IN VIVO EFFECTS OF VANADIUM ON PROTEIN KINASE B AND KEY GLUCONEOGENIC ENZYMES IN ANIMAL MODELS OF DIABETES:
COMPARISON WITH INSULIN

by
LUCY MARZBAN
Faculty of Medicine, University of Tehran, 1994

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ABSTRACT

Insulin resistance is a major characteristic of both Type 1 and Type 2 diabetes. However, despite numerous studies during the past decade the mechanism of insulin resistance is still not clear. Protein kinase B (PKB) has been proposed to be an intermediate protein kinase in the insulin signaling pathway by which insulin controls glucose disposal via stimulation of glycogen synthesis and glucose uptake in insulin sensitive tissues as well as hepatic glucose output. Hence, PKB may play a potential role in the development of insulin resistance. Vanadium compounds are known to have insulin mimetic/enhancing effects both in vitro and in vivo and therefore, are candidates for oral therapy in diabetes. Bis(maltolato)oxovanadium (IV) (BMOV) is an organic vanadium compound which corrects hyperglycemia in streptozotocin (STZ)-diabetic rats and lowers the elevated plasma insulin levels in fatty Zucker rats.

In this study, we investigated 1) the association between PKB activity and insulin resistance 2) the in vivo effects of insulin and chronic BMOV treatment on PKB activity in the skeletal muscle and liver of two animal models of diabetes: STZ-diabetic Wistar rats, an animal model of poorly controlled Type 1 diabetes and fatty Zucker rats, an animal model that represents several characteristics of Type 2 diabetes. Animals were treated with BMOV in the drinking water (0.75-1 mg/ml) for 3 (or 8) weeks and sacrificed with or without insulin injection. Insulin (5 U/kg, i.v.) increased PKBα activity more than 10-fold and PKBβ activity more than 3-fold in both animal models. Despite the development of insulin resistance, PKBα activity was not impaired in STZ-diabetic rats up to 9 weeks of diabetes, thus excluding a
role for PKBα in the development of insulin resistance in Type 1 diabetes. In contrast, insulin-induced PKBα (but not PKBβ) activity was markedly reduced in the skeletal muscle (fatty: 7-fold vs. lean: 14-fold), and significantly increased in the liver (fatty: 15.7-fold vs. lean: 7.6-fold) of fatty Zucker rats. These observations indicated an association between altered insulin-stimulated PKB activity and insulin resistance in this model. Comparison of basal PKBα activity in Zucker fatty and Zucker diabetic fatty rats indicated that basal enzyme activity was not affected by diabetes. BMOV, at doses sufficient to normalize fasting plasma glucose in STZ-diabetic rats and decrease plasma insulin levels in fatty Zucker rats had no detectable effect on basal or insulin-induced PKBα or PKBβ activities, indicating that the glucoregulatory effects of BMOV are independent of PKB activity in vivo.

These findings led to the notion that BMOV may have more selective targets in the metabolic pathways. One potential pathway could be gluconeogenesis in the liver, since elevated hepatic glucose output is shown to be responsible for the fasting hyperglycemia in both types of diabetes. Hence, we tested the hypothesis that in vivo effects of vanadium may be mediated by changes in key gluconeogenic enzymes and inhibition of hepatic glucose output. STZ-diabetic rats were treated with BMOV in the drinking water (0.75-1 mg/ml) for 4 weeks or, for comparison, with insulin implants (4 U/day) for the final week of study. Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two key gluconeogenic enzymes, were measured in the liver and kidney, the main sites of endogenous glucose production. Treatment of STZ-diabetic rats with BMOV led to normalization of PEPCK enzyme activity and mRNA, and G6Pase mRNA levels in
both liver and kidney, indicating that hypoglycemic effects of BMOV in STZ-diabetic rats are at least partially mediated by its direct and/or indirect effects on PEPCK and G6Pase mRNA expression. BMOV had no detectable effect on the expression of PEPCK and G6Pase in either liver or kidney in the non-diabetic rats. Furthermore, insulin treatment of STZ-diabetic rats restored the elevated mRNA levels of PEPCK and G6Pase in both tissues.

In summary, results of this study demonstrated that: 1) In STZ-diabetic rats, both basal and insulin-stimulated PKBα activity were normal up to 9 weeks of diabetes, thus excluding a role for PKB in the development of insulin resistance in Type 1 diabetes. 2) In fatty Zucker rats, insulin-induced activation of PKBα (but not PKBβ) was markedly altered in the skeletal muscle and liver, indicating an association between PKBα activity and insulin resistance in this model. 3) Changes in PKBα activity were tissue specific implying that different mechanisms may be involved in the regulation of PKB. 4) The hypoglycemic effects of BMOV were at least partially mediated by its direct and/or indirect effects on PEPCK and G6Pase mRNA levels in STZ-diabetic rats via a PKB independent pathway.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Schemes</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xiv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xvii</td>
</tr>
<tr>
<td>Dedication</td>
<td>xviii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1. Diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Classification of diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>1.2.1. Type 1 diabetes</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2. Type 2 diabetes</td>
<td>3</td>
</tr>
<tr>
<td>1.3. Mechanisms of insulin resistance</td>
<td>5</td>
</tr>
<tr>
<td>1.3.1. Type 1 diabetes</td>
<td>5</td>
</tr>
<tr>
<td>1.3.2. Type 2 diabetes</td>
<td>6</td>
</tr>
<tr>
<td>1.4. Insulin</td>
<td>9</td>
</tr>
<tr>
<td>1.4.1. Metabolic actions of insulin</td>
<td>9</td>
</tr>
<tr>
<td>1.4.2. Insulin signal transduction</td>
<td>11</td>
</tr>
<tr>
<td>1.4.2.1. The insulin receptor</td>
<td>11</td>
</tr>
<tr>
<td>1.4.2.2. Insulin receptor substrates</td>
<td>13</td>
</tr>
</tbody>
</table>
1.4.2.3. Mitogen activated protein kinase (MAPK)-dependent pathway 15
1.4.2.4. Phosphatidylinositol 3-kinase (PI3-K)-dependent pathway 20
1.4.2.5. Regulation of protein kinase B (PKB) 24
1.4.3. Links between PKB and metabolic effects of insulin 27
1.4.4. Phosphoenolpyruvate carboxykinase (PEPCK) 28
1.4.5. Glucose-6-phosphatase (G6Pase) 29

