoroacetic acid (TFA)) and then with 2 mL of 80% acetonitrile (containing 0.1% TFA) to elute ET-1. The eluent was then dried in a Speed-Vac (Savant Instruments, Farmingdale, NY) overnight and reconstituted in the assay buffer. Immunoreactive ET-1 was measured by a radioimmunoassay (RIA) using a specific anti-serum to ET-1 obtained from Peninsula Laboratories, Inc., Belmont, CA, USA.

**Measurement of thromboxane and prostacyclin levels**

Blood samples for the determination of plasma thromboxane B₂ (TxB₂) and 6-keto-prostaglandin F₁α (6-keto-PGF₁α), the stable metabolites of TxA₂ and prostacyclin, respectively, were obtained via cardiac puncture. Cardiac puncture samples were collected into polypropylene tubes containing 0.95 mL EDTA (0.05 M) and 0.05 mL indomethacin (0.04 M) to inhibit platelet generation of prostaglandins ex vivo. A quantity of 1 mL of plasma was acidified with 0.25 mL HCl (2M). Extraction of TxB₂ and 6-keto-PGF₁α from plasma was performed using Amprep 500mg C2 mini-columns (Amersham International, Little Chalfont, Buckinghamshire, UK). The columns were washed initially with 2 mL methanol and 2 mL water. The acidified plasma samples were then passed through the columns, which were subsequently washed with 5mL water, 5 mL 10% ethanol and 5 mL hexane. TxB₂ and 6-keto-PGF₁α were eluted with 5
mL methyl formate. The eluent was then dried under nitrogen and reconstituted in the assay buffer. TxB\(_2\) and 6-keto-PGF\(_{1\alpha}\) levels were measured using enzyme immunoassay kits from Amersham.

**Measurement of glucose, triglycerides, cholesterol and insulin**

Plasma glucose was measured either with enzymatic colorimetric assay kits (Boehringer Mannheim, Laval, Quebec) or with an automatic Beckman Glucose Analyzer 2 (Beckman Instrumentals, Inc., Diagnostic Systems Group, Brea, CA). Triglyceride and cholesterol levels were determined using enzymatic colorimetric kits from Boehringer Mannheim and Sigma, St. Louis, MO. Plasma insulin levels were measured using double antibody radioimmunoassay kits from Linco Research Inc., St. Charles, MO.

**3.3.6 Systolic blood pressure measurement**

Blood pressure was measured in conscious rats using the indirect tail cuff method without external preheating as previously described [243, 244]. Rats were placed in Plexiglas holders and their tails inserted into an inflatable cuff containing a photoelectric sensor, connected to a multi-sensor manual scanner (Model 65-120) and a blood pressure amplifier with an analog/digital recorder and printer (Model 179 semi-automatic blood pressure analyzer) from IITC Inc., Woodland Hills, CA. The reappearance of pulsations on gradual deflation of the cuff was detected by the photoelectric sensor. Pulsations were amplified and recorded digitally as the blood pressure. A minimum of 5 readings were obtained for each rat and an average of three of those readings taken as the individual blood pressure. Animals were preconditioned
to the procedures before conducting actual measurements. The tail cuff method has
been validated in our laboratory as well as demonstrated by others that recorded
pressures by this method were similar (within 5 mmHg) to those obtained by direct intra-
arterial cannulation [243-246].

3.3.7 Statistical analyses

Values are expressed as mean±SEM. "n" indicates the number of rats in each
group. Statistical analyses were performed using a one-way analysis of variance
(ANOVA) or a repeated measures ANOVA (general linear models ANOVA), followed by
a Newman-Keuls test, with the Number Cruncher Statistical System (Kaysville, Utah).
The level of significance was set at P<0.05.
4. RESULTS

4.1 GENERAL CHARACTERISTICS

Untreated and bosentan-treated control and STZ-diabetic rats

A representation of the general characteristics of the untreated and bosentan-treated rats at basal (i.e. 4 to 7 days after STZ injection in the diabetic groups prior to bosentan treatment), week 5 and week 10 of bosentan treatment is summarized in Table 4.1. Induction of STZ-diabetes in D rats resulted in characteristic symptoms of diabetes including hyperglycemia, hypoinsulinemia, decreased body weight gain, increased food and fluid intake when compared with age-matched controls (C rats). Plasma triglyceride levels were higher in diabetic rats than in control rats while plasma cholesterol levels were not different among groups. Bosentan treatment did not affect the plasma glucose, insulin, triglyceride or cholesterol levels in either control (CT) or diabetic (DT) rats. Nor did it affect body weight gain and food and fluid intake in these rats. The systolic blood pressure of the STZ-diabetic rats was similar to that of the control rats and was not influenced by bosentan treatment.

Untreated and ABT-627-treated control and STZ-diabetic rats

The general features of the untreated and ABT-627-treated rat groups before and after 7 weeks of ABT-627 treatment are depicted in Table 4.2. STZ injection in D rats resulted in characteristic symptoms of diabetes including hyperglycemia, decreased body weight gain, and increased food and fluid consumption when compared with age-matched controls (C rats). These characteristics were unaffected by 7 weeks of ABT-
627 treatment as seen in CA and DA rats. Systolic blood pressure was similar among all groups at the end of 7 weeks of ABT-627 treatment (C 106±2 mmHg, CA 105±2 mmHg, D 103±2 mmHg, DA 102±3 mmHg, P>0.05).
<table>
<thead>
<tr>
<th></th>
<th>C (n=8)</th>
<th>CT (n=8)</th>
<th>D (n=9)</th>
<th>DT (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Week 5</td>
<td>Week 10</td>
<td>Basal</td>
</tr>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>227±2</td>
<td>392±9</td>
<td>482±14</td>
<td>223±3</td>
</tr>
<tr>
<td><strong>Food Intake (g/day)</strong></td>
<td>30±1</td>
<td>29±1</td>
<td>38±5</td>
<td>34±0</td>
</tr>
<tr>
<td><strong>Fluid Intake (mL/day)</strong></td>
<td>71±2</td>
<td>85±2.2</td>
<td>78±5</td>
<td>75±3</td>
</tr>
<tr>
<td><strong>Plasma Insulin (ng/mL)</strong></td>
<td>2.5±0.3</td>
<td>2.8±0.6</td>
<td>2.7±0.3</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td><strong>Plasma Glucose (mM)</strong></td>
<td>7.9±0.2</td>
<td>7.4±0.2</td>
<td>7.1±0.1</td>
<td>8.2±0.1</td>
</tr>
<tr>
<td><strong>Plasma Cholesterol (mM)</strong></td>
<td>2.1±0.1</td>
<td>2.0±0.1</td>
<td>2.1±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td><strong>Plasma Triglyceride (mM)</strong></td>
<td>1.4±0.1</td>
<td>1.6±0.3</td>
<td>1.6±0.1</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure (mmHg)</strong></td>
<td>105±1</td>
<td>118±2</td>
<td>116±3</td>
<td>104±2</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, CT, D and DT denote control, control bosentan-treated, diabetic and diabetic bosentan-treated rats, respectively. Diabetes was induced in D and DT rats by an intravenous injection of 60 mg/kg streptozotocin. The above parameters were measured at basal (i.e. prior to bosentan treatment), week 5 and week 10 of bosentan treatment (100 mg/kg/day). *P<0.05, different from C and CT. #P<0.05, different from C only.
**Table 4.2**

General Characteristics of the Four Experimental Groups before or after 7 Weeks of ABT-627 Treatment

<table>
<thead>
<tr>
<th></th>
<th>C (n=10)</th>
<th>CA (n=10)</th>
<th>D (n=10)</th>
<th>DA (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Week 7</td>
<td>Basal</td>
<td>Week 7</td>
</tr>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>257±3</td>
<td>518±10</td>
<td>270±3</td>
<td>542±7</td>
</tr>
<tr>
<td><strong>Food Intake (g/day)</strong></td>
<td>27±0</td>
<td>30±1</td>
<td>29±1</td>
<td>34±1</td>
</tr>
<tr>
<td><strong>Fluid Intake (mL/day)</strong></td>
<td>49±1</td>
<td>55±4</td>
<td>49±2</td>
<td>68±4</td>
</tr>
<tr>
<td><strong>Plasma Glucose (mM)</strong></td>
<td>8.2±0.1</td>
<td>7.3±0.1</td>
<td>8.2±0.2</td>
<td>7.3±0.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, CA, D and DA denote control, control ABT-627-treated, diabetic and diabetic ABT-627-treated rats, respectively. Diabetes was induced in D and DA rats by an intravenous injection of 60 mg/kg streptozotocin. The above parameters were measured at basal (i.e. before ABT-627 treatment) and week 7 of ABT-627 treatment (5 mg/kg/day). *P<0.05, different from C and CA.
STUDIES IN THE HEART

4.2 STUDY #1

Effects of chronic diabetes and ET receptor blockade with bosentan (a mixed ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist) on the heart weight/body weight ratio and on the tissue level and gene expression of ET-1 in rat hearts

In this study, hearts from untreated and 10-week bosentan-treated control and diabetic rats were obtained and analyzed by immunohistochemistry and RT-PCR, and the chronic effects of diabetes and bosentan treatment on cardiac ET-1 peptide and mRNA expression were evaluated.

The mean absolute heart weight of the 11-week STZ-diabetic (D) rats was lower than that of the control (C) rats (Table 4.3). However, diabetes also resulted in lower body weight. Thus, in order to correct for differences in body mass, the ratio of heart weight to body weight was used as a measure of relative cardiac hypertrophy. Diabetic rats exhibited a greater heart-to-body weight ratio than that of the control rats (Table 4.3). Bosentan treatment did not have any significant effects on this ratio in either control or diabetic rats as noted in CT and DT rats. Hence, this observation suggests that the cardioprotective effects of bosentan in diabetes previously seen in our studies are likely not mediated by blocking the hypertrophic effects of ET-1.

Immunohistochemical analysis revealed a marked increase in ET-1-ir with a higher intensity of anti-ET-1 immunostaining in both left and right ventricular tissues from diabetic rats when compared with the control rats (Figures 4.1 and 4.2). Chronic treatment with bosentan did not appear to alter the ventricular ET-1 peptide levels in
control or diabetic rats (Figures 4.1 and 4.2). Sections incubated with non-immune rabbit serum did not demonstrate immunostaining, indicating the specificity of the ET-1 antiserum (not shown).

RNA analysis by the semi-quantitative RT-PCR assay from the linear phase of amplification showed that ventricular ET-1 mRNA levels were significantly (P<0.001) increased in diabetic rats in comparison with the controls (Figure 4.3). Again, bosentan treatment did not have any effect on the mRNA expression of ET-1 in either control or diabetic ventricles. The mRNA expression of the housekeeping gene β-actin did not differ among groups (data not shown).

Hence, this study demonstrated that the expression of both peptide and mRNA for ET-1 was increased in ventricles from 11-week STZ diabetic rats, but 10 weeks of bosentan treatment did not have any effect on ET-1 expression in rat ventricles.
Table 4.3

Heart-to-Body Weight Ratio of the Rats After 10 Weeks of Bosentan Treatment

<table>
<thead>
<tr>
<th></th>
<th>C (n=10)</th>
<th>CT (n=10)</th>
<th>D (n=10)</th>
<th>DT (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>497±14</td>
<td>509±9</td>
<td>377±16*</td>
<td>383±16*</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>1.25±0.03</td>
<td>1.21±0.03</td>
<td>1.12±0.02#</td>
<td>1.14±0.04</td>
</tr>
<tr>
<td>Heart to Body Weight Ratio (mg/g)</td>
<td>2.53±0.04</td>
<td>2.37±0.05</td>
<td>3.01±0.13*</td>
<td>3.01±0.11*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, CT, D and DT denote control, control bosentan-treated, diabetic and diabetic bosentan-treated rats, respectively. Diabetes was induced in D and DT rats by an intravenous injection of 60 mg/kg streptozotocin (STZ). One week after STZ injection, bosentan treatment was administered to CT and DT rats at a dose of 100 mg/kg/day by oral gavage for 10 weeks. *P<0.05, different from C and CT. #P<0.05, different from C only.
FIGURE 4.1
ET-1-like-immunoreactivity in the Left Ventricle

**Left panel.** Representative figures showing ET-1-like-immunoreactivity (examined by immunohistochemistry) in left ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. **Right panel.** ET-1 peptide level examined by immunohistochemistry in left ventricles from control (C)(n=8), control bosentan-treated (CT)(n=8), diabetic (D)(n=8) and diabetic bosentan-treated (DT)(n=8) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
FIGURE 4.2
ET-1-like-immunoreactivity in the Right Ventricle

**Left panel.** Representative figures showing ET-1-like-immunoreactivity (examined by immunohistochemistry) in right ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. **Right panel.** ET-1 peptide level examined by immunohistochemistry in right ventricles from control (C)(n=3), control bosentan-treated (CT)(n=4), diabetic (D)(n=5) and diabetic bosentan-treated (DT)(n=5) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
FIGURE 4.3
ET-1 mRNA Levels in Ventricles

Upper panel. ET-1 mRNA level as assessed by reverse-transcription-polymerase chain reaction (RT-PCR) in ventricles from untreated control (C)(n=10), control bosentan-treated (CT)(n=10), diabetic (D)(n=8) and diabetic bosentan-treated (DT)(n=9) rats. Each point is presented as the mean±SEM. *P<0.001, different from C and CT. Lower panel. A representative photograph of the PCR products electrophoresed on a 1.5% agarose gel stained with ethidium bromide.
4.3 STUDY #2

**Effects of chronic ET receptor blockade with bosentan on the tissue level and gene expression of ET\textsubscript{A} and ET\textsubscript{B} receptors in diabetic rat hearts**

To study the effects of chronic bosentan treatment on the protein and mRNA expression of cardiac ET receptors in diabetes, hearts from untreated and 10-week bosentan-treated control and diabetic rats were obtained and analyzed by immunohistochemistry and RT-PCR.

The protein expression of ET\textsubscript{A} receptors, as assessed by immunohistochemistry, was increased in both the left and right ventricles from diabetic (D) rats which exhibited a higher intensity of anti-ET\textsubscript{A} receptor immunostaining than those of control (C) rats (Figures 4.4 and 4.5). An increased ET\textsubscript{B}-ir was found in right but not in left ventricular tissues from diabetic rats (Figures 4.6 and 4.7). Bosentan treatment did not seem to have any effect on the immunoreactivity for either ET receptors in either control (CT) or diabetic (DT) animals (Figures 4.6 and 4.7). Sections incubated with non-immune sheep serum did not demonstrate immunostaining, indicating the specificity of the polyclonal antibodies against ET\textsubscript{A} and ET\textsubscript{B} receptors (not shown).

In parallel with the increase in the protein expression, ET\textsubscript{A} receptor mRNA levels (examined by the semi-quantitative RT-PCR assay) were significantly (P<0.05) raised in diabetic rat ventricles when compared with controls (Figure 4.8). Long-term bosentan treatment did not affect the mRNA expression of ventricular ET\textsubscript{A} receptors in either control or diabetic rats. The mRNA expression of both ET\textsubscript{B} receptors (Figure 4.8) and the housekeeping gene \(\beta\)-actin did not differ among groups (data not shown).
Data from this study showed that the expression of both protein and mRNA for ET\textsubscript{A} receptors was increased in ventricles from 11-week STZ diabetic rats. The protein expression of ET\textsubscript{B} receptors was found to be increased in the right but not left ventricles from diabetic rats without any significant changes in the ventricular mRNA expression. Ten weeks of bosentan treatment did not affect the expression of the ET receptors in rat ventricles.
FIGURE 4.4
ET\textsubscript{A} Receptor-like-immunoreactivity in the Left Ventricle

Left panel. Representative figures showing ET\textsubscript{A} receptor-like-immunoreactivity (examined by immunohistochemistry) in left ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. Right panel. ET\textsubscript{A} receptor protein level examined by immunohistochemistry in left ventricles from control (C)(n=9), control bosentan-treated (CT)(n=10), diabetic (D)(n=9) and diabetic bosentan-treated (DT)(n=9) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
FIGURE 4.5
ETₐ Receptor-like-immunoreactivity in the Right Ventricle

Left panel. Representative figures showing ETₐ receptor-like-immunoreactivity (examined by immunohistochemistry) in right ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. Right panel. ETₐ receptor protein level examined by immunohistochemistry in right ventricles from control (C)(n=8), control bosentan-treated (CT)(n=6), diabetic (D)(n=7) and diabetic bosentan-treated (DT)(n=7) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
FIGURE 4.6
ET$_B$ Receptor-like-immunoreactivity in the Left Ventricle

**Left panel.** Representative figures showing ET$_B$ receptor-like-immunoreactivity (examined by immunohistochemistry) in left ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. **Right panel.** ET$_B$ receptor protein level examined by immunohistochemistry in left ventricles from control (C)(n=9), control bosentan-treated (CT)(n=9), diabetic (D)(n=9) and diabetic bosentan-treated (DT)(n=8) rats. Each bar represents the mean±SEM. *P<0.05, different from CT.
FIGURE 4.7

ET_B Receptor-like-immunoreactivity in the Right Ventricle

Left panel. Representative figures showing ET_B receptor-like-immunoreactivity (examined by immunohistochemistry) in right ventricles from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. Right panel. ET_B receptor protein level examined by immunohistochemistry in right ventricles from control (C)(n=7), control bosentan-treated (CT) (n=8), diabetic (D) (n=8) and diabetic bosentan-treated (DT) (n=7) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
FIGURE 4.8
ET_A and ET_B Receptor mRNA Levels in Ventricles

**Upper panel.** Endothelin-A (ET-A) and endothelin-B (ET-B) receptor mRNA level as assessed by reverse-transcription-polymerase chain reaction (RT-PCR) in ventricles from untreated control (C)(n=10), control bosentan-treated (CT)(n=10), diabetic (D)(n=7) and diabetic bosentan-treated (DT)(n=9) rats. Each point is presented as the mean±SEM. *P<0.05, different from C and CT. **Lower panel.** A representative photograph of the PCR products electrophoresed on a 1.5% agarose gel stained with ethidium bromide.
4.4 STUDY #3

**Effects of chronic treatment with a selective ET\(_A\) receptor blocker on isolated working heart function of diabetic rats**

The aim of this study was to investigate if chronic selective blockade of ET\(_A\) receptors would provide protection against diabetes-induced cardiac functional impairment. Hearts were isolated from control and diabetic rats either treated with 7 weeks of the selective ET\(_A\) receptor antagonist ABT-627 or not treated, and cardiac performance was examined using the isolated working heart technique.

Eight weeks of STZ-diabetes resulted in lower heart and body weights in the diabetic (D) rats compared with the nondiabetic control (C) group (Table 4.4). However, the heart-to-body weight ratio of the diabetic animals was significantly higher than that of the control group (Table 4.4), which is suggestive of relative cardiac hypertrophy in diabetic rats. Like bosentan, ABT-627 treatment did not alter the heart mass or the ratio of heart weight to body weight in either control (CA) or diabetic (DA) rats (Table 4.4).

Isolated working heart function was assessed by measuring left ventricular responses to changing left atrial filling pressures in terms of rate of relaxation (-dP/dt), rate of contraction (+dP/dt) and left ventricular developed pressure (LVDP). In control hearts, there was a progressive increase in these indices in responses to increases in left atrial filling pressure (Figure 4.9). In this study, cardiac function in untreated diabetic rats appeared to be impaired at the higher left atrial filling pressures, but significant differences were found between the control and diabetic rats only in the rate of contraction and LVDP (Figure 4.9; +dP/dt at 10 mmHg: C 3923±228 mmHg/s vs. D 2910±347 mmHg/s, P<0.05; LVDP at 10 mmHg: C 129±4 mmHg vs. D 110±6 mmHg,
P<0.05). Previous data from our laboratory demonstrated that the responses for all three parameters (±dP/dt and LVDP) of 8-week STZ-diabetic rat hearts were significantly depressed when compared with those of control rats [225].

At a dose of 5mg/kg/day, ABT-627 treatment did not induce any significant changes in cardiac performance of the diabetic rats when assessed by ±dP/dt and LVDP (Figure 4.9). However, a trend towards improvements in cardiac function after treatment was observed in diabetic rat hearts at 10 mmHg filling pressure. The cardiac performance of control rats was not affected by ABT-627 treatment.

The above data demonstrated that chronic ET\textsubscript{A} receptor blockade with ABT-627 had no effects on the heart size in diabetic rats. Moreover, the treatment was unable to restore cardiac function in diabetic rats.
Table 4.4

Heart-to-Body Weight Ratio of the Rats After 7 Weeks of ABT-627 Treatment

<table>
<thead>
<tr>
<th></th>
<th>C (n=8)</th>
<th>CA (n=8)</th>
<th>D (n=7)</th>
<th>DA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>539±13</td>
<td>567±13</td>
<td>386±19*</td>
<td>368±16*</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>1.61±0.04</td>
<td>1.74±0.09</td>
<td>1.37±0.06*</td>
<td>1.34±0.06*</td>
</tr>
<tr>
<td>Heart to Body Weight Ratio (mg/g)</td>
<td>3.02±0.12</td>
<td>3.06±0.12</td>
<td>3.57±0.08*</td>
<td>3.65±0.13*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, CA, D and DA denote control, control ABT-627-treated, diabetic and diabetic ABT-627-treated rats, respectively. Diabetes was induced in D and DA rats by an intravenous injection of 60 mg/kg streptozotocin (STZ). One week after STZ injection, ABT-627 treatment was administered to CA and DA rats at a dose of 5 mg/kg/day in the drinking water for 7 weeks. *P<0.05, different from C and CA.
Isolated working heart function assessed in terms of rate of relaxation, rate of contraction and left ventricular developed pressure (LVDP) in untreated control (C)(n=8), control ABT-627-treated (CA)(n=8), diabetic (D)(n=7) and diabetic ABT-627-treated (DA)(n=5) rats. Each point is presented as the mean±SEM. *P<0.05, different from C. #P<0.05, different from CA.
4.5 STUDY #4

Effects of chronic diabetes and ET receptor blockade with bosentan on the plasma and vascular tissue level and gene expression of ET-1 in rats

In this study, plasma samples as well as superior mesenteric arteries and renal arteries obtained from untreated and 10-week bosentan-treated control and diabetic rats were analyzed by radioimmunoassay and immunohistochemistry, respectively, to examine the chronic effects of diabetes and bosentan treatment on plasma and vascular ET-1 peptide levels.

Plasma levels of ET-1 for the four rat groups were C 5.40±0.56 pg/mL, CT 8.30±0.67 pg/mL*, D 5.97±0.22 pg/mL, DT 9.96±1.07 pg/mL* (*P<0.05, different from C and D). The plasma levels for ET-1 were similar between untreated control (C) and diabetic (D) rats. Bosentan treatment resulted in an increase in plasma ET-1 levels in both CT and DT groups, in accord with previous reports in human studies [247, 248].

Immunohistochemical analysis of the rat superior mesenteric arteries and renal arteries revealed the presence of a stronger ET-1-ir in both tissues from untreated diabetic (D) rats compared with those of control (C) rats (Figures 4.10 and 4.11). Similar to the observations seen in rat heart tissues, bosentan did not have any effect on the ET-1 levels in the superior mesenteric arteries and renal arteries of either control (CT) or diabetic (DT) rats. Sections incubated with non-immune rabbit serum did not demonstrate immunostaining, indicating the specificity of the ET-1 antiserum (data not shown).
Results from this study showed that diabetes resulted in an increase in ET-1 peptide expression in rat superior mesenteric arteries and renal arteries while plasma ET-1 levels were unaffected by the diabetic state. Bosentan treatment had no effect on the vascular ET-1 levels. Plasma ET-1 levels were raised by bosentan treatment in both control and diabetic rats.
FIGURE 4.10
ET-1-like-immunoreactivity in Superior Mesenteric Arteries

**Left panel.** Representative figures showing ET-1-like-immunoreactivity (examined by immunohistochemistry) in superior mesenteric arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. **Right panel.** ET-1 peptide level examined by immunohistochemistry in superior mesenteric arteries from control (C)(n=5), control bosentan-treated (CT)(n=7), diabetic (D)(n=6) and diabetic bosentan-treated (DT)(n=7) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
**FIGURE 4.11**
ET-1-like-immunoreactivity in Renal Arteries

**Left panel.** Representative figures showing ET-1-like-immunoreactivity (examined by immunohistochemistry) in renal arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats.

**Right panel.** ET-1 peptide level examined by immunohistochemistry in renal arteries from control (C)(n=7), control bosentan-treated (CT)(n=6), diabetic (D)(n=5) and diabetic bosentan-treated (DT)(n=7) rats. Each bar represents the mean±SEM. *P<0.05, different from C and CT.
4.6 STUDY #5

Effects of chronic bosentan treatment on the expression of \( \text{ET}_A \) and \( \text{ET}_B \) receptors in diabetic rat arteries

In this experiment, the effects of chronic bosentan treatment on the protein expression of vascular ET receptors in diabetes were studied. Superior mesenteric arteries and renal arteries taken from untreated and 10-week bosentan-treated control and diabetic rats were examined by immunohistochemistry.

The protein expression of \( \text{ET}_A \) receptors, as analyzed by immunohistochemistry, was increased in the superior mesenteric arteries from diabetic (D) rats which exhibited a higher intensity of anti-\( \text{ET}_A \) receptor immunostaining than those of control (C) rats (Figure 4.12), while there was no change in \( \text{ET}_A \) receptor expression in the renal arteries after 11 weeks of STZ-diabetes (Figure 4.13). Interestingly, treatment with bosentan restored the increased \( \text{ET}_A \) receptor levels in diabetic superior mesenteric arteries to control levels (Figure 4.12). No effect of bosentan treatment on the expression of renal arterial \( \text{ET}_A \) receptor levels was seen in either control or diabetic rats (Figure 4.13).

The immunoreactivity for \( \text{ET}_B \) receptors in the superior mesenteric arteries and renal arteries did not differ between control and diabetic rats (Figures 4.14 and 4.15), indicating that the protein expression of \( \text{ET}_B \) receptors was not altered in either arteries after long-term diabetes. Bosentan treatment did not seem to have any effect on the \( \text{ET}_B \)-ir in either arteries as observed in CT and DT rats (Figures 4.14 and 4.15). The specificity of the polyclonal antibodies against \( \text{ET}_A \) and \( \text{ET}_B \) receptors was
demonstrated by the absence of immunostaining in tissue sections incubated with non-immune sheep serum (data not shown).

This study demonstrated that the protein expression of \( \text{ET}_\text{A} \) receptors was increased in superior mesenteric arteries but not in renal arteries from 11-week STZ diabetic rats. Bosentan treatment was able to normalize the \( \text{ET}_\text{A} \) receptor levels in diabetic superior mesenteric arteries. The \( \text{ET}_\text{B} \) receptor expression in both arteries was similar among the groups.
FIGURE 4.12
ET\textsubscript{A} Receptor-like-immunoreactivity in Superior Mesenteric Arteries

Left panel. Representative figures showing ET\textsubscript{A} receptor-like-immunoreactivity (examined by immunohistochemistry) in superior mesenteric arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. Right panel. ET\textsubscript{A} receptor protein level examined by immunohistochemistry in superior mesenteric arteries from control (C)(n=8), control bosentan-treated (CT)(n=9), diabetic (D)(n=8) and diabetic bosentan-treated (DT)(n=6) rats. Each bar represents the mean±SEM. *P<0.05, different from C, CT and DT.
FIGURE 4.13
ET\textsubscript{A} Receptor-like-immunoreactivity in Renal Arteries

**Left panel.** Representative figures showing ET\textsubscript{A} receptor-like-immunoreactivity (examined by immunohistochemistry) in renal arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. **Right panel.** ET\textsubscript{A} receptor protein level examined by immunohistochemistry in renal arteries from control (C)(n=8), control bosentan-treated (CT)(n=8), diabetic (D)(n=8) and diabetic bosentan-treated (DT)(n=5) rats. Each bar represents the mean±SEM.
**FIGURE 4.14**

**ET₉** Receptor-like-immunoreactivity in Superior Mesenteric Arteries

*Left panel.* Representative figures showing ET₉ receptor-like-immunoreactivity (examined by immunohistochemistry) in superior mesenteric arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats. *Right panel.* ET₉ receptor protein level examined by immunohistochemistry in superior mesenteric arteries from control (C)(n=8), control bosentan-treated (CT)(n=7), diabetic (D)(n=10), and diabetic bosentan-treated (DT)(n=8) rats. Each bar represents the mean±SEM.
**FIGURE 4.15**
ET<sub>B</sub> Receptor-like-immunoreactivity in Renal Arteries

**Left panel.** Representative figures showing ET<sub>B</sub> receptor-like-immunoreactivity (examined by immunohistochemistry) in renal arteries from untreated control, control bosentan-treated, diabetic and diabetic bosentan-treated rats.  **Right panel.** ET<sub>B</sub> receptor protein level examined by immunohistochemistry in renal arteries from control (C)(n=6), control bosentan-treated (CT)(n=6), diabetic (D)(n=7) and diabetic bosentan-treated (DT)(n=7) rats. Each bar represents the mean±SEM.
4.7 STUDY #6

Effects of chronic bosentan treatment on vascular reactivity in diabetic rats

In this study, the long-term effects of bosentan on diabetic vascular function were investigated. Superior mesenteric arteries (SMA) and renal arteries (RA) from untreated and 10-week bosentan-treated control and diabetic rats were isolated, and vascular responses to various agonists were assessed in endothelium-intact as well as endothelium-denuded arteries. Responses to the vasoconstrictors NE and ET-1 were examined. As well, both endothelium-dependent and endothelium-independent relaxation responses were evaluated by studying the responses to Ach and SNP, respectively. To determine if the NO pathway was affected by the diabetic state and by the bosentan treatment, the effects of the NO synthase inhibitor L-NAME on the vascular responses were also appraised.

The absolute tension produced in response to vasoconstrictors was corrected for the cross-sectional area of individual arteries. The cross-sectional area of the superior mesenteric arteries and renal arteries did not differ between control and diabetic rats and was not affected by bosentan treatment (Table 4.5).

Vascular Reactivity in Endothelium-Intact Tissues

The maximum contractile responses of both superior mesenteric arteries and renal arteries to NE were markedly increased in the untreated diabetic rats when compared with the control rats (maximum tension in g/mm²: in SMA: C 7.43±0.95 vs. D 12.86±1.51, P<0.05; in RA: C 5.73±0.91 vs. D 12.67±2.35, P<0.05; Figure 4.16). This exaggerated response to NE was completely rectified following 10 weeks of bosentan
treatment in the DT (maximum tension in g/mm²: in SMA: C 7.43±0.95, D 12.86±1.51*, DT 8.10±0.85, *P<0.05 vs. C and DT; in RA: C 5.73±0.91, D 12.67±2.35*, DT 7.80±0.95, *P<0.05 vs. C and DT; Figure 4.16).

As with NE, endothelium-intact superior mesenteric arteries and renal arteries from untreated diabetic rats exhibited a marked increase in the maximum contractile response to the potent vasoconstrictor ET-1 (Figure 4.17). This increase was completely prevented by chronic bosentan treatment in the DT group (maximum tension in g/mm²: in SMA: C 9.22±1.04, D 14.20±1.44*, DT 9.28±0.53, *P<0.05 vs. C and DT; in RA: C 6.69±1.28, D 13.83±2.96*, DT 7.46±1.00, *P<0.05 vs. C and DT; Figure 4.17). Bosentan did not affect the contractile responses to either NE or ET-1 in the CT groups (Figure 4.17). The agonist sensitivity to NE or ET-1 was not altered by the diabetic state in both superior mesenteric arteries and renal arteries (Table 4.6). Furthermore, bosentan treatment did not affect the agonist sensitivity to NE or ET-1 in any group (Table 4.6).

In superior mesenteric arteries, incubation with the NO synthase inhibitor L-NAME increased the basal tone in all groups to a similar extent (changes in tension from the basal tone in g/mm²: C 2.06±0.97, CT 2.37±0.72, D 3.77±0.50, DT 2.11±0.59, P>0.05), and it also tended to increase the maximum contractile responses to NE although the increase did not reach statistical significance (Figures 4.16 and 4.18). As well, the diabetic superior mesenteric arteries became more sensitive to NE after being incubated with L-NAME (pD₂ values in diabetic SMA: NE 7.24±0.06 vs. NE+L-NAME 7.77±0.10, P<0.05; Table 4.6A). In contrast, L-NAME did not affect the renal arterial basal tone (changes in tension from the basal tone in g/mm²: C 0.25±0.11, CT
0.07±0.06, D 0.28±0.17, DT 0.13±0.12, P>0.05) nor did it cause any significant changes in the renal arterial responses to NE (Figures 4.16 and 4.18). The maximum contractile response to NE (in the presence of L-NAME) was increased in D vs. C superior mesenteric arteries and renal arteries and was normalized following long-term bosentan treatment (maximum tension in g/mm²: in SMA: C 10.48±1.60, D 16.76±1.91*, DT 9.68±1.06, *P<0.05 vs. C and DT; in RA: C 7.68±1.06, D 15.88±3.02*, DT 9.32±1.14, *P<0.05 vs. C and DT; Figure 4.18).

The responses and agonist sensitivity to Ach were not different between C and D groups (percent maximum relaxation responses at 10⁻⁴ M Ach: in SMA: C 71.2±9.2 vs. D 59.3±4.0, P>0.05; in RA: C 79.1±8.1 vs. D 54.5±11.0, P>0.05; pD₂ values for Ach: in SMA: C 7.16±0.27 vs. D 7.24±0.17, P>0.05; in RA: C 7.55±0.48 vs. D 6.73±0.29, P>0.05). Bosentan treatment did not affect these responses in the diabetic rats (percent maximum relaxation responses at 10⁻⁴ M Ach: in SMA: D 59.3±4.0 vs. DT 68.7±4.2, P>0.05; in RA: D 54.5±11.0 vs. DT 53.2±4.1, P>0.05; pD₂ values for Ach: in SMA: D 7.24±0.17 vs. DT 7.11±0.19, P>0.05; in RA: D 6.73±0.29 vs. DT 6.12±0.34, P>0.05).