1.5. Vanadium 31

1.5.1. Chemical and biological characteristics 31
1.5.2. Insulin-mimetic/enhancing effects of vanadium in vivo 32
1.5.3. Effects of vanadium on Type 1 diabetes 33
1.5.4. Effects of vanadium on Type 2 diabetes 34
1.5.5. Mechanism of action of vanadium 35

1.6. Other insulin sensitizing drugs: comparison with vanadium 37

1.6.1. History and pharmacokinetic properties 38
1.6.2. Anti-hyperglycemic effects of metformin 38

1.6.2.1. Effects of metformin on hepatic glucose output 39
1.6.2.2. Effects of metformin on peripheral glucose utilization 39
1.6.2.3. Effects of metformin on lipid metabolism 40
1.6.2.4. Effects of metformin on the insulin signaling pathway 41
1.6.2.5. How does vanadium differ from metformin 42

1.7. Experimental models 43

1.7.1. The streptozotocin (STZ) diabetic rat 43
1.7.2. The fatty Zucker rat 45
1.7.3. The Zucker diabetic fatty (ZDF) rat 46

1.8. Research strategy 47

1.8.1. Rationale 47

1.8.2. Hypotheses 48

2. MATERIAL AND METHODS

2.1. Materials 50

2.2. Experimental protocols 51

2.2.1. Studies on protein kinase B 51

2.2.1.1. Time course studies: STZ-diabetic rats and fatty Zucker rats (Study # 1) 51

2.2.1.2. Basal PKB activity in ZDF rats: comparison with fatty Zucker rats (Study # 2) 52

2.2.1.3. Effects of BMOV on PKB activity in STZ-diabetic rats (Short-term study) (Study # 3) 52

2.2.1.4. Effects of BMOV on PKB activity in STZ-diabetic rats (Long-term study) (Study # 4) 54

2.2.1.5. Effects of BMOV on PKB activity in Zucker rats (Study # 5) 54

2.2.2. Studies on the effects of BMOV on gluconeogenesis in STZ-diabetic rats (Study # 6) 55

2.3. Methods 57

2.3.1. Oral glucose tolerance test 57

2.3.2. Tissue extract preparation 57
2.3.3. PKB immunoprecipitation assay  58
2.3.4. Electrophoresis and immunoblotting  59
2.3.5. PEPCK enzyme assay  60
2.3.6. RNA extraction  61
2.3.7. Semi-quantitative transcriptase polymerase chain reaction  61
2.3.8. Plasma parameters  63
2.4. Statistical analyses  63

3. RESULTS

3.1. Studies on protein kinase B  66

3.1.1. Time course studies: STZ-diabetic rats and fatty Zucker rats  
(Study # 1)  66

3.1.2. Basal PKBα activity in ZDF rats: comparison with fatty Zucker Rats  (Study # 2)  76

3.1.3. Effects of BMOV on PKB activity in the STZ-diabetic rats 
(Short-term study) (Study # 3)  79

3.1.4. Effects of BMOV on PKB activity in the STZ-diabetic rats 
(Long-term study) (Study # 4)  80

3.1.5. Effects of BMOV on PKB activity in Zucker rats  
(Study # 5)  94

3.2. Effects of BMOV on gluconeogenesis in STZ-diabetic 
rats (Study # 6)  107

3.2.1. PEPCK activity and mRNA expression  108
3.2.2. G6Pase mRNA expression  109
4. DISCUSSION

4.1. Overview 120
4.2. Time course studies 122
4.3. Basal PKB activity in ZDF rats 123
4.4. PKB activity in STZ-diabetic rats 124
4.5. PKB activity in fatty Zucker rats 127
    4.5.1. Skeletal muscle 127
    4.5.2. Liver 130
4.6. Effects of BMOV treatment on PKB activity 133
4.7. Effects of insulin and BMOV on PEPCK and G6Pase in STZ-diabetic rats 137
    4.7.1. Effects of insulin on PEPCK and G6Pase 138
    4.7.2. Effects of BMOV on PEPCK and G6Pase 140
4.8. Summary 143

5. CONCLUSIONS 148

6. BIBLIOGRAPHY 151
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The subunit structure of the insulin receptor.</td>
<td>12</td>
</tr>
<tr>
<td>2. The major signaling pathways involved in mediating the physiologic</td>
<td>19</td>
</tr>
<tr>
<td>effects of insulin.</td>
<td></td>
</tr>
<tr>
<td>3. Potential sites of vanadium action.</td>
<td>147</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Optimization of PCR assay for liver and kidney.</td>
<td>65</td>
</tr>
<tr>
<td>3.1</td>
<td>PKBα activity in basal state or at different time points after insulin</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>injection in the skeletal muscle and liver of control and STZ-diabetic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wistar rats.</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>PKBα activity in basal state or at different time points after insulin</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>injection in the skeletal muscle and liver of lean and fatty Zucker rats.</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Comparison of PKBα activity in the skeletal muscle and liver in basal state</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>or after insulin injection.</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Basal and insulin-induced PKBβ activity in the skeletal muscle and liver</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>of Wistar rats and Zucker rats.</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Basal PKBα activity in skeletal muscle and liver of Wistar, Zucker fatty</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>and Zucker diabetic fatty (ZDF) rats.</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>Plasma glucose levels in different treatment groups at the beginning and</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>the end of the study: Wistar rats (Short-term study).</td>
<td></td>
</tr>
<tr>
<td>3.7</td>
<td>The effect of BMOV on basal and insulin-stimulated PKBα activity in the</td>
<td>87</td>
</tr>
<tr>
<td>3.8</td>
<td>Plasma glucose levels in different treatment groups at the beginning and</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>the end of the study: Wistar rats (Long-term study).</td>
<td></td>
</tr>
<tr>
<td>3.9</td>
<td>The effect of BMOV on basal and insulin-stimulated PKBα</td>
<td></td>
</tr>
</tbody>
</table>
activity in the skeletal muscle of STZ-diabetic rats after 8 weeks of treatment.