**Vascular Reactivity in Endothelium-Denuded Tissues**

The exaggerated responses to NE and ET-1 observed in endothelium-intact arteries from the D group (above) were completely abrogated by denudation of the endothelium (NE: maximum tension in g/mm²: in SMA: C 7.97±0.99, CT 9.38±2.47, D 9.03±1.01, DT 7.00±1.21, P>0.05; in RA: C 6.34±1.00, CT 4.79±0.72, D 6.92±1.10, DT 5.72±1.20, P>0.05; Figure 4.19; and ET-1: in SMA: C 9.37±1.39, CT 10.05±2.83, D
10.66±1.60, DT 8.27±1.50, P>0.05; in RA: C 6.76±1.73, CT 3.37±1.01, D 6.45±0.97, DT 5.47±1.39, P>0.05; Figure 4.20). In contrast, the responses in the denuded arteries from the other three treatment groups were not different from those of their corresponding vessels with an intact endothelium. L-NAME did not affect the maximum contractile responses (Figure 4.21) nor the sensitivity to NE (Table 4.6) in endothelium-denuded arteries from all groups. Ach responses were abolished in rings denuded of the endothelium (data not shown).

Vascular Responses to SNP

The nitrovasodilator SNP caused a complete relaxation in all endothelium-intact and -denuded vessels from all rat groups, and no significant difference was found in the sensitivity to SNP among different groups (Figure 4.22), indicating that the responsiveness of the vascular smooth muscle to NO remained intact in the superior mesenteric arteries and renal arteries with or without endothelium from all rat groups.
Table 4.5

Cross-Sectional Area of the Superior Mesenteric Arteries and Renal Arteries from the Four Rat Groups

<table>
<thead>
<tr>
<th></th>
<th>Cross-Sectional Area (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Superior Mesenteric Arteries</strong></td>
<td></td>
</tr>
<tr>
<td>C with Endo (n=6)</td>
<td>0.124 ± 0.011</td>
</tr>
<tr>
<td>CT with Endo (n=5)</td>
<td>0.151 ± 0.021</td>
</tr>
<tr>
<td>D with Endo (n=6)</td>
<td>0.133 ± 0.020</td>
</tr>
<tr>
<td>DT with Endo (n=6)</td>
<td>0.143 ± 0.012</td>
</tr>
<tr>
<td>C without Endo (n=7)</td>
<td>0.138 ± 0.009</td>
</tr>
<tr>
<td>CT without Endo (n=5)</td>
<td>0.135 ± 0.021</td>
</tr>
<tr>
<td>D without Endo (n=6)</td>
<td>0.126 ± 0.006</td>
</tr>
<tr>
<td>DT without Endo (n=6)</td>
<td>0.121 ± 0.014</td>
</tr>
<tr>
<td><strong>Renal Arteries</strong></td>
<td></td>
</tr>
<tr>
<td>C with Endo (n=7)</td>
<td>0.090 ± 0.011</td>
</tr>
<tr>
<td>CT with Endo (n=6)</td>
<td>0.108 ± 0.009</td>
</tr>
<tr>
<td>D with Endo (n=6)</td>
<td>0.085 ± 0.012</td>
</tr>
<tr>
<td>DT with Endo (n=6)</td>
<td>0.074 ± 0.013</td>
</tr>
<tr>
<td>C without Endo (n=6)</td>
<td>0.080 ± 0.005</td>
</tr>
<tr>
<td>CT without Endo (n=7)</td>
<td>0.090 ± 0.007</td>
</tr>
<tr>
<td>D without Endo (n=8)</td>
<td>0.079 ± 0.009</td>
</tr>
<tr>
<td>DT without Endo (n=6)</td>
<td>0.088 ± 0.011</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, control; CT, control bosentan-treated; D, diabetic; DT, diabetic bosentan-treated rats; Endo, endothelium. No significant differences in the cross-sectional area of the superior mesenteric arteries and renal arteries were observed among different rat groups.
Table 4.6

A. Sensitivities to Various Agents in the Superior Mesenteric Arteries from the Four Rat Groups

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (pD₂)</th>
<th>C (n=6)</th>
<th>CT (n=5)</th>
<th>D (n=6)</th>
<th>DT (n=6)</th>
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<tbody>
<tr>
<td><strong>With Endothelium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>7.50 ± 0.12</td>
<td>8.03 ± 0.29</td>
<td>7.24 ± 0.06</td>
<td>7.86 ± 0.25</td>
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<tr>
<td>NE+L-NAME</td>
<td>7.51 ± 0.13</td>
<td>7.68 ± 0.10</td>
<td>7.77 ± 0.10*</td>
<td>7.85 ± 0.25</td>
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<tr>
<td>ET-1</td>
<td>8.18 ± 0.19</td>
<td>8.03 ± 0.25</td>
<td>8.08 ± 0.19</td>
<td>8.14 ± 0.15</td>
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<tr>
<td>SNP</td>
<td>8.21 ± 0.09</td>
<td>8.01 ± 0.04</td>
<td>8.25 ± 0.07</td>
<td>8.00 ± 0.09</td>
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<tr>
<td><strong>Without Endothelium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>7.71 ± 0.16</td>
<td>7.61 ± 0.26</td>
<td>8.70 ± 0.77</td>
<td>8.27 ± 0.43</td>
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<tr>
<td>NE+L-NAME</td>
<td>7.45 ± 0.13</td>
<td>7.44 ± 0.38</td>
<td>7.43 ± 0.25</td>
<td>7.46 ± 0.65</td>
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<tr>
<td>ET-1</td>
<td>7.92 ± 0.11</td>
<td>8.22 ± 0.10</td>
<td>7.93 ± 0.58</td>
<td>8.32 ± 0.25</td>
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<tr>
<td>SNP</td>
<td>8.31 ± 0.04</td>
<td>8.02 ± 0.07</td>
<td>8.14 ± 0.09</td>
<td>7.94 ± 0.09</td>
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B. Sensitivities to Various Agents in the Renal Arteries from the Four Rat Groups

<table>
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<tr>
<th></th>
<th>Sensitivity (pD₂)</th>
<th>C (n=7)</th>
<th>CT (n=6)</th>
<th>D (n=6)</th>
<th>DT (n=6)</th>
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<td></td>
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<tr>
<td>NE</td>
<td>6.59 ± 0.23</td>
<td>6.62 ± 0.13</td>
<td>6.99 ± 0.21</td>
<td>7.13 ± 0.25</td>
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<tr>
<td>NE+L-NAME</td>
<td>6.91 ± 0.17</td>
<td>6.59 ± 0.13</td>
<td>6.98 ± 0.22</td>
<td>7.24 ± 0.24</td>
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</tr>
<tr>
<td>ET-1</td>
<td>8.34 ± 0.35</td>
<td>7.89 ± 0.32</td>
<td>8.12 ± 0.28</td>
<td>8.16 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>8.25 ± 0.11</td>
<td>8.05 ± 0.18</td>
<td>8.47 ± 0.81</td>
<td>7.77 ± 0.03</td>
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</tr>
<tr>
<td><strong>Without Endothelium</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>NE</td>
<td>6.45 ± 0.30</td>
<td>6.32 ± 0.14</td>
<td>7.07 ± 0.18</td>
<td>6.96 ± 0.22</td>
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<td>NE+L-NAME</td>
<td>6.60 ± 0.24</td>
<td>6.41 ± 0.15</td>
<td>6.71 ± 0.12</td>
<td>6.77 ± 0.10</td>
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<tr>
<td>ET-1</td>
<td>7.90 ± 0.33</td>
<td>7.63 ± 0.45</td>
<td>7.83 ± 0.40</td>
<td>7.95 ± 0.35</td>
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<tr>
<td>SNP</td>
<td>8.09 ± 0.14</td>
<td>8.20 ± 0.32</td>
<td>8.10 ± 0.19</td>
<td>7.59 ± 0.09</td>
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</table>

Values are expressed as mean±SEM. pD₂ represents -log ED₅₀. *P<0.05, different from NE alone in the same rat group.

**Abbreviations:** C, control rats; CT, control bosentan-treated rats; D, diabetic; DT, diabetic bosentan-treated rats; NE, norepinephrine; L-NAME, L-arginine-methyl ester; ET-1, endothelin-1; SNP, sodium nitroprusside.
FIGURE 4.16
NE Concentration Response Curve in Endothelium-intact Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

Renal Arteries

Norepinephrine (NE) concentration response curve in endothelium-intact superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT) diabetic (D), and diabetic bosentan-treated (DT) rats. n=5-7 per group. Each point represents the mean±SEM. *P<0.05, versus C; # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.17
ET-1 Concentration Response Curve in Endothelium-intact Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

Renal Arteries

Endothelin-1 (ET-1) concentration response curve in endothelium-intact superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-7 per group. Each point represents the mean±SEM. *P<0.05, versus C; # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.18
NE Concentration Response Curve in Endothelium-intact Arteries from Control and Diabetic Rats in the Presence of L-NAME

Superior Mesenteric Arteries

Renal Arteries

Norepinephrine (NE) concentration response curve after incubation with N^G-nitro-L-arginine-methyl ester (L-NAME) (10^{-4} M for 30 minutes) in endothelium-intact superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-7 per group. Each point represents the mean±SEM. *P<0.05, versus C; # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.19
NE Concentration Response Curve in Endothelium-denuded Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

Renal Arteries

Norepinephrine (NE) concentration response curve in endothelium-denuded superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. No significant differences were found among groups.
FIGURE 4.20
ET-1 Concentration Response Curve in Endothelium-denuded Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

<table>
<thead>
<tr>
<th>Tension (g/mm²)</th>
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<tr>
<td>20.0</td>
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</tbody>
</table>

Log ET-1 Concentration (M)

- C
- CT
- D
- DT

Renal Arteries

<table>
<thead>
<tr>
<th>Tension (g/mm²)</th>
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</table>

Log ET-1 Concentration (M)

- C
- CT
- D
- DT

Endothelin-1 (ET-1) concentration response curve in endothelium-denuded superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. No significant differences were noted among groups.
FIGURE 4.21

NE Concentration Response Curve in Endothelium-denuded Arteries from Control and Diabetic Rats in thePresence of L-NAME

Superior Mesenteric Arteries

Renal Arteries

Norepinephrine (NE) concentration response curve after incubation with N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME) (10\textsuperscript{-4} M for 30 minutes) in endothelium-denuded superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. No significant differences were observed among groups.
FIGURE 4.22
A. SNP Concentration Response Curve in Endothelium-intact Arteries from Control and Diabetic Rats
Superior Mesenteric Arteries
Renal Arteries

B. SNP Concentration Response Curve in Endothelium-denuded Arteries from Control and Diabetic Rats
Superior Mesenteric Arteries
Renal Arteries

Sodium nitroprusside (SNP) concentration response curve in superior mesenteric and renal arteries with A) intact endothelium or B) endothelium denuded from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. No significant differences were noted among groups.
4.8 STUDY #7

Role of thromboxane A₂ in endothelin-induced hyper-reactivity in diabetic arteries

Our earlier studies demonstrated that arteries from diabetic rats exhibited an enhanced contractile response to vasoconstrictors, which was corrected by chronic treatment with the ET receptor blocker, bosentan and was also abolished by the removal of the endothelium. In addition, the increased vascular responses to vasoconstrictors in the diabetic arteries do not seem to be related to decreased endothelium-derived NO production, since endothelium-dependent vasorelaxation to Ach was preserved in the diabetic versus control groups. These findings suggest that an endothelium-derived vasoconstrictor such as TxA₂ may be important in mediating the changes in vascular responses in the diabetic arteries. This notion is supported by previous observations from our laboratory that inhibition of cyclooxgenases with indomethacin abrogated the exaggerated contractile responses to NE in diabetic arteries [116], and that ET-1 is a potent inducer of TxA₂ synthesis/release [233]. Hence, we hypothesized that the hyper-responsiveness to vasoconstrictors in diabetic arteries was related to an increase in ET-1-induced TxA₂ synthesis/release and/or action, and blockade of the ET-1-mediated effects on the TxA₂ system with bosentan would improve vascular responses in diabetes.

In the present study, we examined both the acute (i.e. in vitro incubation) and long-term (i.e. chronic treatment) effects of ET receptor blockade on the vascular reactivity of diabetic arteries. Moreover, the effects of thromboxane synthase inhibition with dazmegrel on vascular responses were studied. Nondiabetic and diabetic male Wistar rats were chronically treated with bosentan (100mg/kg/day po) for 10 weeks or
not treated. At the end of the treatment, superior mesenteric and renal arteries were isolated from all rats. Isometric tension responses to NE in the presence and absence of bosentan, the selective ET$_A$ receptor blocker BQ123, or dazmegrel as well as to ET-1 with or without dazmegrel were examined. Vascular responses to U46619, a TxA$_2$ analogue, were also studied. In addition, plasma levels of thromboxane B$_2$ (TxB$_2$) and 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$), the stable metabolites of TxA$_2$ and prostacyclin, respectively, were determined in untreated and bosentan-treated control and diabetic rats.

**Plasma Thromboxane and Prostacyclin Metabolite Levels**

Table 4.7 depicts the plasma levels of TxB$_2$ and 6-keto-PGF$_{1\alpha}$ in the four rat groups. Control and diabetic rats had similar levels of plasma TxB$_2$ and 6-keto-PGF$_{1\alpha}$; likewise, they had comparable TxB$_2$-to-6-keto-PGF$_{1\alpha}$ ratios. Chronic bosentan treatment did not seem to have any effect on the plasma levels of TxB$_2$ or 6-keto-PGF$_{1\alpha}$ in either control or diabetic rats (Table 4.7).

**Vascular Reactivity – Superior Mesenteric Arteries**

In agreement with the results obtained in Study #6, the maximum contractile responses of superior mesenteric arteries to NE and ET-1 were markedly increased in the untreated diabetic rats when compared with the control rats (Figure 4.23). This hyper-reactivity towards NE and ET-1 was completely corrected after long-term bosentan treatment in the DT rats (Figure 4.23).
Similar to the effects produced by the long-term bosentan treatment, *in vitro* incubation with bosentan in untreated diabetic superior mesenteric arteries also abolished the exaggerated NE response, in that the diabetic arteries exhibited similar contractile responses towards NE as the arteries from the control rats (maximum tension in g/mm²: C 12.67±1.20 vs. D 14.90±1.20, P>0.05; Figure 4.24). However, the maximum contractile response to NE of the diabetic group was significantly higher than that of the bosentan-treated groups (i.e. CT and DT; Figure 4.24). Similarly, following the *in vitro* incubation with the selective ETₐ blocker, BQ123, the hyper-reactivity of the D group towards NE was normalized, with the maximum contractile response to NE in the diabetic group being restored to the control level (maximum tension in g/mm²: C 11.54±1.26, D 11.36±.20, DT 12.50±1.61, P>0.05; Figure 4.24).

Figures 4.26 and 4.27 illustrate the effects of the *in vitro* incubation with the thromboxane synthase inhibitor dazmegrel on the vasoconstrictor responses to NE and ET-1, respectively. Dazmegrel incubation resulted in the normalization of the increased response to NE in the untreated diabetic arteries to the control values (maximum tension in g/mm²: C 12.51±1.20 vs. D 14.68±2.73, P>0.05; Figure 4.26). The maximum contractile response to NE of the diabetic group, however, remained significantly higher than that of the CT rats (maximum tension in g/mm²: D 14.68±2.73 vs. CT 7.18±1.12, P<0.05; Figure 4.26).

As with NE responses, the exaggerated contractile response towards ET-1 of the untreated diabetic arteries was abolished following *in vitro* incubation with dazmegrel when compared with control arteries (maximum tension in g/mm²: C 13.42±1.21 vs. D 16.33±2.79, P>0.05; Figure 4.27). However, the maximum tension responses to ET-1
were still significantly higher in D arteries compared with CT and DT arteries (Figure 4.27).

As for the contractile responses towards the TxA$_2$ analogue, U46619, no difference in the maximum contractile responses was observed between the untreated diabetic and control arteries (Figure 4.28). Diabetic arteries exhibited a greater response to U46619 at concentrations higher than 3x10$^{-7}$ M when compared with CT arteries (Figure 4.28).

As observed in Study #6, the percent maximum relaxation response to Ach in superior mesenteric arteries did not differ between C and D rats (Figure 4.29). Long-term bosentan treatment did not affect the Ach responses in either C or D rat group (Figure 4.29).

Chronic bosentan treatment did not affect the contractile response to NE, ET-1 nor U46619 in control rats (i.e. CT rats). The sensitivity to NE, ET-1 and Ach did not differ among the superior mesenteric arteries from different rat groups (Table 4.8A). Intriguingly, in contrast to results on the maximum contractile responses, data from the nonlinear regression analysis revealed a lower sensitivity to the thromboxane analogue, U46619, in D than in CT arteries (pD$_2$ values for U46619: CT $9.51\pm0.35$ vs. D $8.14\pm0.26$, P<0.05; Table 4.8A). Chronic bosentan treatment did not affect the agonist sensitivity to NE, Ach, U46619 nor ET-1 in the superior mesenteric arteries from all rats. As well, in vitro incubation with bosentan, BQ123 or dazmegrel did not appear to have any effect on the sensitivity to NE or ET-1 in the arteries from all groups.
**Vascular Reactivity – Renal Arteries**

Similar to the observations made in the superior mesenteric arteries, renal arteries from the untreated diabetic rats exhibited an exaggerated contractile response to NE and ET-1, and chronic bosentan treatment normalized this hyper-reactivity in the diabetic treated group (Figure 4.23).

However, unlike the results obtained in the superior mesenteric arteries, *in vitro* incubation with bosentan or BQ123 did not normalize the exaggerated response to NE in the untreated diabetic renal arteries (maximum tension in g/mm²: NE+bosentan: C 11.80±1.63 vs. D 24.11±3.53, P<0.05; and NE+BQ123: C 18.64±2.82 vs. D 31.35±5.50, P<0.05; Figure 4.25). *In vitro* incubation with dazmegrel also did not correct the maximum contractile response to NE in the untreated diabetic arteries (maximum tension in g/mm²: C 10.75±21.19 vs. D 23.43±3.79, P<0.05; Figure 4.26).

Inhibition of thromboxane synthase with dazmegrel resulted in a slight but significant decrease in the maximum contractile responses to ET-1 in diabetic renal arteries (maximum tension of D renal arteries in g/mm²: ET-1 30.54±4.97 vs. ET-1+dazmegrel 21.18±4.28, P<0.05; Figures 4.23B and Figure 4.27); however, these responses in diabetic arteries remained significantly greater than those of the control group in the presence of dazmegrel (maximum tension in g/mm²: C 8.65±1.04 vs. D 21.18±4.28, P<0.05; Figure 4.27).

In response to U46619, the untreated diabetic arteries had a significantly higher maximum contraction compared with the control, and this exaggerated response was completely normalized with chronic bosentan treatment in the DT group (maximum
tension in g/mm²: C 11.54 ± 2.29, D 21.62 ± 2.81*, DT 6.65 ± 1.40, *P < 0.05 vs. C and DT; Figure 4.28).

Renal arteries from all four rat groups had similar percent maximum relaxation response to Ach (Figure 4.29). In the diabetic group and diabetic-treated group, maximum relaxation occurred at 3x10⁻⁷ and 10⁻⁶ M Ach, respectively. After the maximum relaxation was attained, progressive contraction was observed with increased concentration of Ach added. Similar observations on the vascular responses to Ach in rat renal arteries have been reported by other investigators [249]. They showed that Ach evoked endothelium-dependent contraction in both quiescent and pre-contracted rat renal arteries at concentration greater than 10⁻⁷M [249].

Chronic bosentan treatment did not cause any changes to the contractile responses to NE, ET-1 or U46619 in the control rats. The agonist sensitivity to NE, Ach and U46619 did not differ among the renal arteries from different rat groups, indicating that chronic bosentan treatment did not affect the sensitivity to these vasoactive substances in the renal arteries (Table 4.8B). In vitro incubation with bosentan elicited no effect on the sensitivity of all arteries to NE while BQ123 was shown to lower the sensitivity to NE in DT renal arteries (pD₂ values in DT: NE 6.84±0.10 vs. NE+BQ123 6.48±0.10, P<0.05; Table 4.8B). Moreover, dazmegrel incubation resulted in a small decrease in the sensitivity to NE in both C and DT renal arteries (pD₂ values: in C: NE 6.65±0.09 vs. NE+dazmegrel 6.35±0.08, P<0.05; in DT: NE 6.84±0.10 vs. NE+dazmegrel 6.54±0.04, P<0.05; Table 4.8B). Data from the nonlinear regression analysis indicated a higher sensitivity to ET-1 in untreated diabetic arteries as compared with control arteries; this increase in the sensitivity remained significant after chronic
bosentan treatment in DT rats (pD$_2$ values for ET-1: C 8.25±0.04, D 8.66±0.11*, DT 8.65±0.07*, P<0.05 vs. C; Table 4.8B). In the presence of dazmegrel, the sensitivity to ET-1 was different between CT and DT arteries (Table 4.8B).
Table 4.7

Plasma Levels of Thromboxane and Prostacyclin Metabolites in the Untreated and Bosentan-treated Control and Diabetic Rats

<table>
<thead>
<tr>
<th></th>
<th>C (n=6)</th>
<th>CT (n=7)</th>
<th>D (n=7)</th>
<th>DT (n=6)</th>
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</thead>
<tbody>
<tr>
<td>TxB₂ (pg/mL)</td>
<td>200±19</td>
<td>203±22</td>
<td>226±37</td>
<td>215±10</td>
</tr>
<tr>
<td>6-keto-PGF₁₆ (pg/mL)</td>
<td>154±9</td>
<td>156±15</td>
<td>158±24</td>
<td>134±5</td>
</tr>
<tr>
<td>TxB₂ to 6-keto-PGF₁₆ Ratio</td>
<td>1.36±0.14</td>
<td>1.32±0.12</td>
<td>1.45±0.10</td>
<td>1.59±0.07</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. C, CT, D and DT denote control, control bosentan-treated, diabetic and diabetic bosentan-treated rats, respectively. Diabetes was induced in D and DT rats by an intravenous injection of 60 mg/kg streptozotocin (STZ). One week after STZ injection, bosentan treatment was administered to CT and DT rats at a dose of 100 mg/kg/day by oral gavage for 10 weeks. After treatment, blood samples were obtained via cardiac puncture for determination of plasma thromboxane B₂ (TxB₂) and 6-keto-prostaglandin F₁₆ (6-keto-PGF₁₆), the stable metabolites of TxA₂ and prostacyclin, respectively. No significant differences in the plasma levels of thromboxane and prostacyclin metabolites were detected among the groups.
Table 4.8

A. Sensitivities to Various Agents in the Superior Mesenteric Arteries from the Four Rat Groups

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (pD₂)</th>
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<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>NE</td>
<td>6.84 ± 0.06</td>
</tr>
<tr>
<td>Ach</td>
<td>7.06 ± 0.23</td>
</tr>
<tr>
<td>NE + Bos</td>
<td>6.78 ± 0.10</td>
</tr>
<tr>
<td>NE + BQ123</td>
<td>6.59 ± 0.09</td>
</tr>
<tr>
<td>NE + Daz</td>
<td>6.58 ± 0.07</td>
</tr>
<tr>
<td>U46619</td>
<td>9.09 ± 0.38</td>
</tr>
<tr>
<td>ET-1</td>
<td>8.40 ± 0.06</td>
</tr>
<tr>
<td>ET-1 + Daz</td>
<td>8.37 ± 0.20</td>
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</table>

B. Sensitivities to Various Agents in the Renal Arteries from the Four Rat Groups

<table>
<thead>
<tr>
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<th>Sensitivity (pD₂)</th>
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<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>NE</td>
<td>6.65 ± 0.09</td>
</tr>
<tr>
<td>Ach</td>
<td>7.73 ± 0.29</td>
</tr>
<tr>
<td>NE + Bos</td>
<td>6.55 ± 0.05</td>
</tr>
<tr>
<td>NE + BQ123</td>
<td>6.42 ± 0.07$</td>
</tr>
<tr>
<td>NE + Daz</td>
<td>6.35 ± 0.08$</td>
</tr>
<tr>
<td>U46619</td>
<td>7.40 ± 0.74</td>
</tr>
<tr>
<td>ET-1</td>
<td>8.25 ± 0.04</td>
</tr>
<tr>
<td>ET-1 + Daz</td>
<td>8.29 ± 0.06</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. pD₂ represents -log ED₅₀. n=5-8 per group.

Abbreviations: C, control rats; CT, control bosentan-treated rats; D, diabetic; DT, diabetic bosentan-treated rats; NE, norepinephrine; Ach, acetylcholine; Bos, bosentan; Daz, dazmegrel; ET-1, endothelin-1.

*P<0.05, different from C
#P<0.05, different from CT
$P<0.05, different from NE alone in the same rat group
FIGURE 4.23

A. NE Concentration Response Curve in Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

Renal Arteries

B. ET-1 Concentration Response Curve in Arteries from Control and Diabetic Rats

Superior Mesenteric Arteries

Renal Arteries

A) Norepinephrine (NE) and B) endothelin-1 (ET-1) concentration response curve in superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. *P<0.05, versus C; # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.24
NE Concentration Response Curve in Control and Diabetic Rat Superior Mesenteric Arteries in the Presence of Endothelin Receptor Blocker

NE+Bozantara CRC

NE+BQ 123 CRC

Norepinephrine (NE) concentration response curve (CRC) in the presence of bosentan or BQ123 (1µM for 30 minutes) in superior mesenteric arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=6-8 per group. Each point represents the mean±SEM. # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.25
NE Concentration Response Curve in Control and Diabetic Rat Renal Arteries in the Presence of Endothelin Receptor Blocker

Norepinephrine (NE) concentration response curve (CRC) in the presence of bosentan or BQ123 (1μM for 30 minutes) in renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. *P<0.05, versus C; # P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.26
NE Concentration Response Curve in Control and Diabetic Rat Arteries in the Presence of Dazmegrel

Superior Mesenteric Arteries

Renal Arteries

Norepinephrine (NE) concentration response curve in the presence of dazmegrel (1μM for 30 minutes) in superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=6-8 per group. Each point represents the mean±SEM. * P<0.05, versus C; $P<0.05, versus D; @ P<0.05, versus DT.
FIGURE 4.27
ET-1 Concentration Response Curve in Control and Diabetic Rat Arteries in the Presence of Dazmegrel

Superior Mesenteric Arteries

Renal Arteries

Endothelin-1 (ET-1) concentration response curve in the presence of dazmegrel (1μM for 30 minutes) in superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=6-8 per group. Each point represents the mean±SEM. * P<0.05, versus C; #P<0.05, versus CT; @ P<0.05, versus DT.
U46619 concentration response curve in superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean ± SEM. * P<0.05, versus C; #P<0.05, versus CT; @ P<0.05, versus DT.
FIGURE 4.29
Ach Concentration Response Curve in Control and Diabetic Rat Arteries

Acetylcholine (Ach) concentration response curve in superior mesenteric and renal arteries from untreated control (C), control bosentan-treated (CT), diabetic (D) and diabetic bosentan-treated (DT) rats. n=5-8 per group. Each point represents the mean±SEM. No significant differences were found among groups.
5. DISCUSSION

5.1 OVERVIEW

Diabetic patients are afflicted with a predisposition to various cardiovascular diseases. Data from the Framingham study revealed a two- and fivefold increase in the incidence of congestive heart failure in men and women, respectively, in the diabetic population [250]. Despite the increasing number of interventive pharmacological and surgical therapies made available in recent years, mortality among diabetic patients with heart failure and vascular complications has remained high [251, 252]. As much as 80% of all diabetic deaths have directly been attributable to cardiovascular causes [18, 250, 253]. It is now recognized that diabetes mellitus can be considered "a cardiovascular disease" [19] and that diabetes is an independent risk factor for a variety of cardiovascular diseases, including hypertension, atrial fibrillation and arrhythmia, cardiomyopathy and heart failure [250].

The development of the diabetic cardiovascular complications is largely attributed to atherosclerosis, coronary artery disease and autonomic neuropathy commonly encountered in the diabetic population [15]. However, a subset of diabetic patients, which includes approximately 30% of all type 1 diabetic patients, exhibit a specific cardiac muscle disease (diabetic cardiomyopathy) in the absence of clinically detectable atherosclerosis and coronary artery disease [24-29]. This distinct form of cardiomyopathy is generally characterized by early impairments in diastolic function (increased left ventricular end diastolic pressure, prolonged isovolumic relaxation times, increased myocardial stiffness), followed by later reductions in systolic function.
(increased left ventricular end systolic volume, decreased left ventricular ejection fractions) without any evidence of microvascular ischemia [28, 31, 32].

The etiologies of the diabetic cardiac and vascular diseases are complex and appear to involve an array of factors including specific vascular, neuropathic and myopathic alterations. Altered vascular responses to vasoactive substances, defective cardiac autonomic function, suboptimal myocardial substrate metabolism, structural alterations leading to decreased ventricular compliance and hypertrophy have been implicated in diabetic cardiomyopathy [16, 48-51]. Changes in several transmembrane and sarcomembranous proteins involved in Ca$^{2+}$ handling have been suggested to have an important role in cardiac dysfunction of diabetes [52, 53]. Excess oxidative stress, one of the sequelae of hyperglycemia, has also received much attention for its potential role in the etiology of diabetic cardiovascular diseases [54-57]. As well, abnormal activities of G-proteins have been postulated to mediate diabetic cardiac and vascular dysregulation [60-68]. Furthermore, the role of an activated DAG-PKC pathway in diabetic complications has been extensively explored [69-76, 254].

The powerful endothelium-derived peptidic vasoconstrictor-mitogen, ET-1, may also play a role in the development and/or reinforcement of the cardiovascular complications of chronic diabetes. Since its first description as a vasoconstrictor in 1988, there has been a marked interest in the role of ET-1 in mediating physiological and/or pathophysiological cardiovascular function. It is now believed that ET-1 may generate a whole spectrum of haemodynamic aberrations including hypertension, renal failure, myocardial ischemia, increased microvascular cell permeability, profound mitogenesis, atherosclerosis and cardiac shock [153, 178, 201-208]. In view of the
powerful cardiovascular and trophic effects of this peptide, an increased level of ET-1 can be perceived as adversely affecting cardiovascular function. Indeed, this view is advocated by studies demonstrating beneficial effects of ET receptor antagonists in hypertension, atherosclerosis, renal failure and ischemia-reperfusion injury [201-204, 206, 255].

The aforementioned pathological changes in the cardiovascular system during diabetes fit with many of the known biological effects of ET-1. Consequently, substantial interest has been generated toward determining the role of this peptide in diabetes and its complications. Multiple reports have found an increase in ET-1 levels in both human and experimental diabetes [80, 82, 85, 212-218]. The vascular responses to ET-1 have also been noted to be altered in diabetes [77-79, 112, 113, 119, 221-224]. Furthermore, an increasing number of studies have revealed that the blockade of ET system may have favourable effects in either preventing or slowing down the progression of diabetic-induced damage in various organs [82, 85, 195, 198, 199, 211, 225, 256].

The main focus of the work presented in this thesis was to investigate the potential role of ET-1 in modulating diabetic cardiac and vascular dysfunction. In addition, we evaluated the effects of chronic ET receptor blockade on the cardiovascular system in diabetes. Using STZ-diabetic rats, a widely employed rodent model of type 1 diabetes, we examined the effects of diabetes on the expression of ET-1 and ET receptors in the heart and arteries. The effects of diabetes on cardiac performance and vascular reactivity were also assessed. Moreover, the effects of
chronic treatment with an ET receptor antagonist on the expression of the endothelin system as well as the cardiovascular function in diabetic rats were appraised.

Rats injected with STZ consistently showed many of the classical features seen in patients suffering from uncontrolled type 1 diabetes, including hyperglycemia, hypoinsulinemia, hyperlipidemia, polyphagia, polyuria and decreased weight gain. Cardiovascular function was also demonstrated to be compromised in those rats with depressed cardiac performance and abnormal vascular reactivity. Although we did not examine if STZ directly affected the heart and arteries, a previous study reported that no effect on vascular responses was seen in the rat arteries when STZ was directly added to the tissue bath medium [257]. Moreover, histological studies failed to discover any sign of a direct toxic effect of STZ on organs other than pancreatic β-cells [258]. The short serum half-life of the drug, which is reported to be 15 minutes [259], also rules out any prolonged exposure of the tissues to STZ. Additionally, studies have repeatedly shown that correction of the hyperglycemic state with insulin treatment or other glucose-lowering agents was able to prevent and reverse the defects found in the cardiovascular system of STZ-diabetic rats [52, 99, 105, 108, 113, 130, 260]. Hence, it is reasonable to conclude that any changes seen in the STZ-induced diabetic rat were not likely due to a direct effect of STZ, but rather, were the consequences of the long-term hyperglycemic and hypoinsulinemic diabetic state.

As alluded to earlier, the pathogenesis of diabetic complications is clearly multifactorial and complex; it would be difficult, if not impossible, to cover every aspect that may be associated with diabetes-induced cardiovascular complications in the present thesis. The purpose of the following discussion is to reiterate the primary
findings from the studies in the present thesis that demonstrate the involvement of ET-1 in diabetic cardiovascular dysfunction and to highlight some of the potential pathways that may link ET-1 to diabetic cardiovascular complications.
5.2 EFFECTS OF DIABETES AND CHRONIC ENDOTHELIN RECEPTOR BLOCKADE ON THE EXPRESSION OF ET-1

Elevated glucose levels have been shown to trigger the gene expression and release of ET-1 [215, 219, 220]. However, the data on plasma ET-1 levels in diabetes are conflicting; studies have demonstrated increased, decreased or no change in ET-1 levels in diabetes [79, 84, 113, 222]. Hopfner et al. have attributed these discrepancies among studies to the temporal variation in ET-1 release at different stages of STZ-diabetes [79]. They have concluded that while ET-1 release is decreased in early diabetes, it is increased in the later stages. The disagreement between different reports on plasma ET-1 levels in diabetes may also due to the fact that plasma ET-1 level is not a reliable indicator in assessing the contribution of this peptide towards local vascular homeostasis. Most (75%) of ET-1 produced by the endothelial cells is released towards the vascular smooth muscle [154, 158], and ET-1 in plasma represents the spillover of locally produced ET-1. Moreover, since ET-1 binds tightly to its receptors with a slow rate of dissociation [261], circulating ET-1 levels may not reflect the local tissue levels or rates of production. Given this preamble, the lack of difference in plasma ET-1 levels between our control and diabetic rats is not surprising [262] and is consistent with reports from other laboratories [84].