3.10. Plasma glucose levels in different treatment groups at the beginning and the end of the study and insulin sensitivity indices: Zucker rats (4-week study).

3.11. The effect of BMOV on basal and insulin-stimulated PKBα activity in the skeletal muscle of fatty Zucker rats after 3 weeks of treatment.


3.15 Effects of BMOV and insulin on PEPCK mRNA levels in the liver and kidney of control and STZ-diabetic rats.

3.16 Effects of BMOV and insulin on G6Pase mRNA levels in the liver and kidney of control and STZ-diabetic rats.
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.</td>
<td>Wistar rats (Short-term study): General characteristics of animals at the beginning and the end of study <em>(Study #3).</em></td>
</tr>
<tr>
<td>3.2.</td>
<td>Wistar rats (Short-term study): Plasma insulin levels during the study <em>(Study #3).</em></td>
</tr>
<tr>
<td>3.3.</td>
<td>Wistar rats (Long-term study): General characteristics of animals at the beginning and the end of study <em>(Study #4).</em></td>
</tr>
<tr>
<td>3.4.</td>
<td>Wistar rats (Long-term study): Plasma insulin levels during the study <em>(Study #4).</em></td>
</tr>
<tr>
<td>3.5.</td>
<td>Zucker rats (4-week study): General characteristics of animals at the beginning and the end of study <em>(Study #5).</em></td>
</tr>
<tr>
<td>3.6.</td>
<td>Zucker rats (4-week study): Plasma insulin levels at the beginning and the end of study <em>(Study #5).</em></td>
</tr>
<tr>
<td>3.7.</td>
<td>Wistar rats (PEPCK and G6Pase study): General characteristics of animals at the beginning and the end of study <em>(Study #6).</em></td>
</tr>
<tr>
<td>3.8.</td>
<td>Wistar rats (PEPCK and G6Pase study): Plasma glucose levels during the study <em>(Study #6).</em></td>
</tr>
<tr>
<td>3.9.</td>
<td>Wistar rats (PEPCK and G6Pase study): Plasma insulin levels during the study <em>(Study #6).</em></td>
</tr>
<tr>
<td>3.10.</td>
<td>Wistar rats (PEPCK and G6Pase study): Effects of BMOV on plasma glucagon and triglycerides <em>(Study #6).</em></td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMOV</td>
<td>Bis(maltolato)oxovanadium (IV)</td>
</tr>
<tr>
<td>CAP</td>
<td>c-Cbl-associated protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>4E-BP</td>
<td>4E-binding protein</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>Eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBPase</td>
<td>Fructose 1,6-bisphosphatase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GAB-1</td>
<td>Grb2-associated binder-1</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor binding protein 2</td>
</tr>
<tr>
<td>Grb10</td>
<td>Growth factor receptor binding protein 10</td>
</tr>
<tr>
<td>GS</td>
<td>Glycogen synthase</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HGO</td>
<td>Hepatic glucose output</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
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<td>---------</td>
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</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>ISI</td>
<td>Insulin sensitivity index</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH₂-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKK</td>
<td>MAP Kinase kinase</td>
</tr>
<tr>
<td>MKKK</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>ERK kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</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>p70⁷⁰ sk</td>
<td>p70 ribosomal S6 kinase</td>
</tr>
<tr>
<td>p90⁷⁰ sk</td>
<td>p90 ribosomal S6 kinase</td>
</tr>
<tr>
<td>PDK</td>
<td>3-Phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PDE3B</td>
<td>Phosphodiesterase type 3B</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PIF</td>
<td>PDK1-interacting fragment</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
</tbody>
</table>
PKA  cAMP-dependent protein kinase
PKB  Protein kinase B
PKC  Protein kinase C
PP-1  Protein phosphatase-1
PP1_G  Glycogen bound form of protein phosphatase-1
PP2A  Protein phosphatase 2A
PRK2  PKC-related kinase 2
PTB  Phosphotyrosine binding domain
PTPs  Protein tyrosine phosphatases
PTP1B  Protein tyrosine phosphatase 1B
Ras  Rat sarcoma protein
SAPK  Stress-activated protein kinases
SH2  Src homology-2 domain
SHIP  SH2-containing inositol 5-phosphatase
SHPS-1  SHP substrate-1
SHP2  SH2-domain containing protein tyrosine phosphatase 2
SIRP  Signal-regulatory protein
mSOS  Mammalian homologue of the Drosophila son of sevenless protein
Shc  Src homology/α-collagen protein
STZ  Streptozotocin
TNF-α  Tumor necrosis factor-α
ZDF  Zucker diabetic fatty
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DEDICATION

To my parents

who have been

a guiding light throughout my life

my source of strength and inspiration

whose love gave me encourage to continue my way

To my brother, Ali

for always being there for me and his continuous support

without which I would have never succeeded.
1.1. DIABETES MELLITUS

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. Because of its high incidence, diabetes has emerged as a serious public health issue in many countries. In the recent report of the World Health Organization (WHO) it has been predicted that the number of diabetic patients will rise to about 300 million by 2025 [2]. Chronic complications of diabetes associated with hyperglycemia, including retinopathy, neuropathy, nephropathy and atherosclerotic macrovascular disease are among the major causes of morbidity and mortality in diabetic patients [3, 4]. According to the revised criteria of American Diabetes Association (ADA) and WHO, diabetes is diagnosed by replicate fasting plasma glucose levels $\geq 126$ mg/dl (7 mM) and/or plasma glucose levels $\geq 200$ mg/dl (11.1 mM) at 2 h after ingestion of oral glucose (75 g) in the absence of symptoms, or a random plasma glucose level $\geq 200$ mg/dl in the presence of symptoms such as polyuria, polydipsia and weight loss [1, 5].

1.2. CLASSIFICATION OF DIABETES MELLITUS

The classification of diabetes mellitus was modified in the recent report of WHO and ADA [5, 6]. In the revised classification, the terms Type 1 and Type 2 which refer to pathologic mechanisms (immune-mediated and non-immune-mediated, respectively) were used instead of terms insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM), which
describe physiologic states (ketoacidosis-prone and ketoacidosis-resistant, respectively). Based on the new criteria, diabetes is classified into four etiological categories: (1) Type 1 (absolute insulin deficiency); (2) Type 2 (insulin resistance with an insulin secretory defect); (3) gestational diabetes; and (4) other specific types [1, 5].

1.2.1. Type 1 Diabetes

Type 1 diabetes is an autoimmune disease characterized by selective destruction of pancreatic β-cells in genetically prone individuals, and is strongly influenced by environment [1, 7, 8]. Immune dysregulation that usually precedes overt clinical onset of the disease involves both humoral (B cells) and cell-mediated (T cells) mechanisms [1, 7]. The presence of inflammatory lesions (insulitis) within the islets of diabetic patients and the finding that diabetes is transferable by CD4+ and CD8+ cells and can be inhibited by immunosuppressive agents in experimental models of diabetes, supports an important role for immune dysregulation in Type 1 diabetes [7]. Furthermore, autoantibodies to islet cells (ICAs), insulin (IAAs), glutamic acid decarboxylase (GAD65) and tyrosine phosphatase IA-2 and IA-2B are present in most Type 1 diabetic patients [6, 9]. The disease also has strong associations with class II Major Histocompatibility Complex (MHC) genes, with linkage to the DQA and B genes, and is influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective and indicate the importance of genetic factors in the pathogenesis of Type 1 diabetes [7, 8].

Although a genetic component and/or dysregulation of immune system are important for Type 1 diabetes to be expressed, it seems that health is maintained
until an environmental factor (e.g. viruses, toxins, nutrients) triggers the autoimmune response perhaps through a molecular mimicry mechanism (e.g., Coxsackie B virus and GAD65) or by direct damage of β-cells [1, 7].

The development of Type 1 diabetes can be divided into six stages: (1) genetic susceptibility; (2) triggering agents; (3) activation of autoimmunity; (4) decrease in glucose-induced insulin secretion; (5) overt diabetes associated with β-cell destruction; and (6) complete β-cell destruction [10]. Insulin deficiency associated with Type 1 diabetes results in a starvation-like state including (a) excessive hepatic and renal gluconeogenesis, (b) decreased peripheral utilization of glucose with glucosuria, (c) proteolysis in muscle liberating amino acids for gluconeogenesis and (d) uncontrolled lipolysis with formation of ketone bodies [11]. Since Type 1 diabetes is primarily caused by insulin deficiency, these patients are susceptible to ketoacidosis in the absence of exogenous insulin [1].

1.2.2. Type 2 Diabetes

Type 2 diabetes, the most common form of diabetes [5], is a polygenic disease caused by a combination of two physiological defects: insulin resistance and impaired insulin secretion [12, 13]. It appears that both of these defects are caused by primary (i.e., genetic), secondary (i.e., hyperglycemia-induced) and environmental (i.e., dietary) factors [12-16]. Insulin resistance and impaired insulin secretion are both required for diabetes to be expressed and in most cases the superimposition of obesity-related insulin resistance, upon a β-cell with a genetically limited capacity to compensate, leads to Type 2 diabetes [12, 13]. The etiology of changes observed in β-cells in Type 2 diabetic patients is not well understood.
These changes include an impairment in insulin secretion associated with a decrease in islet mass (probably due to amyloid deposition) and a defect in the conversion of proinsulin to insulin [12].

The importance of genetic factors in Type 2 diabetes is strongly supported by the finding that monozygotic twins demonstrate extremely high concordance rates (~90%) for the development of Type 2 diabetes [11]. Although several genes have been reported to cause rare syndromes associated with insulin resistance, these mutations do not appear to make an important contribution to the development of the common form of Type 2 diabetes. Maturity onset diabetes of the young (MODY) is the only form of diabetes in which a definite mode of inheritance has been determined [1, 5].

The development of Type 2 diabetes can be divided into three phases: 1) normoglycemic hyperinsulinemic state: during this phase peripheral insulin resistance, which develops relatively early in the course of disease, is compensated by a rise in the plasma insulin levels; 2) postprandial hyperglycemic state: the first phase is followed by a progressive impairment in the ability of the β-cells to secrete adequate insulin in order to compensate for the developing insulin resistance; and 3) onset of overt diabetes: In this phase insulin resistance in the liver and peripheral tissues (muscle and adipose tissue) combined with impaired insulin secretion from the β-cells results in severe hyperglycemia and decompensated diabetes [12, 13]. Unlike Type 1, Type 2 diabetes is usually associated with relatively mild hyperglycemia, and ketoacidosis seldom occurs spontaneously in Type 2 diabetic patients [1].
1.3. MECHANISMS OF INSULIN RESISTANCE

1.3.1. Type 1 Diabetes

The term insulin resistance, describes an impaired biological response to insulin [17] and is a major characteristic of both Type 1 [18, 19] and Type 2 diabetes [20, 21]. In contrast to Type 2 diabetes, insulin resistance in Type 1 seems to be a secondary phenomenon associated with chronic hyperglycemia [18]. However, the cellular mechanisms of impaired insulin action may be different at various stages of Type 1 diabetes [22]. Development of antibodies against exogenous insulin has been shown to decrease insulin action during the early stages of insulin therapy in Type 1 diabetic patients, while the insulin resistance observed in long-term diabetes appears to be associated with the chronic hyperglycemia [22]. Wide clinical use of recombinant human insulin during the recent years has significantly lowered the risk of development of the antibodies against insulin.