An increase in plasma ET-1 levels was observed following bosentan treatment in both of our control and diabetic rats [262]. This phenomenon has also been noted in other studies after treatment with either a mixed ET\textsubscript{A}/ET\textsubscript{B} selective antagonist or an ET\textsubscript{B} selective antagonist, with the rise in plasma ET-1 levels occurring as early as minutes to
a few hours after dosing with the blockers [247, 248, 263, 264]. Conversely, plasma ET-1 level was found to be unaffected by blockade with the selective ET\textsubscript{A} antagonists BQ123 and FR139317 [263]. These observations led to the notion that the ET\textsubscript{B} receptor may participate in the clearance of ET-1 [165]. One recent study, however, reported a modest increase in plasma ET-1 after treatment with the selective ET\textsubscript{A} receptor antagonists A-216546 (in rats) and ABT-627 (in human) for at least 7 days [264]. Since the increase could only be detected after a prolonged period of ET\textsubscript{A} receptor blockade, those authors proposed that a sustained inhibition of the ET\textsubscript{A} receptor may lead to an up-regulation of expression of the ET-1 gene via induction of a feedback mechanism [264]. The increase in plasma ET-1 levels may also be explained by dissociation of ET-1 from ET receptors following ET receptor blockade [247, 248, 263].

Tissue levels are a better indicator of local ET-1 production and regulation. To circumvent the problems associated with the interpretation of plasma ET-1, we assessed immunoreactive ET-1 levels (by immunohistochemistry) in ventricles and superior mesenteric and renal arteries from diabetic and control rats. Our data reveal an enhanced peptide expression of ET-1 in these tissues from 11-week diabetic rats (Figures 4.1, 4.2, 4.10 and 4.11) [225, 262]. In addition, results from RT-PCR assays indicated an increased ET-1 mRNA expression in diabetic rat heart ventricles (Figure 4.3). Other studies have also observed an increased ET-1 expression in different tissues from diabetic animals [84, 85, 199, 200, 215, 218, 265, 266]. Wu and Tang [84] reported an elevated level of vascular ET-1 in aortae and mesenteric arteries from 2-week STZ-diabetic rats (without concomitant increases in plasma ET-1 levels).
Mesenteric arteries from rats exhibited an increase in both the immunoreactivity and mRNA expression for ET-1 after 3 weeks of diabetes when compared with age-matched controls [199]. Similarly, an exaggerated vascular production or release of ET-1 from mesenteric arteries has been shown in STZ-induced diabetic rats [213]. Chakrabarti's group found a raised level of immunoreactive ET-1 and mRNA for ET-1 in the retinal and cardiac tissues from diabetic rats [85, 200, 218, 265]. Results obtained from quantitative RT-PCR and Northern blot analyses demonstrated that the mRNA expression for ET-1 was also increased in the retina and brain of diabetic rats when compared with those of control rats [266]. Furthermore, an up-regulation of the ET-1 gene was observed in the renal tissues from diabetic rats [82, 215], with the mRNA for ET-1 in the glomeruli of diabetic rats increasing in association with the progression of diabetic nephropathy [215]. The above observations suggest that an augmented expression of tissue ET-1 appear to be a generalized phenomenon in diabetes.

A few studies have shown that ET-1 augments its own production, possibly via stimulation of the ET$_B$ receptor, in rat mesangial cells [228], human endothelial cells [229] and human proximal tubular cells [230]. This has led to the proposition that ET receptor blockers exert their beneficial effects by preventing the autoinduction of ET-1. Benigni et al. [82] demonstrated that treatment with a mixed ET$_A$ and ET$_B$ receptor antagonist was able to improve renal function as well as normalize the elevated renal ET-1 levels in diabetes. However, this effect of ET receptor blockade on the expression of ET-1 seems to be confined only to the kidney. Findings from our laboratory show an increased expression of ET-1 in diabetic rat hearts and arteries; however, chronic bosentan treatment did not seem to have any effect, at least in those tissues, on ET-1
levels in diabetes (Figures 4.1, 4.2, 4.3, 4.10 and 4.11) [225, 262]. Other studies also did not observe any changes in tissue ET-1 expression in diabetic rat hearts, retina and mesenteric arteries after treatment with bosentan [85, 199, 200]. Moreover, as bosentan did not alter glucose levels in any group, the primary stimulus for ET-1 release (i.e. elevated glucose levels) was left intact.

Hyperglycemia is postulated to be the main determinant responsible for the elevation in ET-1 levels in diabetes [219, 220, 267], although other factors such as hyperinsulinemia and hyperlipidemia may also be involved when present [268-270]. Reversal of STZ-induced hyperglycemia by insulin treatment has been shown to ameliorate the increase in ET-1 levels in diabetic glomeruli [215] and arteries [84]. Perfusion of isolated mesenteric arterial beds from normal rats with high glucose promoted a time-dependent increase in the release of ET-1 while no detectable amount of ET-1 was released in response to perfusion with normal glucose [267]. Furthermore, in vitro studies have demonstrated that exposure of the cultured cells to increased levels of glucose enhanced the protein and mRNA expression of ET-1 [219, 220, 271]. The absence of effect of an elevated mannitol level on ET-1 expression led to the conclusion that the induction of ET-1 expression by glucose is not due to an osmotic effect [219, 220]. Stimulation of ET-1 synthesis by elevated glucose levels has been shown to be attained by activation of PKC [220]. Park et al. [220] demonstrated that the glucose-mediated increase in ET-1 expression was blocked by a general PKC inhibitor GF109203X (bisindolylmaleimide I) in capillary bovine retinal endothelial cells and bovine retinal pericytes. Moreover, overexpression of PKCβ1 or δ, isoforms which are present in the rat heart and vascular tissues, by infecting cells with adenovirus vectors
containing the respective cDNA resulted in an increase in total PKC activities accompanied by an enhancement in basal and glucose-induced ET-1 mRNA expression [220]. Other possible mediators for diabetes-induced increase in ET-1 expression include nuclear factor-kappa B (NF-κB) [159], a sustained activation and an increased expression of which was found in diabetes [272]; oxidative stress, which is believed to be elevated in diabetes [56] and has been demonstrated to promote the synthesis of preproET-1 mRNA and big ET-1 by inducing the ET-1 promoter activity; and vascular endothelial growth factor (VEGF), which has been reported to be increased in diabetic patients [273] and shown to be involved in the stimulation of ET-1 synthesis [271, 274].
5.3 EFFECTS OF DIABETES AND CHRONIC ENDOTHELIN RECEPTOR BLOCKADE ON THE EXPRESSION OF ENDOTHELIN RECEPTORS

While a wealth of information has been acquired on diabetes-induced alterations in the expression of ET-1, there is a limited literature with regard to the effects of diabetes on the expression of its two receptors (ET\textsubscript{A} and ET\textsubscript{B}) in the cardiovascular system. The increases in ET-1 expression in our STZ-diabetic rat tissues included both mRNA and peptide levels, suggesting that changes in ET-1 expression may be functionally important. In the presence of the increased tissue ET-1 levels in the diabetic group, one would expect, according to the rule for the regulation of G-protein-coupled receptors, a down-regulation of the ET receptors in those animals. \textit{In vitro} studies demonstrated that prolonged exposure to ET-1 reduced the ET receptor number without changing its binding affinity in cultured mesangial cells [275] as well as in cultured rat vascular smooth muscle cells [276].

An earlier study demonstrated a reduced ET-1 receptor density in the heart from rats with a short diabetes period of three days [83]. In contrast, an increase in cardiac ET-1 binding sites, with no change in affinity of ET-1, has been observed in 2, 4, and 6-week STZ-diabetic rats [86]. Kakoki et al. [277] reported that 4-week diabetic renal arteries exhibited less immunostaining of the ET\textsubscript{B} receptor in the endothelium than was observed in the vascular smooth muscle, whereas staining of the ET\textsubscript{B} receptor in the endothelium was greater than that in the vascular smooth muscle in the control arteries, thus suggesting that the alteration in the expression of the ET receptors in diabetes would favour the vasoconstrictor effects of ET-1 over its vasodilator effects. However,
others demonstrated that the mRNA levels for ET\textsubscript{A} and ET\textsubscript{B} receptors were not altered in glomeruli from diabetic and control rats in the presence of increased ET-1 mRNA levels [215].

Our eleven-week diabetic rat hearts displayed an increase in immunoreactive level of the ET\textsubscript{A} receptor in both left and right ventricles (Figure 4.4 and Figure 4.5) with the level of the ET\textsubscript{B} receptor being increased only in the right ventricles (Figure 4.6 and Figure 4.7). A corresponding increase in the mRNA expression of the ET\textsubscript{A} receptor was also found in the diabetic rat heart ventricles while that of the ET\textsubscript{B} receptor remained unchanged (Figure 4.8). The discrepancy between the immunohistochemical and mRNA data for the ET\textsubscript{B} receptor in diabetic rats may be due to the fact that the immunoreactive levels were separately assessed in the left and right ventricles whereas the mRNA expression was examined in both ventricles together. Since the immunohistochemical data suggest that the expression for the ET\textsubscript{B} receptor was increased only in the right ventricles of the diabetic rats, this increase might not be detected in the mRNA expression when both ventricles were jointly assessed for the RT-PCR analysis. Long-term bosentan treatment did not have any effect on the expression of the ET receptors in either control or diabetic rat hearts. In parallel with our findings, an increase in ET\textsubscript{A} receptor mRNA expression was also noted by Chen et al. [200] in 6-month diabetic rat myocardium. However, they also found an increase in ET\textsubscript{B} receptor mRNA expression in their diabetic rat hearts. In addition, they demonstrated that bosentan treatment was able to prevent the increased ET\textsubscript{A} receptor expression. The discrepancies between our data and theirs may be due to the difference in the duration (11 weeks vs. 6 months) of diabetes and bosentan treatment.
Nonetheless, the above findings suggest that ET receptor expression may be increased in the heart during diabetes, which may contribute towards the development of ET-1-induced cardiac dysfunction in diabetes.

In the vasculature, we found an increase in the immunoreactivity of the ET\textsubscript{A} receptor in the superior mesenteric arteries from diabetic rats (Figure 4.12); interestingly, this increase was prevented by bosentan treatment. No change in the immunoreactivity for the ET\textsubscript{B} receptor was observed in the superior mesenteric arteries (Figure 4.14) or either receptor in the renal arteries (Figure 4.13 and Figure 4.15) from diabetic rats. As well, these levels were not affected by bosentan treatment.

Our data demonstrate that the ET receptor levels were either increased or unchanged in the heart and arteries from diabetic rats even though there was an increase in ET-1 levels in those diabetic tissues. The research team of Chakrabarti has also demonstrated an up-regulation of ET receptor (both ET\textsubscript{A} and ET\textsubscript{B}) mRNA in the presence of elevated levels of ET-1 in retina isolated from long-term diabetic rats [85]. As well, parallel to our findings, they reported an increase in ET-1 mRNA expression in the diabetic myocardium coinciding with the increase in ET\textsubscript{A} and ET\textsubscript{B} receptors mRNA [200]. Similar effects have been noted in rats with congestive heart failure wherein increased ET-1 levels are associated with parallel and paradoxical increases in ET-1 binding sites [175, 231, 278]. Hence, the emerging evidence from animal studies indicates that the general rules for the down-regulation of receptors do not always seem to be applicable to the endothelin system \textit{in vivo}. In addition, a number of factors such as cytokines and angiotensin II have been shown to have modulatory effects on the
expression of the ET receptors [279], some of which are known to be affected by diabetes.

The above findings indicate that both the cardiac and vascular endothelin system was up-regulated in diabetic rats. However, except in the superior mesenteric arteries, the beneficial effects of bosentan on heart and vascular function in STZ-diabetic rats (see later) did not seem to be related to the normalization of ET receptor levels.
Cardiac dysfunction in diabetes is well documented in the literature, and a number of excellent reviews, based on evidence from both clinical and experimental studies, have been written on the subject [28, 51, 280-282]. In a previous study, we demonstrated that long-term administration of the mixed ET$_A$/ET$_B$ receptor blocker bosentan at a dose of 100 mg/kg/day for 7 weeks partially improved the isolated working heart function in the diabetic group when assessed by the rate of relaxation (-dP/dt) and left ventricular developed pressure (LVDP) without affecting the rate of contraction (+dP/dt) (Figure 1.1) [225]. The parameter -dP/dt is a very sensitive index of diabetic diastolic dysfunction which appears to manifest first in STZ-induced diabetes. In our hands, +dP/dt is the last factor to be corrected (possibly following improvement in diastolic function). Prolonging the treatment protocol may have uncovered beneficial effects on this parameter as well; however, this remains to be investigated.

The use of a mixed ET$_A$ and ET$_B$ receptor such as bosentan enabled us to examine the role of endogenous ET-1 in diabetic complications. However, the non-specific nature of bosentan prevented us from determining which specific receptor subtype(s) may be responsible for mediating the pathogenic role of ET-1. Moreover, the increased expression in ET$_A$ receptors in both the left and right ventricles of our diabetic rats (Figures 4.4, 4.5 and 4.8) suggests that ET$_A$ receptors may have an important role in the development of diabetic cardiac failure. Hence, to test if the blockade of ET$_A$ receptors per se would provide cardioprotective effects in diabetes, we
had examined the effects of chronic treatment with the oral selective ET$_A$ receptor antagonist ABT-627 on isolated working heart function of diabetic and control rats.

At a dose of 5 mg/kg/day, seven weeks of ABT-627 treatment did not cause any significant changes in cardiac performance of the diabetic rats when assessed by ±dP/dt and LVDP (Figure 4.9). However, there appeared to be a trend towards improvements in those parameters of cardiac function after treatment in diabetic hearts. The above data suggest that ET-1-induced aberrations to the heart are not mediated solely via ET$_A$ receptors but also through ET$_B$ receptors. Another possible explanation is that a higher dose of ABT-627 may be required to provide cardioprotective effects in diabetic rats. However, the latter is a less likely possibility. Although ABT-627 is approximately 200 times as potent as bosentan in blocking the ET$_A$ receptor [209, 210], bosentan at 100 mg/kg/day conferred beneficial effects on diabetic rat heart function while ABT-627 at 5 mg/kg/day did not provide any significant improvement. Furthermore, preliminary results from our recent experiments showed that oral treatment with 10 mg/kg/day of ABT-627 for 10 weeks did not ameliorate the depressed cardiac function in STZ-diabetic rats (Arikawa, Yuen, Song and McNeill, unpublished observations).

The above observations imply that blockade of the ET$_B$ receptor is required to prevent the development of diabetic cardiac functional impairments. The involvement of the ET$_B$ receptor has also been suggested in other cardiovascular disease states. For instance, the vasoconstrictor effect of sarafotoxin S6c, an ET$_B$ receptor agonist, is enhanced in patients with chronic heart failure [283], indicating an up-regulation of vascular smooth muscle (constricting) ET$_B$ receptors in those patients. Similar
outcomes were observed in dogs with experimental congestive heart failure which exhibit an increase in coronary vasoconstriction to sarafotoxin [284]. As well, the observation that bosentan, but not the ET\textsubscript{A} selective receptor blocker BQ123, resulted in an increase in cardiac output in dogs with myocardial infarction suggests that blockade of ET\textsubscript{B} receptors is necessary to improve cardiac function [285]. However, the use of a selective ET\textsubscript{B} receptor antagonist in long-term systemic treatment of cardiac failure is not recommended as it may block ET\textsubscript{B} receptor-mediated vasodilation and may unmask ET\textsubscript{A} receptor-induced vasoconstriction. Indeed, administration of the ET\textsubscript{B} selective receptor antagonist A-192621 to rats significantly increases blood pressure [286, 287].

It has been suggested that both ET\textsubscript{A} and ET\textsubscript{B} receptors may contribute to the deleterious effects of ET-1 [288]. In addition, several studies including ours have demonstrated that blockade of both ET\textsubscript{A} and ET\textsubscript{B} receptors effectively ameliorated cardiac, vascular, retinal and renal abnormalities in diabetes [82, 85, 127, 199, 200, 225, 262]. Our data demonstrate that selective ET\textsubscript{A} receptor antagonism did not offer functional benefits to the diabetic rat heart. However, since we had not studied the effects of ABT-627 on other diabetes-induced changes in the heart or other organs, the answer to the important question whether a nonselective ET\textsubscript{A} and ET\textsubscript{B} receptor blockade will confer greater or fewer benefits than selective ET receptor blockade in treatment of diabetic complications must await future research.

The exact mechanisms underlying the beneficial effects of bosentan on diabetic heart function are unknown. Although it is not covered in the scope of studies in the present thesis, a few potential mechanisms can be proposed here. The effects of bosentan appear to be unrelated to glycemic status or lipid metabolism; no changes in
glucose, triglyceride or cholesterol levels were noted after bosentan treatment in our studies (Table 4.1) [225]. Endothelin-1 has been shown to induce a direct toxic effect on cardiac myocytes [289] and bosentan may serve to counter this effect.