Studies have shown that hyperglycemia-induced peripheral insulin resistance in Type 1 diabetes is associated with down-regulation of GLUT4 in the plasma membrane in insulin responsive tissues [23-25] and a defect in non-oxidative glucose disposal (glycogen synthesis) in the muscle [18, 24], suggesting that both glucose-transport and post-transport abnormalities contribute to the impaired insulin action on carbohydrate metabolism [24]. It has been shown that the elevated levels of basal hepatic glucose production observed in Type 1 diabetic patients are suppressed by insulin administration, indicating that response to the inhibitory effects of insulin on hepatic glucose output is not impaired [22]. However, this finding cannot exclude the possibility that hepatic sensitivity to lower insulin
concentrations might be altered. As with muscle, it appears that in the liver glucagon-stimulated hepatic glucose production results, at least in part, from glucose toxicity [26].

1.3.2. Type 2 Diabetes

Insulin resistance in the peripheral tissues is one of the earliest detectable metabolic defects observed in Type 2 diabetes [17, 20, 27]. Resistance to insulin action at the level of the liver results in an increase in hepatic glucose production, while in the skeletal muscle it causes a decrease in glucose uptake [20], the rate-limiting step for glucose utilization [18]. Reduced level of glucose uptake in the muscle in Type 2 diabetes is associated with a decrease in glycogen synthesis [21, 28], the main pathway of glucose disposal in this tissue [29, 30]. The increased hepatic glucose output is a key factor responsible for fasting hyperglycemia, whereas decreased muscle glucose uptake and impaired suppression of hepatic glucose production play major roles in postprandial hyperglycemia in Type 2 diabetic patients [31].

The molecular mechanism(s) that cause insulin resistance in Type 2 diabetes are not yet entirely clarified and several factors have been proposed to contribute to the pathogenesis of this metabolic defect. Mutations in the insulin receptor gene have been reported in several uncommon syndromes of insulin resistance which result in (a) impaired receptor biosynthesis, (b) impaired transport of the receptors to the cell membrane, (c) decreased affinity of insulin binding, and (d) impaired tyrosine kinase activity [11]. However, these syndromes, due to their low incidence, do not seem to make a significant contribution to the development of insulin resistance in
the overall population. Several defects at insulin receptor and post-receptor levels have been reported in the skeletal muscle in Type 2 diabetes including a decrease in the number of insulin receptors [17], phosphorylation of insulin receptor β-subunit and insulin receptor substrate (IRS) proteins [32, 33], activation of PI3-K [34] and membrane translocation of glucose transporters [21]. Although it is not clear whether these defects are primary to the development of insulin resistance or are secondary to this metabolic disorder.

The vast majority of Type 2 diabetic patients are obese. A growing body of evidence indicates that obesity (central obesity) is closely associated with insulin resistance and the development of Type 2 diabetes [2, 13, 17]. Several factors released from adipose tissue have been proposed to link obesity to insulin resistance including tumor necrosis factor-α (TNF-α) [35], leptin [36] and the recently identified hormone, termed resistin [37]. There is evidence from recent studies indicating a role for TNF-α in the pathogenesis of insulin resistance. An increase in the expression of TNF-α in adipose tissue has been reported in different animal models of insulin resistance and Type 2 diabetes [38]. It has been shown that TNF-α decreases insulin-induced translocation of GLUT1 and GLUT4 and glucose uptake in adipocytes via activation of sphingomyelinases and the production of ceramides [35]. Leptin is another peptide released from adipocytes. Defects in leptin synthesis or leptin receptor function in rodents results in obesity, hyperinsulinemia and insulin resistance [36]. Effects of Leptin are mediated through the central nervous system (CNS) by suppression of food intake and an increase in the metabolic rate. In addition to its effects on CNS, leptin also acts directly on the peripheral tissues by
inhibition of insulin secretion and gene expression in pancreatic β-cells and stimulation of fatty acid oxidation in adipocytes [36, 39]. It was shown that leptin can mimic some of the anabolic actions of insulin such as increase in glucose uptake in muscle and adipose tissue, decrease in glycogenolysis and gluconeogenesis in the liver [36]. Recently, a new hormone was identified and proposed to link obesity and diabetes. This hormone, termed resistin, is produced by adipocytes and is shown to antagonize the action of insulin. It has been proposed that resistin mediates its effects via a resistin receptor in insulin responsive tissues [37].

Fatty acids released from adipose tissue have also been proposed as a link between obesity and insulin resistance [13, 40]. The phenomenon that free fatty acid oxidation inhibits glucose oxidation was first described in 1963 by Randle and co-workers, who named it the “glucose-fatty acid cycle”, now known as the Randle cycle [41, 42]. According to this theory, increased levels of fatty acids can interfere with glucose metabolism at the level of glycolysis, pyruvate oxidation, and glucose transport. Acetyl CoA and NADH, two products of fatty acid oxidation, are both competitive inhibitors of pyruvate dehydrogenase (PDH), the enzyme which catalyzes the oxidation of pyruvate produced during glycolysis [40, 43]. Furthermore, acetyl CoA which is produced by oxidation of fatty acids, combines with oxaloacetate to form citrate. Citrate is an inhibitor of phosphofructokinase-1 (PFK-1), a key regulatory enzyme in glycolysis, and an activator of acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis [40]. Inhibition of PFK increases the level of its substrate glucose-6-phosphate (G-6-P), which is also an allosteric inhibitor of hexokinase II [43]. Thus, fatty acid oxidation can cause
inhibition of the two kinases that catalyze regulatory reactions in the glycolytic pathway.

In the liver, increase in fatty acid oxidation promotes endogenous glucose production by elevating the levels of acetyl CoA, which in turn stimulates pyruvate carboxylase, a key gluconeogenic enzyme, and providing continued source of energy (ATP) and substrate to drive gluconeogenesis [40, 44]. Hence, fatty acids interfere with several steps in glucose metabolism in the liver and muscle resulting in peripheral insulin resistance.

1.4. INSULIN

1.4.1. Metabolic Actions of Insulin

Insulin is the main anabolic hormone in mammals, mediating its metabolic effects by stimulating the storage of carbohydrates, lipids and proteins [27]. The major pathways for glucose metabolism within the cell include glycogen synthesis, glycolysis and metabolism through the pentose cycle [30, 45, 46]. On the one hand, insulin stimulates glycogen formation by activating glycogen synthase, the rate-limiting enzyme in glycogen synthesis [47-49]. On the other hand, insulin inhibits glycogenolysis by inhibition of glycogen phosphorylase, the key enzyme in glycogen breakdown [40, 50]. Stimulatory effects of insulin on the glycolytic pathway in the insulin sensitive tissues are mediated via increasing the flux through the key enzymes that catalyze the three irreversible reactions in this pathway. These include hexokinase, phosphofructokinase-1 (PFK-1) and pyruvate kinase, the latter enzyme leads to formation of ATP [51, 52]. Gluconeogenesis, which is functionally the reverse of glycolysis, is inactivated by insulin through its inhibitory effects on the
activity and/or gene expression of the key metabolic enzymes that catalyze the irreversible steps in this pathway including fructose 1,6-bisphosphatase, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) [53-55]. Furthermore, insulin activates the pentose cycle, a side pathway which leads to synthesis of NADPH, and provides ribose for the synthesis of RNA [46].

Triglyceride is the major form in which fuel is stored in the human body. Catecholamines stimulate lipolysis by increasing intracellular cAMP and activation of cAMP-dependent protein kinase (PKA), which leads to stimulation of hormone-sensitive lipase, the rate-limiting enzyme in intracellular triglyceride hydrolysis [56]. Glycerol derived from hydrolysis of triglycerides undergoes further metabolism and is converted to glucose by gluconeogenesis in the liver, while fatty acids can either be metabolized in peripheral tissues or converted to ketone bodies within the liver [46]. Insulin inhibits lipolysis by activating phosphodiesterase type 3B (PDE3B), leading to a decrease in cAMP levels and concomitant decrease of PKA activity [56, 57]. Pyruvate dehydrogenase (PDH), the enzyme which catalyzes the conversion of pyruvate into acetyl CoA, is another target for metabolic regulation by insulin. Its activation by insulin favors the synthesis of fatty acids and triglycerides in the liver and adipose tissue, while in the skeletal muscle activation of PDH provides acetyl CoA for oxidation in the Krebs cycle [43, 58].

Insulin plays an important role in cell growth and differentiation by stimulation of amino acid uptake and protein synthesis and inhibition of protein degradation [30, 45]. A major protein that is regulated by insulin is eukaryotic initiation factor 4E (eIF-4E), which binds eIF-4A and eIF-4G to form the so-called cap binding complex
which initiates mRNA translation. eIF-4E is negatively regulated by 4E-binding protein (4E-BP)1/2 which binds to it and prevents the formation of the cap binding complex [30, 45]. Insulin promotes formation of this complex and mRNA translation by phosphorylation and release of 4E-BP from eIF-4E [59]. Furthermore, insulin regulates expression of many genes through both positive (e.g. glucokinase) and negative (e.g. PEPCK and G6Pase) effects [54, 55].

The metabolic actions of insulin are opposed by several counterregulatory hormones including epinephrine, glucagon, growth hormone, and cortisol, among which glucagon plays the most important acute role. Growth hormone and cortisol appear to be more important during chronic hypoglycemia rather than in acute situations and epinephrine does not play a major role in the presence of glucagon [60]. Glucagon acts primarily on the liver by stimulating its G protein-coupled receptors on the surface of hepatocytes, promoting glycogenolysis and gluconeogenesis via activation of PKA [50, 60].

1.4.2. Insulin Signal Transduction

1.4.2.1. The Insulin Receptor

The biological effects of insulin are initiated by activation of its receptor on the surface of the cell. Insulin receptors are present in virtually all the vertebrate tissues, although the concentration varies from as few as 40 to more than 200,000 receptors per cell [61]. This receptor is a heterotetrameric glycoprotein (350,000 Da) consisting of 2α and 2β subunits, which are held together by disulfide bonds and non-covalent interactions [62, 63]. The α-subunit is entirely extracellular, whereas the β-subunit contains an extracellular portion, a transmembrane domain, and an
intracellular part that includes a tyrosine domain [63, 64]. Binding of insulin to its receptor removes the inhibitory effect of the α-subunit upon the receptor tyrosine kinase leading to transphosphorylation of at least 7 tyrosine residues in the intracellular domain of the β-subunit [61, 62, 65]. These tyrosine residues are located in three clusters referred to as juxtamembrane domain, activation loop and C-terminal domain. Phosphorylation of three tyrosine residues in the activation loop leads to a conformational change permitting the active site of the receptor to bind its substrates [61]. Furthermore, phosphorylation of a motif containing Tyr$^{972}$ in the juxtamembrane domain creates a binding site for phosphotyrosine binding (PTB) domains of insulin receptor substrates (IRS) and Src homology/α-collagen (Shc) proteins. Finally, phosphorylation of a motif containing Tyr$^{1334}$ in the C-terminal domain provides a binding site for Src homology 2 (SH2) domains in the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase [61, 66, 67].

Scheme 1. The Subunit Structure of the Insulin Receptor (Adapted from Ref 11)
Several pathways have been proposed to terminate the insulin-induced activation of the receptor. It has been suggested that phosphorylation of serine/threonine sites in the insulin receptor may lead to its inactivation [61, 68]. Although these phosphorylation sites are not well characterized, there is convincing evidence indicating that phosphorylation of serine/threonine residues in the insulin receptor by several protein kinase C (PKC) isoforms leads to inhibition of tyrosine kinase activity, indicating a potential role for PKC in terminating the insulin signals [68]. Recent studies indicate that protein tyrosine phosphatase (PTP) 1B, which becomes activated via tyrosine phosphorylation by the insulin receptor, is involved in deactivation of the receptor tyrosine kinase by initiating its dephosphorylation [68, 69]. The importance of PTP1B in the regulation of insulin receptor activity is supported by the finding that knockout mice lacking PTP1B exhibits enhanced insulin sensitivity [70]. Furthermore, it has been reported that phosphorylation of tyrosine residues in the insulin receptor initiates its release from a microvillus domain of the plasma membrane. This results in endocytosis of the insulin receptor complex through a clathrin-coated pit mechanism and insulin degradation, while most of the unoccupied receptors recycle to the plasma membrane [71].