Bosentan may improve diabetic heart function by preventing ET-1-mediated coronary vasoconstriction thereby improving coronary blood flow. This is substantiated by our previous findings that diabetic rat hearts exhibited enhanced coronary pressor responses to ET-1 which was restored by chronic bosentan treatment to control values (Verma, Lee and McNeill, in press [226]; see Section 1.3 and Figure 1.2). Since bosentan, but not the selective ET$_A$ receptor blocker ABT-627, conferred cardioprotection in diabetic rats, we speculate that the exaggerated coronary reactivity to ET-1 in the diabetic rats might be due to an increase in ET$_B$ receptor-mediated coronary vasoconstriction, which may be blocked by bosentan. As alluded to earlier, in the failing myocardium of other disease models, data have demonstrated an increased coronary vasoconstriction to ET$_B$ receptor activation [284]. Whether such an event takes place in diabetic rat hearts remains to be investigated.

Under normal conditions, ET-1 has positive inotropic and chronotropic actions on heart muscle cells [153, 290]. The positive chronotropic and inotropic responses to ET-1 have been reported to be reduced in isolated rat atria after 8 weeks of STZ-diabetes [291]. Interestingly, however, under conditions of β-adrenergic stimulation, ET-1 has been shown to trigger a negative modulatory effect on isolated myocytes through a G$_i$-mediated pathway [292, 293]. An inhibitory effect by ET-1 on the positive chronotropic and inotropic responses to sympathetic nerve stimulation and to noradrenaline has been demonstrated in isolated rat atria [291]. Endothelin-1 has been reported to
hyperpolarize the membrane potential and shorten the duration of the action potential in mammalian atrial myocytes, leading to suppression of electrical excitability of the heart and decrease in heart rate [292]. Endothelin-1 also elicits a potent inhibitory effect against isoproterenol-enhanced L-type Ca$^{2+}$ current in both atrial and ventricular myocytes; this ET-1-induced effect has been shown to be mediated via a pertussis toxin (PTX)-sensitive G-protein [292, 293]. In agreement with the above observations, ET-1 is reported to inhibit cAMP formation in response to isoproterenol and forskolin in adult cardiomyocytes, an effect which is PTX-sensitive and appears to be mediated via G$_i$ [294, 295]. Thus, increased ET-1 levels in the diabetic heart may therefore attenuate cardiac responses to β-adrenergic agents. Whether bosentan improves β-adrenoceptor mediated contractility is speculative at the present time.

Another mechanism through which an elevated level of ET-1 may adversely affect cardiac function in diabetes is the activation of PKC. PKC consists of at least 12 isoforms: calcium- and DAG-dependent PKC (α, β1/2, γ); calcium-independent and DAG-dependent PKC (δ, ε, η, θ, μ); and calcium- and DAG-independent atypical PKC (ζ, ι/λ) [296]. The distribution of different PKC isoforms is tissue and species dependent. PKC isoforms α, β1/2, δ and ε have been identified in both rat and human myocardium [296]. Activation of PKC is another key feature of diabetes mellitus, which appears to be related to the elevation of DAG levels primarily as a result of de novo synthesis induced by high glucose [69, 74]. Studies by King's group and others provide strong evidence for the involvement of PKC activation in diabetic complications [69, 72-75, 297]. King's group demonstrated that STZ-diabetic rats exhibit a preferential activation of PKCβ2 isoform in the heart and aorta [69], and that treatment with a specific
PKCβ inhibitor (LY333531) ameliorated renal function and retinal circulation in diabetic rats [72]. Other investigators reported an increase in total PKC activities in STZ-diabetic rat heart homogenate and cytosolic fractions with corresponding increases in the protein contents of PKCα, β, ε and ζ isozymes [298]. Furthermore, PKC activities have also been found to be increased in spontaneously diabetic BB rats [69, 75] with an impairment in myocardial contractility [75]. Furthermore, in Langendorff perfused hearts, PKC inhibition with staurosporine or chelerythrine has been shown to improve the decreased left ventricular developed pressure and coronary flow rate of STZ-diabetic rats [254]. PKC may exert a negative inotropic effect in the myocardium via phosphorylation of tropinin I and troponin T, thereby reducing calcium sensitivity and inhibiting MgATPase activity of myofilaments [299, 300]. In addition, PKC may phosphorylate phospholamban and inhibit Ca\(^{2+}\) transport by sarcoplasmic reticulum (SR) [301]. Moreover, as mentioned earlier, recent work has demonstrated that stimulation of ET-1 synthesis by elevated glucose levels is mediated by activation of PKC [220]. A study has also reported a negative inotropic effect elicited by ET-1 in the mouse right ventricle; this effect is mediated through the activation of ET\(_A\) receptors and is PKC-dependent since the ET\(_A\) receptor antagonist BQ123 and the PKC inhibitor bisindolylmaleimide I were able to inhibit ET-1-induced negative inotropy [302]. Since ET-1 is known to activate PKC, an increase in ET-1 levels may alter cardiac function by contributing towards the activation of PKC in diabetes. Further studies, however, will be needed to clarify whether the favourable effects of bosentan on diabetic rat heart function are achieved by blocking ET-1-induced activation of PKC.
Cardiac dysfunction in diabetes has also been ascribed to alterations in myocardial energy utilization and the exclusive reliance of the heart on free fatty acids as a source of ATP [280]. Increased free fatty acids exert adverse electrophysiological, biochemical and mechanical effects on the heart with consequent changes in intracellular calcium handling, membrane permeability and eventual cell death and cardiac dysfunction [280]. It is possible that the effects of bosentan are mediated through improvement in myocardial energetics and it will be valuable to examine this proposition. Endothelin-1 is also a potent arrhythmogen [153, 303]. Ventricular arrhythmias following ET-1 are well documented [303]. Inhibition of ET-1-induced arrhythmias by bosentan may exert myocardial protective effects in diabetes.

Findings suggest that ET-1 may stimulate neutrophil accumulation in the heart and may enhance the production of oxidants such as superoxide anions [304]. As increased oxidative stress has been implicated in diabetic cardiomyopathy, the beneficial effect of bosentan may be secondary to a reduction in the level of oxidants. Indeed, agents that decrease oxidative stress have been shown to improve diabetic myocardial function [305]. Moreover, it has been suggested that increased generation of superoxide anions may be a contributing factor in the impairment of endothelium-dependent regulation of coronary flow in diabetes. Supportive evidence is provided by studies demonstrating that perfusion of hearts with superoxide dismutase or treatment of the rats with a tocopherol-acetate enriched diet prevented the defects in endothelium-dependent vasodilation observed in diabetic rats [54]. However, further experiments will be required to investigate whether the cardioprotective effect of bosentan involves its effects on oxidative stress.
Our untreated diabetic rats displayed relative cardiac hypertrophy compared with the untreated controls when the heart weight was corrected for the differences in the body mass between the two rat groups (Table 4.3 and Table 4.4). This is a relatively crude index of cardiac hypertrophy. In a study by Fiordaliso et al., results from morphometric assessment have demonstrated that at the cellular level, there was an increase in myocyte volume in the viable cardiomyocytes from 4-week diabetic rats [306]. However, an increase in myocyte loss was also observed in those diabetic rat hearts, which may account for the reduction in cardiac mass in diabetic rats [306]. Cardiac hypertrophy occurs secondary to a variety of stimuli and ET-1 exhibits potent hypertrophic effects on cardiac myocytes [192]. Antagonism of cardiac hypertrophy by ET receptor blockade has been demonstrated in experimental models of heart failure [175, 307]. Our data in diabetic rat hearts do not unmask a similar effect; heart weight to body weight ratios remained unchanged following bosentan or ABT-627 treatment. This suggests that growth factors other than ET-1 may contribute towards the development of cardiac hypertrophy in STZ-induced diabetes.
5.5 EFFECTS OF CHRONIC ENDOTHELIN RECEPTOR BLOCKADE ON DIABETIC VASCULAR FUNCTION

Abnormalities in vascular reactivity are common findings in diabetes [51, 78, 110-126, 308-311]. However, results from different laboratories are not always in agreement. Some investigators have reported diminished responses to vasoconstrictors including NE, phenylephrine, methoxamine, 5-hydroxytryptamine (5-HT) and potassium in STZ-treated rats [110, 111, 308], although they found no change in sensitivity to those vasoconstrictors [111, 308]. In contrast, a number of studies including ours indicate that arteries from STZ-diabetic animals exhibit enhanced contractile responses to a variety of vasoconstrictor agents [78, 112-114, 116-122, 225, 262, 309-311]. Although the reasons for these discrepancies remain unclear, the interstudy variability is generally, albeit inadequately, explained by differences in the induction of diabetes (e.g. the type, dose and route of administration of diabetogen used), the duration of diabetes, the gender of the animals, animal strains, the type of vessel preparation selected, bathing medium, and the methods used for the measurement and expression of the contractile force [51, 123].

**Vascular responses to norepinephrine (NE)**

Contractile responses to α-adrenoceptor agonists have been shown to be increased in different diabetic vessels. NE-induced vasoconstriction has been reported to be enhanced in the rat renal arteries 6 weeks after induction of STZ-diabetes [118]. Superior mesenteric arteries from 12-week diabetic rats were more responsive to
methoxamine, phenylephrine as well as NE than the control arteries [116, 117]. In addition, other studies have demonstrated an increase in maximum response to NE of aortae and mesenteric arteries from chronic diabetic rats with either an increase or no change in the sensitivity to NE [120-122, 310, 311].

The observations from our studies (Figure 4.16 and Figure 4.23) [262] that NE-mediated contractile responses in diabetic superior mesenteric and renal arteries were increased are in agreement with the above reports. Strikingly, chronic endothelin receptor blockade with bosentan was able to ameliorate the exaggerated NE-induced vascular responses of the diabetic arteries (Figure 4.16 and Figure 4.23) [262]. This suggests that ET-1 may be involved in the enhancement of NE-induced vasoconstriction in diabetes. Indeed, ET-1 has been shown to potentiate the vasoconstrictor effect of NE in vascular smooth muscle [312-314]. Since this endothelin-induced amplification of NE responses was shown to be prevented by the PKC inhibitors and occurred without changes in stimulated Ca\(^{2+}\) entry, it has been suggested that this ET-1 effect is accomplished by activating PKC-dependent mechanisms which increases the sensitivity of the contractile apparatus to Ca\(^{2+}\) [312]. Bosentan may therefore exert its beneficial effects in the diabetic arteries by blocking the effects of ET-1 on NE-mediated responses \emph{in vivo}.

**Vascular responses to endothelin-1 (ET-1)**

A variety of studies have demonstrated alterations in ET-1 reactivity in diabetic vessels [77-79, 112, 113, 119, 221-224]. However, there is considerable amount of controversy surrounding the effects of diabetes on ET-1-induced vascular responses.
While some studies have shown an attenuation in vascular responses to ET-1 [119, 221-224], an equal number of studies (including our studies) suggest that diabetes serves to potentiate the vasoconstrictor responses evoked by ET-1 [41, 78, 112, 113, 119, 225, 262]. As mentioned above, reasons for these inconsistent results between vascular studies are not completely understood. A high degree of variability in the methodology employed in the measurement of vasoconstriction may partially account for the conflicting results. As well, since there are regional differences in vascular responses to ET-1 [182], tissue-specific changes in the reactivity to ET-1 may occur during diabetes, which may contribute to the variable data obtained in vessels isolated from different vascular beds.

Similar to the responses to NE, we found an augmented contractile response to ET-1 in superior mesenteric and renal arteries from diabetic rats when compared to the controls; this enhanced response is corrected after chronic bosentan treatment (Figure 4.17 and Figure 4.23) [225, 262]. However, preliminary data from our laboratory showed no differences between control and diabetic rats in relation to ET-1 pressor response on the mean arterial pressure in intact anaesthetized rats (Arikawa, Sandhu, Yao, McNeill, unpublished observations). In contrast, other investigators found in conscious chronically instrumented rats that ET-1 produced a greater decrease in renal and mesenteric vascular conductances in diabetic compared to control animals [315], agreeing with our findings obtained from an isolated vessel preparation. The presence of confounding factors such as influence of anaesthesia on the baroreceptor reflex and regional differences in vascular responsiveness in our study with the intact
anaesthetized rats may explain the lack of difference in ET-1 pressor response between our diabetic and control rats.

In the face of increased tissue ET-1 levels in the diabetic arteries, an increased ET-1 reactivity is difficult to construe (since down regulation of ET receptors would be expected). However, as noted earlier, superior mesenteric arteries and renal arteries isolated from our diabetic rats showed no significant change in the expression of either ET receptors except an increase in the immunoreactive ET_A receptor levels in superior mesenteric arteries in comparison with those of control rats. Furthermore, long-term bosentan treatment resulted in the normalization of the ET_A receptor level in parallel with the correction of the vascular responses in diabetic superior mesenteric arteries, suggesting that an up-regulation of the ET_A receptor may be partly responsible for the vascular hyper-responsiveness to vasoconstrictors in diabetes.

**Vascular responses to acetylcholine (Ach)**

Contrary to the previous observations in long-term diabetes that endothelium-dependent relaxation is impaired in diabetic arteries [51, 123, 127, 128, 316], we did not see any difference in Ach-induced relaxation of the arteries from the untreated control and diabetic rats (Figure 4.29). The discrepancies seen in the results can likely be attributed to differences in the duration and/or severity of diabetes induced in various studies. Bosentan treatment did not affect Ach-mediated responses in arteries from our control and diabetic groups, suggesting that blockade of the ET_B receptor by the mixed ET receptor blocker did not affect endothelium-dependent relaxation responses. Interestingly, a recent study has demonstrated that chronic treatment with a mixed ET_A
and ET\textsubscript{B} receptor antagonist, J-104132, significantly attenuated the impairment in Ach-induced relaxation and the increase in superoxide anion levels in diabetic rat aortae [127]. The data suggest that ET-1 may be participate in impairing endothelium-dependent relaxation via increased superoxide anion production which is believed to result in inactivation of NO [127].

The observed increases in NE- and ET-1-mediated contractile responses in diabetes may be due to a non-specific elevation in vascular smooth muscle contractility, as a result of hyperglycemia-induced PKC activation [120, 123]; this would explain the consistent enhancement of contractile responses to a variety of vasoactive agents in the presence of diabetes. Studies have suggested that an increased PKC-activated process may account for the enhanced responsiveness to vasoconstrictors in diabetes [119, 120]. Indeed, PKC inhibitors such as calphostin C or staurosporine are able to eliminate the enhanced vasoconstrictor responses in diabetic rat aortae and mesenteric arteries [119, 120, 317]. Furthermore, PKC may induce other vascular abnormalities such as changes in permeability, proliferation and basement membrane [74]. As ET-1 can activate PKC, bosentan treatment may correct vascular dysfunction via blockade of ET-1-induced PKC-activated processes.

An increased phosphoinositide turnover and/or altered intracellular calcium flux [123, 309-311, 317-319] have been implicated in diabetes-induced enhancement of vascular reactivity. Altered vascular function in diabetes may be attributed to altered expression or function of proteins relevant for calcium homeostasis [123]. Both increases in intracellular Ca\textsuperscript{2+} release [310, 311, 318, 319] and extracellular Ca\textsuperscript{2+} influx
[311, 317] have been suggested to mediate the enhanced contractile responses to vasoconstrictors found in diabetes. However, in a recent study, when simultaneous measurement of $[\text{Ca}^{2+}]_i$ and tension development was performed, the augmented maximum contractile response of diabetic arteries to NE was not found to be associated with a corresponding rise in $[\text{Ca}^{2+}]_i$ levels [126]. This led to the proposition that the contractile proteins of diabetic arteries may be more sensitive to $\text{Ca}^{2+}$ in response to NE than those of the control arteries [126]. As mentioned earlier, ET-1 can potentiate NE-induced vasoconstriction; interestingly, this effect has been proposed to be mediated via the activation of PKC-dependent mechanisms which increases the $\text{Ca}^{2+}$ sensitization of the contractile apparatus [312]. In aortae from STZ-diabetic rats, inhibition of extracellular $\text{Ca}^{2+}$ influx with the calcium channel blocker verapamil has been shown to lower the exaggerated contractile responses to ET-1 to the respective control values, suggesting that a rise in ET-1-induced $\text{Ca}^{2+}$ entry through the L-type channel may contribute to the hyper-reactivity to ET-1 in diabetic arteries [112]. Taken together, the beneficial effects of bosentan may be related to improvements in intracellular calcium handling; future studies examining $[\text{Ca}^{2+}]_i$ in bosentan-treated vascular smooth muscle will be required to validate such a hypothesis.

The possibility that the beneficial effects of bosentan may be ascribed to putative reductions of blood pressure and hence vascular hypertrophy, merits attention. Vascular hypertrophy may in and of itself enhance responses to vasoconstrictors. The STZ-induced model of diabetes in rodents is not generally associated with hypertension [199, 320]. In this model we have found that neither induction of diabetes nor treatment with bosentan affected blood pressure significantly (Table 4.1), in agreement with the
results reported by Gilbert et al. [199]. However, in that study, it was found that the mesenteric arteries from rats with STZ-diabetes induced 3 weeks earlier had significant increases in vessel weight and wall-to-lumen ratio with associated increases in extracellular matrix deposition [199]. Bosentan treatment led to amelioration of these vascular abnormalities. Since no morphometric assessments were made in our studies, it is not known whether similar occurrence took place in our eleven-week STZ diabetic rats. Thus, while the beneficial effects of bosentan on vascular function in STZ-induced diabetic rats appears to be unrelated to effects on blood pressure, further studies will be required to elucidate the effects of bosentan on vascular hypertrophy in our diabetic rats.