1.4.2.2. Insulin Receptor Substrates

During the past decade several proteins have been identified which are directly phosphorylated by insulin receptor tyrosine kinase. However, the physiological role of some of them in mediating the effects of insulin is still not clear. Two major classes of insulin receptor substrates have been identified including PTB-containing substrates (IRS and Shc proteins) and integral membrane protein
substrates [11, 61, 72, 73]. Three integral membrane proteins are known so far: signal-regulatory protein (SIRP), pp120/HA4 and SHP substrate-1 (SHPS-1). These proteins consist of an extracellular domain containing an “immunoglobulin-like loop”, a single transmembrane domain, and a short intracellular domain containing sites for tyrosine phosphorylation [11, 72, 73]. Unlike their structure, the physiological importance of these proteins is not well understood.

Among insulin receptor substrates, IRS proteins are the main family mediating intracellular insulin signaling. Since the discovery of IRS-1 [74], three other members of this family have been identified namely IRS-2 [75], IRS-3 [76] and IRS-4 [77]. While there is considerable evidence for a direct role of IRS-1 and IRS-2 in insulin’s metabolic actions, the roles of IRS-3 and IRS-4 are less clear [45]. Studies using knockout mice have provided direct evidence that lack of IRS-1 and IRS-2 results in insulin resistance and diabetes, respectively [78, 79]. IRS-1 and IRS-2 are widely distributed, while IRS-3 has been demonstrated in adipocytes, hepatocytes, fibroblasts and IRS-4 has been observed only in cultured embryonic kidney cells [45]. IRS proteins consist of a phosphotyrosine binding (PTB) domain recognizing the NPXY motif at the insulin receptor juxtamembrane region, an N-terminal pleckstrin-homology (PH)-domain which can bind to membrane phospholipids, and a C-terminal domain that contains multiple tyrosine phosphorylation sites that can bind to SH2 domains [45, 68]. IRS-1 is a 185 kDa protein which contains 21 potential tyrosine phosphorylation sites [61]. At least 8 tyrosines in IRS-1 undergo phosphorylation by the activated insulin receptor [71, 80]. The molecular mechanism by which IRS proteins interact with the tyrosine phosphorylated
cytoplasmic tail of the insulin receptor is not clear and it has been suggested that this process might be facilitated by the cytoskeletal elements in the cell [81]. Similar to the insulin receptor, IRS proteins are phosphorylated on serine residues by many proteins including PKC, MAPK, GSK-3, mTOR and PI3-K which negatively regulate their function and PKB which has a positive regulatory effect [68, 82, 83]. IRS proteins bind several SH2 containing substrates including PI3-K and growth factor receptor binding protein 2 (Grb2) which initiate two main insulin-signaling cascades, referred to as the phosphatidylinositol 3-kinase (PI3-K) pathway and mitogen activated protein kinase (MAPK) pathway, respectively. Shc proteins are thought to be involved in the activation of MAPK cascade by several growth factor and cytokine receptors including the insulin receptor [61, 68].

Besides the two mentioned families, there are also other proteins that act as substrates for the insulin receptor such as growth factor receptor binding protein-10 (Grb-10/Grb-IR), and Grb2-associated binder-1 (GAB-1) [45, 68]. Although these proteins all contain SH2- and PH-domains through which they can bind to the insulin receptor and membrane phospholipids, their functions are not yet well characterized. Grb-IR seems to inhibit insulin signaling at the level of IRS-1 phosphorylation and PI3-K activation [68]. Also, GAB-1 is reported to direct insulin signal to PI3-K and the protein tyrosine phosphatase 2 (SHP2) [45, 68].

1.4.2.3. Mitogen Activated Protein Kinase (MAPK)-Dependent Pathway

MAP kinases are common participants in signal transduction pathways from the membrane to the nucleus and hence are known to be important in mediating the mitogenic effects of insulin on DNA synthesis and cell growth [84-86]. The MAPK
pathway is activated by insulin following the phosphorylation of IRS proteins. Phosphorylated IRS and Shc proteins can bind to SH2 domains of downstream signaling molecules such as PI3-K, Grb2 and SHP2. Activation of PI3-K initiates the PI3-K-dependent pathway, while activation of Grb2 initiates the MAPK-dependent pathway. The role of phosphorylation and activation of SH2-domain containing protein tyrosine phosphatase 2 (SHP2) in the insulin signaling is less clear [30, 85].

Grb2 is a small cytoplasmic protein devoid of enzymatic activity, which consists of one SH2 domain that binds to IRS or Shc proteins and two SH3 domains [85]. The Src homology-3 (SH3) domain of Grb2 binds to the proline rich sequences in a guanine nucleotide exchange protein named son of sevenless (SOS), which promotes binding of GTP to p21\textsuperscript{ras}, in exchange for release of GDP, thereby activating this protein [61, 80, 85]. P21\textsuperscript{ras} is a small molecular weight G-protein that indirectly leads to the phosphorylation and activation of Raf or MAPK kinase kinase (M KK K K ), which subsequently phosphorylates and activates MAP kinase kinase (M K K ) or ERK kinase (MEK). MEK is a dual specificity protein kinase that catalyzes the phosphorylation of tyrosine and threonine residues on MAP kinases leading to their activation [30, 85, 86].

The details of mechanism(s) by which activated p21\textsuperscript{ras} directs Raf activation are still not clear. Although the interaction between these two proteins is necessary for the recruitment of Raf to the plasma membrane, it does not seem to be enough for its full activation, indicating that additional mechanism(s) are involved in the activation of Raf [30, 86]. It has been proposed that phosphorylation by protein kinase C, tyrosine kinases, and the p21-activated protein kinase PAK as well as
binding to a group of proteins termed 14-3-3 proteins are involved in the activation of Raf [86].

MAP kinases, also known as extracellular signal-regulated kinases (ERKs) have several isoforms, which are categorized into at least three distinct groups including ERKs (ERK1-ERK5 and ERK7), c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), and the p38 group of protein kinases (α, β, γ/ERK6/SAPK3 and δ) [86, 87]. Among MAPK isoforms, ERK1 and ERK2 (also known as p44 and p42, respectively) are the most studied isoforms of this family and are shown to be activated by insulin both in vitro [88-90] and in vivo [91-93]. Activated MAPK translocates into the nucleus, where it is thought to phosphorylate transcription factors, while a large fraction of the activated enzyme remains in the cytoplasm [80].