The exaggerated vascular responses to vasoconstrictors in the diabetic arteries do not seem to be related to decreased endothelium-derived nitric oxide production, since inhibition of NO synthase with L-NAME did not affect these vasoconstrictor responses to NE in control and diabetic arteries. Although the effect of L-NAME on ET-1 responses was not examined in the current study, findings from a previous study in hamsters showed that constrictor responses to ET-1 remained enhanced in the diabetic arterioles when compared with the controls during inhibition of NO synthase [78].

Data from the present study showed that the removal of the endothelium abolished the increases seen in the vasoconstrictor responses of the diabetic arteries while no significant effects were observed in the arteries from other rat groups (Figures 4.19, 4.20, 4.21). Similar observations were previously reported in both the aortae and mesenteric arteries from 3-month diabetic rats [114, 321, 322]. However, as with data on other vascular responses in diabetes, controversies exist regarding the contribution
of the vascular endothelium towards the altered vasoconstrictor responses in diabetes, with some studies showing enhanced constrictor responses in endothelium-denuded arteries from diabetic arteries [126, 323].

The endothelium dependence of the enhanced responses to vasoconstrictors observed in our diabetic rats suggests that an endothelium-derived vasoconstrictor such as TxA$_2$ and the endoperoxide PGH$_2$ may be important in mediating this effect [116, 232]. We have previously demonstrated that the hyper-responsiveness to NE in superior mesenteric arteries from diabetic rats was corrected by in vitro inhibition of cyclooxygenase with indomethacin [116], implying that an altered synthesis of endogenous vasoconstrictor eicosanoids could be a factor in the enhanced NE responses of the diabetic vessels. Indeed, an increase in TxA$_2$ release from diabetic aorta has been previously demonstrated [232, 324]. Additionally, experimental diabetes has been shown to increase contractile responses to U46619, a TxA$_2$ analogue [119].

The effects of bosentan on the prostanoid system in diabetes are unclear. However, accumulating evidence has shown an interaction between the ET-1 and TxA$_2$ systems. Endothelin-1 has been shown to stimulate the production of TxA$_2$ [233-235, 325]. Furthermore, ET-1-evoked vasoconstriction is shown to be dependent on TxA$_2$ in human placental vessels [326]. Thromboxane A$_2$ appears to play a role in the vasoconstrictor effect of ET-1 in the animal models of two different diseases, the spontaneously hypertensive rat and the postischemic rat heart, but not in their control counterparts (i.e. the Wistar-Kyoto rat and the nonischemic rat heart, respectively) [326, 327]. The functional interaction between ET-1 and TxA$_2$ has also been demonstrated in fructose-induced hypertensive rats where chronic treatment with either bosentan or the
thromboxane synthase inhibitor dazmegrel can prevent the development of hypertension in those rats [202, 233]. Additionally, Moreau et al. [328] demonstrated that bosentan was able to produce a parallel rightward shift of the contractions to U46619, indicating the antagonism of the direct stimulation of TxA$_2$ receptors by the mixed ET receptor blocker. Based on the above discussion, it is plausible that bosentan improved vascular responses in diabetes by blocking ET-1-induced TxA$_2$ synthesis/release and/or action. We therefore carried out studies to determine if thromboxane is involved in mediating ET-1-induced changes in diabetic vasoconstrictor responses.
5.6 ROLE OF THROMBOXANE A\textsubscript{2} IN ENDOTHELIN-INDUCED HYPER-REACTIVITY IN DIABETIC ARTERIES

Our earlier observation that the increased vascular responsiveness of diabetic rat arteries to NE and ET-1 was diminished by chronic bosentan treatment suggests a role for ET-1 in this vascular defect. Moreover, this hyper-responsiveness to vasoconstrictors was demonstrated to be endothelium-dependent and could be eradicated by cyclooxygenase inhibition, implying that an endothelium-derived vasoconstrictor eicosanoids such as TxA\textsubscript{2} could be involved in this effect. Therefore, experiments were conducted to elucidate (1) if hyper-reactivity in diabetic arteries was mediated by direct vascular actions of ET-1 through its receptors, (2) if TxA\textsubscript{2} is involved in this hyper-responsiveness to vasoconstrictors in diabetes, and (3) to examine if the beneficial effects of chronic bosentan treatment observed in diabetic arteries were related to its blocking actions of the direct effects of ET-1 and/or its effects on the interaction between the endothelin and TxA\textsubscript{2} systems. Thus, contractile responses of arteries from control and diabetic rats, treated or not treated with bosentan for 10 weeks, to vasoconstrictors were assessed following \textit{in vitro} incubation with (1) bosentan, (2) a selective ET\textsubscript{A} receptor blocker, BQ123, and (3) a thromboxane synthase inhibitor, dazmegrel. In addition, to evaluate the vascular reactivity to TxA\textsubscript{2} in diabetic arteries, vascular responses to U46619, a TxA\textsubscript{2} analogue, were examined.
Data from the present study demonstrated that the augmented NE and ET-1 contractile responses in both superior mesenteric and renal arteries from the diabetic rats were normalized with chronic treatment with the mixed ET\textsubscript{A} and ET\textsubscript{B} receptor antagonist, bosentan (Figure 4.23). In superior mesenteric arteries, \textit{in vitro} incubation with bosentan as well as with the selective ET\textsubscript{A} receptor blocker, BQ123, alleviated the exaggerated NE and ET-1 contractile responses in diabetic arteries (Figure 4.24). In addition, \textit{in vitro} incubation with the thromboxane synthase inhibitor, dazmegrel, abolished the exaggerated NE and ET-1 contractile responses in diabetic superior mesenteric arteries (Figure 4.26). In contrast to the superior mesenteric arteries, acute incubation with bosentan, BQ123 or dazmegrel did not result in the abolition of the increased contractile responses to NE observed in diabetic renal arteries (Figure 4.25 and Figure 4.26). Dazmegrel incubation attenuated the maximum contractile responses to ET-1 in diabetic renal arteries; however, these responses in diabetic renal arteries remained significantly greater than those of other groups (Figure 4.27). Results from this study also showed that the U46619 contractile response was enhanced in diabetic renal arteries which were corrected by chronic bosentan treatment (Figure 4.28); however, no significant difference in the response in superior mesenteric arteries was observed between the control and diabetic groups. Furthermore, in the present study, comparable levels of plasma TxB\textsubscript{2} and 6-keto-PGF\textsubscript{1\alpha} (respective stable metabolites of TxA\textsubscript{2} and prostacyclin) as well as similar TxB\textsubscript{2}-to-6-keto-PGF\textsubscript{1\alpha} ratios were observed in control and diabetic rats; no apparent effect of chronic bosentan treatment on the levels of those plasma constituents was seen (Table 4.7).
**Effects of in vitro incubation with bosentan/BQ123/dazmegrel on vascular responses to vasoconstrictors in superior mesenteric arteries**

Observations from this study confirm our earlier findings that chronic bosentan treatment corrected the exaggerated NE response in diabetes, implicating an involvement of ET-1 in the hyper-responsiveness to NE in diabetes. In superior mesenteric arteries, the enhanced vasoconstrictor effects of NE in diabetes could also be abolished by acute blockade with either bosentan or BQ123. Hence, the diabetic-induced increase in the contractile response to NE appears to be mediated by the ET$_A$ receptor in this vessel. This finding can be reconciled with the increase in immunoreactive ET$_A$ receptor levels which we observed in the superior mesenteric arteries from diabetic rats (Figure 4.12). Moreover, the amelioration of the enhanced NE response in diabetes after long-term treatment with bosentan may be attributable to the normalization of the ET$_A$ receptor level as seen in the mesenteric arteries from bosentan-treated diabetic rats.

As mentioned previously, we proposed that the enhanced contractile response to NE in diabetes may be mediated through an increase in ET-1-induced TxA$_2$ synthesis and/or action. Indeed, this hypothesis is supported by our findings that inhibition of thromboxane synthase with dazmegrel abrogated the differences seen in the NE response between the diabetic and control superior mesenteric arteries. Taken together, the above results suggest that in this vessel diabetes-associated hyper-reactivity to NE may be mediated via an increase in ET$_A$ receptor activation and subsequent stimulation of thromboxane synthesis in the endothelium.
In parallel with the findings on NE responses, the exaggerated ET-1-induced contractile responses in diabetic arteries, which was corrected by chronic bosentan treatment, was also shown to be alleviated by inhibiting thromboxane synthesis with dazmegrel. Hence, similar to the responses to NE, the enhanced response to ET-1 in diabetic arteries is probably due to an increase in ET-1-induced thromboxane synthesis.

Similar interactions between NE, ET-1 and TxA₂ has been noted in another rodent model, the spontaneously hypertensive rats (SHR) [329]. Zerrouk et al. [329] have shown that in SHR aortae (but not in Wistar Kyoto (WKY) rat aortae), ET-1 at a subthreshold concentration (3x10⁻¹⁰ M) potentiated the contractile responses to NE. This potentiation was endothelium-dependent and was suppressed by a cyclooxygenase inhibitor (piroxicam) as well as by a PGH₂-TxA₂ receptor antagonist (SQ29548). As well, this effect of ET-1 was elicited by the ETₐ receptor since it was blocked by BQ123, but not BQ788 (an ETₐ receptor selective antagonist). Furthermore, they have demonstrated that the TxA₂ mimetic U46619 also amplified the contractile responses to NE in denuded aortae from both SHR and WKY rats [329], thus providing evidence for this intricate relationship between NE, ET-1 and TxA₂. Taddei and Vanhoutte [327, 330] also reported that the contractile response to ET-1 in SHR aortae involved the release of endothelium-derived TxA₂ evoked by stimulation of the ETₐ receptor; this phenomenon was not observed in WKY rat aortae [327, 330].

The notion that the hyper-reactivity to vasoconstrictors in diabetic superior mesenteric arteries is dependent on an ETₐ receptor-activated process in the endothelium is intriguing, since it would suggest that ETₐ receptors, which are considered to be primarily vascular smooth muscle receptors, may also be present in
the endothelium. The above findings from SHR aortae support this idea since the ET-1-induced effects noted in those aortae (including the stimulation of the release of TxA₂) were abrogated by the blockade of ETₐ receptors as well as removal of the endothelium [327, 329, 330]. Data from one study also provided evidence for the presence of functioning ETₐ receptors and their mRNA expression in the endothelial cells from porcine aortic valve* in situ [331]. Since our studies for the expression of ET receptors in control and diabetic rat arteries had not been designed to assess the distribution of ET receptors in different cell types, further studies will be required to clarify whether ETₐ receptors are present in the endothelium of diabetic superior mesenteric arteries.

**Effects of in vitro incubation with bosentan/BQ123/dazmegrel on vascular responses to vasoconstrictors in renal arteries**

Although chronic treatment with bosentan equally ameliorated the abnormal vasoconstrictor responses in both the superior mesenteric arteries and renal arteries from diabetic rats, results obtained from superior mesenteric arteries and renal arteries were different when acute effects of different blockers were examined. In contrast to the observations in the diabetic superior mesenteric arteries, the contractile response to NE in diabetic rat renal arteries remained significantly greater than that of the control rats after the incubation with bosentan, BQ123 or dazmegrel. In addition, in the renal arteries from diabetic rats, the exaggerated response to ET-1 remained apparent after *in vitro* incubation with dazmegrel. However, dazmegrel incubation partially attenuated the maximum contractile responses to ET-1 in diabetic renal arteries, indicating the presence of an interaction between ET-1 and TxA₂ in those arteries.
The differences observed between the different arteries may possibly be due to a difference in ETₐ/ETₐ receptor density in the renal arteries and the superior mesenteric arteries. This notion is supported by the observation of the regional differences in vascular response to ET-1 [182]. In addition, diabetes has been shown to result in a decrease in the ETₐ receptor level in the endothelium compared with that of vascular smooth muscle cell in rat kidney, indicating that in diabetic states there may be a preferential increase in ETₐ receptor-induced vasoconstriction to vasodilation [277]; however, whether such change occurs in the mesenteric arteries during diabetes remains to be elucidated. Moreover, since results from the experiments involving the *in vitro* incubation of bosentan cannot fully explain the effects of chronic treatment with the ET blocker on vascular reactivity in diabetes, it clearly demonstrates that both long-term and short-term effects of the blocker on the endothelium as well as the vascular smooth muscle are attributable to the beneficial effects of chronic bosentan treatment in the vasculature of diabetes.

**Vascular responses to U46619**

In diabetes, prostanoid metabolism as well as vascular responsiveness to prostanoids appears to be altered. There seems to be a shift from vasodilator to vasoconstrictor prostanoid production in the diabetic state [332]. Vascular production of prostacyclin in diabetic animals has been shown to be decreased [333-335]. Conversely, both the plasma levels and platelet synthesis of TxA₂ have been reported in diabetic patients [336, 337]. An increase in TxA₂ release from the endothelium of diabetic aorta has also been previously demonstrated [232, 324]. As well, femoral
arterial rings from diabetic dogs exhibited an increased production of TxA₂ which was proposed to be responsible for the increased vasoconstrictor response to NE seen in those animals [338]. Additionally, experimental diabetes has been demonstrated to increase contractile responses to the TxA₂ analogue U46619 [119, 333, 339]. Thus, it is possible that vasoconstrictor prostanoid such as TxA₂ may contribute to vascular dysfunction in diabetes.

In our rats, plasma levels of TxA₂ and prostacyclin metabolites did not seem to be affected by diabetes. The lack of change in the plasma levels of these eicosanoids may be explained by the suggestion that diabetes may influence the biosynthesis of TxA₂ and prostacyclin in a tissue-specific manner [124]. As noted later, there appears to be a difference in the effect of diabetes on thromboxane synthesis between the superior mesenteric arteries and renal arteries from our rats.

To determine if the vasoreactivity to TxA₂ was altered in our diabetic rats, we examined the contractile responses to U46619, a TxA₂ mimetic. In rat superior mesenteric arteries, there was no significant change in the contractile response to U46619 after long-term diabetes, and chronic bosentan treatment did not affect this response in either control or diabetic groups. On the contrary, in renal arteries, the contractile response to U46619 in diabetic rats was enhanced, and chronic bosentan treatment was able to correct this abnormality. In parallel with our findings in isolated renal arteries, Quilley and McGiff [339] found that the perfused kidneys from diabetic rats also exhibited an elevated constrictor response to U46619. How diabetes causes a greater renal vasoconstrictor response to TxA₂ and how bosentan treatment corrects this vascular defect is not clear. One possibility is that there may be an increase in TxA₂
receptor levels in diabetic renal arteries, and chronic bosentan treatment may act to counter this increase, although this is speculative at present.

Taken together, our results indicate that an increase in ET\textsubscript{A} receptor-mediated synthesis/release of TxA\textsubscript{2} may account for the diabetes-induced enhancement of vasoconstrictor responses to NE and ET-1 in the superior mesenteric arteries. Bosentan treatment may exert its beneficial effects on diabetic vascular function by normalizing the ET\textsubscript{A} receptor levels and blocking the ET\textsubscript{A} receptor-induced effect on TxA\textsubscript{2} synthesis in this vessel. The exact mechanism by which ET-1 stimulates the production of TxA\textsubscript{2} in the vascular endothelium is not clearly defined. Several in vitro studies have shown that ET-1 increases the release of arachidonate metabolites by increasing both the activity and expression of PLA\textsubscript{2} [340-343]. There is also evidence that ET-1 stimulates the expression of COX-2 in rat mesangial cells [344-346]. While it has been shown in human endothelial cells that an up-regulation of COX-2 results in an increased production of PGI\textsubscript{2} and PGE\textsubscript{2} and that TxA\textsubscript{2} synthesis appears to be solely mediated by COX-1 [347], it is not known exactly how the activity of COX-1 and COX-2 is related to the production of each individual eicosanoid in the vascular endothelium under the pathological conditions of diabetes. Whether the PLA\textsubscript{2}/COX system is altered in the vascular endothelial cells from STZ-diabetic rats and whether the elevated ET-1 levels in this diabetic model increase TxA\textsubscript{2} synthesis by affecting the activity and/or expression of the PLA\textsubscript{2}/COX system remain to be elucidated. As well, the effects of long-term bosentan treatment on the PLA\textsubscript{2}/COX system in diabetic vascular endothelium await further investigations.
In contrast to the superior mesenteric arteries, in the renal arteries, an increase in vascular responses to TxA$_2$, rather than a change in ET-1-induced TxA$_2$ synthesis, may contribute to the augmented responses to NE and ET-1 in diabetes. Chronic bosentan treatment may normalize the responses to NE and ET-1 in diabetic renal arteries by correcting TxA$_2$ responses. How bosentan may attenuate the increased vasoconstrictor responses to TxA$_2$ in diabetic renal arteries is not known. It has been suggested that the alterations in TxA$_2$ response in diabetes may be due to changes in the affinity and number of TxA$_2$ receptors [124, 348]; hence, we propose that bosentan may prevent diabetic-induced changes in TxA$_2$ receptors. Further studies aiming at examining the effects of TxA$_2$ receptor blockade on NE and ET-1 responses and determining the expression and properties of TxA$_2$ receptors in untreated and bosentan-treated diabetic renal arteries are necessary to validate such a hypothesis.

In conclusion, the above study demonstrated a role for ET-1 in mediating the exaggerated vascular responses to vasoconstrictors in diabetic superior mesenteric arteries and renal arteries. The data indicate that an interaction between ET-1 and TxA$_2$ is involved in this vascular defect in diabetes. Furthermore, the underlying mechanism(s) appear to be tissue specific as the ET receptor blockers and the thromboxane synthase inhibitor exerted different effects on vascular responses in different arteries. Also, both long and short-term effects of bosentan may contribute to the beneficial effects of the blocker on the diabetic vasculature.
5.7 CONCLUDING REMARKS

Over the past decade, ET-1 has been receiving much attention for its potential pathogenic roles in several cardiovascular diseases. A burgeoning body of evidence now suggests that ET-1 may also be involved in the development of the cardiovascular complications in diabetes mellitus. This notion is supported by previous studies reporting abnormal ET-1 levels and reactivity in diabetes [77-80, 82, 85, 112, 113, 119, 213-218, 221-224] and the fact that ET-1 has potent cardiac, vasoconstrictor and hypertrophic effects. The main aim of the present thesis was to examine the role of ET-1 in the development of the cardiovascular diseases in diabetes.

The primary observations that originate from the work in the present thesis were that STZ-induced type 1 diabetes resulted in cardiac and vascular dysfunction in association with an up-regulation of the expression of the local ET system. Chronic ET receptor blockade with bosentan improved functional cardiac performance and corrected vascular hyper-reactivity to vasoconstrictors in STZ-diabetic rats. These data are important since they uncover the beneficial effects of ET antagonism on cardiac and vascular function in experimental diabetes and suggest that exaggerated ET-1 production and/or action may play a role in the development of cardiovascular dysfunction in chronic experimental diabetes. The mechanisms underlying the effects of bosentan treatment on the functional changes in the heart and arteries from diabetic rats were also briefly explored in the present thesis.

In the diabetic heart, the cardioprotective effects of bosentan were shown to require the blockade of ET_B receptors, since selective ET_A receptor antagonism did not
provide significant improvement to diabetic rat heart function. Moreover, we previously demonstrated that there was an increase in ET-1-evoked coronary vasoconstriction in diabetes which was blocked by chronic bosentan treatment (Verma et al. in press [226]). In experimental models of congestive heart failure, an enhanced ET\textsubscript{B} receptor-mediated coronary vasoconstriction has been reported [284]; we propose that similar changes may occur in the diabetic rat heart. Hence, a potential mechanism underlying the beneficial effects of bosentan may be the blockade of ET\textsubscript{B} receptor-mediated coronary vasoconstriction.

In the vasculature, while chronic treatment with bosentan equally ameliorated the hyper-responsiveness to NE and ET-1 in superior mesenteric arteries and renal arteries from diabetic rats, the underlying mechanisms for this effect appear to be tissue-specific. In the superior mesenteric arteries, the enhanced vasoconstrictor responses in diabetes seem to be related to an increase in ET\textsubscript{A} receptor-activated thromboxane synthesis/release in the diabetic vascular endothelium, and bosentan may act by blocking the activation of the ET\textsubscript{A} receptor and/or normalizing the increased expression of the receptor. In the renal arteries, however, the beneficial effects of bosentan treatment on the diabetic responses to NE and ET-1 are not likely to be due to an effect on thromboxane synthesis, but rather, may be exerted through the normalization of the vascular responses to TxA\textsubscript{2}. At the present time, it is unclear how bosentan treatment corrected the responses to TxA\textsubscript{2}. The proposed mechanisms underlying the exaggerated vasoconstrictor responses in diabetic arteries are illustrated in Scheme 3.
SCHEME 3

Hypothetical Mechanisms for the Hyper-reactivity of Diabetic Arteries to Vasoconstrictors

In diabetes, an increased production of ET-1 occurs as a result of hyperglycemia-induced activation of diacylglycerol-protein kinase C pathway. The elevated ET-1 level then mediates a series of events that may lead to the increase in the contractile responses to vasoconstrictors such as NE and ET-1 in diabetic rat arteries. ET-1 may stimulate the endothelial synthesis of TxA₂. Activation of PGH₂-TxA₂ (TP) receptors by TxA₂ in turn potentiates NE- and ET-1-evoked vasoconstriction. This involvement of ET-1 is supported by the observation that chronic treatment with the mixed ETₐ/ET₆ receptor blocker bosentan ameliorated the hyper-responsiveness to NE and ET-1 in both superior mesenteric arteries and renal arteries from diabetic rats. However, the underlying mechanisms for this effect appear to be tissue-specific.

A) In diabetic superior mesenteric arteries, the ETₐ receptor level is increased. The enhanced vasoconstrictor responses to NE and ET-1 may be due to an increase in endothelin-induced synthesis/release of endothelial TxA₂, as removal of the endothelium, inhibition of TxA₂ synthesis with indomethacin or dazmegrel and acute blockade of endothelin receptors with bosentan or selective ETₐ receptor blocker BQ123 are all able to abolish the exaggerated vasoconstrictor responses. Furthermore, our findings suggest that ET-1 may stimulate TxA₂ synthesis in the endothelium via activation of ETₐ receptors. Whether ETₐ receptors are present in the endothelium of diabetic rat superior mesenteric arteries is not clear. Bosentan treatment may exert its beneficial effects on diabetic vascular function by normalizing the ETₐ receptor levels and blocking the ETₐ receptor-induced effect on TxA₂ synthesis in this vessel.

B) In contrast to the superior mesenteric arteries, in the renal arteries, in vitro incubation with bosentan, BQ123 or dazmegrel was not able to alleviate the increase in NE and ET-1-evoked vasoconstriction. Hence, an increase in vascular responses to TxA₂, rather than an alteration in ET-1-induced TxA₂ synthesis, may contribute to the augmented responses to NE and ET-1 in diabetes. Chronic bosentan treatment may normalize the responses to NE and ET-1 in diabetic renal arteries by correcting TxA₂ responses. How bosentan may attenuate the increased vasoconstrictor responses to TxA₂ in diabetic renal arteries is not known. One possibility is that diabetes may result in an increase in TP receptor levels and bosentan may prevent diabetes-induced changes in TxA₂ receptors.
A. Superior Mesenteric Artery

Hyperglycemia → ↑ Diacylglycerol → Protein Kinase C → ↑ ET-1 → ↑ TxA₂ → ETA? → ET-1 → ↑ Vasoconstriction

ENDOTHELUM

NE → TP → ↑ ETA

VASULAR SMOOTH MUSCLE

B. Renal Artery

Hyperglycemia → ↑ Diacylglycerol → Protein Kinase C → ↑ ET-1 → ↑ TxA₂ → ETA? → ET-1 → ↑ Vasoconstriction

ENDOTHELUM

NE → TP → ↑ ETA

VASULAR SMOOTH MUSCLE

Abbreviations: α, alpha 1 adrenoceptor; ET-1, endothelin-1; ETA, ET₁ receptor; ET R, ET receptor; NE, norepinephrine; TP, PGH₂-TxA₂ receptor; TxA₂, thromboxane A₂

⊕ activation/potentiation

↑ increase

→ blockade/inhibition resulting in abolition of exaggerated vasoconstriction

..... blockade/inhibition without abolition of exaggerated vasoconstriction
Future directions

Further investigation will be required to elucidate the exact mechanisms underlying the cardiovascular protective effects of bosentan in diabetes. Future experiments directed at elucidating a number of possible mechanisms have been proposed in the present thesis.

An activated DAG-PKC pathway has been implicated in various complications of diabetes [69, 72-75, 297]. Moreover, many ET-1-induced effects are mediated by activation of PKC. Hence, PKC activation may play a permissive role in mediating ET-1-induced aberrations in the cardiovascular system of diabetes. Therefore, it may be worthwhile to investigate if bosentan exerts its restorative effects via blockade of ET-1-mediated PKC activation in the heart and vasculature of diabetes. Furthermore, as more sophisticated molecular techniques that allow identification of functional roles of individual PKC isoforms are becoming available, the effects of bosentan on the expression and activity of the specific PKC isoforms that are reported to be increased in diabetic cardiovascular tissues (e.g. PKCβ2 or δ) [69, 72, 349] can be examined.

Studies for determining the effects of chronic diabetes and bosentan treatment on the coronary vascular response to ET_B receptor agonists as well as the expression of coronary ET_B receptor in diabetes are also warranted. As mentioned above, ET_B receptor-mediated coronary vasoconstriction may be increased in diabetes and may thus play a role in diabetic cardiomyopathy.

In addition, determination of the effects of diabetes and bosentan on the COX and TxA_2 system would be important in elucidating the role of ET-1-TxA_2 interaction in the augmented vasoconstrictor responses in different diabetic arteries. Specifically, the
effects of diabetes and bosentan treatment on the production and release of TXA$_2$ from diabetic superior mesenteric arteries as well as on the expression of PLA$_2$, COX-1, COX-2 and the thromboxane synthase, enzymes that are involved in the synthesis of TXA$_2$, need to be evaluated in the endothelium of diabetic superior mesenteric arteries. We propose that an up-regulation of those enzymes may account for the putative increase in ET-1-induced TXA$_2$ synthesis in those arteries. As well, in diabetic renal arteries, the expression of TXA$_2$ receptors needs to be examined and the effects of bosentan on their expression determined.

Results presented in this thesis may, therefore, provide a key link (i.e. ET-1) between diabetes mellitus and its various cardiovascular complications as well as adding to the therapeutic repertoire available for the treatment of cardiovascular complication encountered in long-term diabetes.
6. SUMMARY AND CONCLUSIONS

1. Chronic endothelin (ET) receptor blockade with bosentan has previously been shown to improve the isolated working heart function of STZ-diabetic rat hearts, suggesting that an activated endothelin system, such as an up-regulation of ET-1 expression, may be involved in diabetic cardiomyopathy. In hearts from STZ-diabetic rats, an increased tissue level of ET-1, as assessed by immunohistochemistry, was seen in both left and right ventricles when compared to the controls. Diabetic rat heart ventricles also exhibited an increased mRNA expression of ET-1. Ten weeks of bosentan treatment did not affect the immunoreactivity or mRNA expression in either diabetic or control hearts. Therefore, the cardioprotective effects of bosentan treatment are not related to an effect on the ET-1 expression.

2. In STZ-diabetic rat hearts, an increase in ET\textsubscript{A} receptor-like immunoreactivity (ET\textsubscript{A}-ir) was found in both the left and right ventricles while ET\textsubscript{B}-ir was increased only in the right ventricles. Heart ventricles from diabetic rats showed a small but significant increase in mRNA expression of ET\textsubscript{A} receptors while no changes were found in the mRNA expression of ET\textsubscript{B} receptors. Long-term bosentan treatment did not have any effect on either protein or mRNA expression of ET receptors. The above findings indicate that the cardiac endothelin system was up-regulated in diabetic rats. However, the beneficial effects of bosentan on heart function in STZ-diabetic rats did not seem to be related to the normalization of ET receptor levels.
3. The increased expression in ETA receptors in both the right and left ventricles of diabetic rats suggests that ETA receptors may have an important role in the development of diabetic cardiac failure. Hence, to determine if selective blockade of ETA receptors per se would provide cardioprotective effects in diabetes, the effects of selective ETA receptor antagonist ABT-627 on heart function of STZ-diabetic rats were examined. Seven weeks of ABT-627 treatment did not induce any significant changes in cardiac performance of the diabetic rats when assessed by ±dP/dt and LVDP. This suggests that ET-1-induced aberrations to the heart are not mediated solely via ETA receptors but also through ETB receptors.

4. Superior mesenteric arteries (SMA) and renal arteries (RA) from diabetic rats exhibited an increase in the immunoreactivity for ET-1. Bosentan did not appear to have any effects on the tissue ET-1 level in arteries. The expression of ET-1 is increased in diabetic rat arteries, implicating a role for ET-1 in diabetic angiopathy.

5. Alterations in vascular action of diabetes (see conclusion #6) may be due to changes in the expression of vascular ET receptors. In superior mesenteric arteries isolated from the diabetic rats, there was an increased ETA-ir while ETB-ir was not different between control and diabetic rat groups. Bosentan normalized the ETA receptor expression in diabetic SMA while it had no effects on ETB receptor expression. In RA, the expression of both ET receptors was not affected by the diabetic state, and bosentan did not have any effect on the ET receptor levels in those arteries. Our findings indicate that the vascular
endothelin system was up-regulated in diabetic rats. Normalization of the \( E_{TA} \) receptor levels may partly account for the observed beneficial effects of bosentan on SMA in diabetic rats (see conclusion #6). In contrast, the restorative effects of bosentan on diabetic rat RA vascular reactivity (see conclusion #6) did not seem to be related to a correction of ET receptor levels.

6. Contractile responses to norepinephrine (NE) \((10^{-9}-10^{-4})\) prior to and after incubation with the nitric oxide synthase inhibitor \( N^G \)-nitro-L-arginine-methyl ester (L-NAME) as well as to ET-1 \((10^{-12}-3\times10^{-8})\) were markedly increased in both SMA and RA isolated from diabetic rats when compared to control rats. These increases in NE and ET-1 responses were prevented by chronic bosentan treatment. The exaggerated responses to NE and ET-1 observed in endothelium-intact SMA and RA from the diabetic group were completely abrogated by denudation of the endothelium. Therefore, the enhanced vasconstrictor responses in diabetic rat arteries may involve ET-1, and an endothelium-derived contracting factor such as thromboxane A\(_2\) (TxA\(_2\)) may play a role in endothelin-induced hyper-reactivity in diabetic arteries.

7. *In vitro* incubation with bosentan as well as with the selective \( E_{TA} \) receptor blocker, BQ123, abolished the exaggerated NE contractile responses in diabetic SMA. *In vitro* incubation with the thromboxane synthase inhibitor, dazmegrel, abolished the exaggerated NE and ET-1 contractile responses in diabetic SMA. No significant *in vitro* effect of bosentan, BQ123 or dazmegrel on vascular responses to NE was observed in diabetic RA. Dazmegrel incubation attenuated the maximum contractile responses to ET-1 in diabetic RA; however, these
responses in diabetic RA remained significantly greater than those of control rats. The TxA$_2$ analogue U46619 contractile response was enhanced in the diabetic RA and was corrected by chronic bosentan treatment. The present study demonstrated an involvement of ET-1 in mediating the exaggerated vasoconstrictor responses in diabetic arteries. Our data indicate that an interaction between ET-1 and TxA$_2$ is involved in this vascular defect of diabetes. Furthermore, the underlying mechanism(s) appear to be tissue specific as the ET receptor blockers and the thromboxane synthase inhibitor exerted different effects on vascular responses in different arteries. Also both long and short-term effects (i.e. chronic vs. acute treatment) of bosentan may contribute to the beneficial effects of the blocker on the diabetic vasculature.
7. REFERENCES


164. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR: Pressor effects of circulating endothelin are limited by its removal in the


204. Han H, Neubauer S, Braeker B, Ertl G: Endothelin-1 contributes to ischemia/reperfusion injury in isolated rat heart-attenuation of ischemic injury by


235. Zaugg CE, Hornstein PS, Zhu P, Simper D, Luscher TF, Allegrini PR, Buser PT: Endothelin-1-induced release of thromboxane A2 increases the vasoconstrictor


placebo supplementation on TxB2 and lipid peroxide levels in type 1 diabetic

337. Chen SY, Yu BJ, Liang YQ, Lin WD: Platelet aggregation, platelet cAMP levels

vasoconstrictor response to noradrenaline in femoral vascular bed of diabetic

339. Quilley J, McGiff JC: Renal vascular responsiveness to arachidonic acid in

340. Resink TJ, Scott-Burden T, Buhler FR: Activation of phospholipase A2 by
endothelin in cultured vascular smooth muscle cells. Biochem Biophys Res

341. Schramek H, Wang Y, Konieczkowski M, Rose PM, Sedor JR, Dunn MJ:
Endothelin-1 stimulates cytosolic phospholipase A2 in Chinese hamster ovary
cells stably expressing the human ETA or ETB receptor subtype. Biochem

stimulates cytosolic phospholipase A2 activity and gene expression in rat

343. Husain S, Abdel-Latif AA: Role of protein kinase C alpha in endothelin-1
stimulation of cytosolic phospholipase A2 and arachidonic acid release in
cultured cat iris sphincter smooth muscle cells. Biochim Biophys Acta 1998;
1392(1):127-44.

344. Kester M, Coroneos E, Thomas PJ, Dunn MJ: Endothelin stimulates
prostaglandin endoperoxide synthase-2 mRNA expression and protein synthesis
through a tyrosine kinase-signaling pathway in rat mesangial cells. J Biol Chem

345. Hughes AK, Padilla E, Kutcher WA, Michael JR, Kohan DE: Endothelin-1