Although many proteins have been shown to act as substrates for MAPKs only a few of them have been shown to be in vivo substrates including several transcription factors such as c-Myc, c-fos, c-jun, ATF-2, Elk-1 and 90-kDa ribosomal S6 kinase II (p90<sup>rsk</sup>) [30]. P90<sup>rsk</sup> is one of the important substrates of MAPK since it phosphorylates transcription factors such as cAMP-response element binding protein (CREB), which is critical for the expression of immediate early genes [30]. P90<sup>rsk</sup> contains two protein kinase domains in a single polypeptide, named C-terminal and N-terminal kinase domains, which are phosphorylated by MAPK on Thr<sup>574</sup> and Ser<sup>364</sup> respectively, resulting in its activation [94].
Scheme 2.

The major signaling pathways involved in mediating the physiologic effects of insulin. Two main pathways are activated following the stimulation of insulin receptor referred to as the PI3-K-dependent pathway and MAPK-dependent pathway. Activation of the insulin receptor by insulin leads to phosphorylation of tyrosine residues on insulin receptor substrates (IRSs). These phosphorylated tyrosine residues in turn interact with SH2 domain containing proteins such as PI3-K and Grb2. Activation of PI3-K leads to the activation of PDK1/2, which results in phosphorylation of PKB. Activation of PKB initiates several metabolic pathways including glycogen synthesis, protein synthesis and glucose uptake. It is believed that most of the metabolic actions of insulin are mediated by PI3-K via PKB.
1.4.2.4. Phosphatidylinositol 3-Kinase (PI3K)-Dependent Pathway

Several studies have indicated that most of the metabolic effects of insulin are mediated via a signaling cascade which is initiated by the activation of PI3-K referred to as the PI3-K-dependent pathway [30, 45]. PI3-K is a heterodimeric enzyme, composed of two subunits, a 110 kDa catalytic (p110) and an 85 kDa regulatory (p85) subunit with two SH2 domains through which it binds to IRS proteins following insulin stimulation [45, 71, 95]. Three highly homologous isoforms of the catalytic subunit (i.e. p110α, p110β and p110δ) and several isoforms of the regulatory subunit (i.e. p85α, p85β, p55α and p50α and p55Pik) for PI3-K have been described [30, 95]. Class I PI3-kinases phosphorylate PtdIns-4-P or PtdIns(4,5)P₂ on the free 3'-position leading to the formation of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ which mediate the effects of PI3-K on its downstream enzymes [45, 95]. In addition to their lipid kinase activity, class I PI3-kinases also possess an intrinsic protein serine kinase activity which results in autophosphorylation of the serine residues in the enzyme associated with a decrease in the lipid kinase activity of the complex in vitro [95].

Insulin-induced activation of PI3-K and the subsequent increase in its PIP₃ products leads to phosphorylation and activation of two downstream enzymes, 3-phosphoinositide-dependent kinase (PDK1/PDK2) and protein kinase B (PKB) [30, 45, 96]. PKB is a 60 kDa serine/threonine protein kinase containing a pleckstrin homology (PH)-domain. This enzyme was originally identified and cloned in 1991 as a kinase with sequence similarity to cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) and was thus named PKB or RAC for "related to A and C
kinase" [97, 98]. Furthermore, this enzyme was also found to be a cellular homologue of the protooncogene v-Akt, so PKB is also known as c-Akt [99]. Three isoforms of mammalian PKB have been identified and are known as PKBα (Akt1) [97], PKBβ (Akt2) [98, 100] and PKBγ (Akt3) [101]. All isoforms of PKB possess an N-terminus PH-domain (amino acids 1-106), followed by a catalytic domain (amino acids 148-411) and a short C-terminus tail (amino acids 412-480) [102, 103]. Insulin activates PKBα by its phosphorylation on Thr\textsuperscript{308} in the activation loop and Ser\textsuperscript{473} at the C-terminus [104-106]. PKBβ (β1 and β2) appears to be regulated in a similar manner by phosphorylation on two sites (Thr\textsuperscript{309} and Ser\textsuperscript{474}) [103, 105, 107]. While rat PKBγ is missing the second serine residue and is activated by phosphorylation on only Thr\textsuperscript{305}, raising the idea that it might be regulated by a distinct mechanism [30, 103]. However, human PKBγ was recently cloned and two regulatory phosphorylation sites corresponding to Thr\textsuperscript{308} and Ser\textsuperscript{473} in PKBα were identified in this isoform, indicating that PKBγ in humans is also regulated similarly to PKBα and PKBβ [108, 109]. Phosphorylation of both residues is required for the maximum activation of PKB [104, 110]. In addition, insulin-induced activation of PKB is associated with its rapid translocation to the plasma membrane [111-115].

PDK1 is a ubiquitously expressed 556-amino acid protein that contains an N-terminus kinase domain and a C-terminus PH-domain which binds with high affinity to PI(3,4,5)P\textsubscript{3} and to a lesser extent to PI(3,4)P\textsubscript{2} [30, 96, 116]. This enzyme was identified a few years ago and introduced as the kinase responsible for phosphorylating Thr\textsuperscript{308} on PKBα [117, 118]. Shortly after that, it was found that PDK1 also phosphorylates the corresponding site of PKBβ (Thr\textsuperscript{309}) and PKBγ
PDK2, the kinase proposed to phosphorylate Ser\textsuperscript{473} on PKB\textsubscript{α} (or Ser\textsuperscript{474} on PKB\textsubscript{β}) is still not cloned. Integrin-linked kinase (ILK) has been suggested as a candidate for PDK2, since it is activated by insulin via a PI3-K-dependent manner and is able to phosphorylate PKB on Ser\textsuperscript{473} \textit{in vitro} [30, 116]. However, the recent finding that interaction of PDK1 with the carboxy-terminus region of PKC-related kinase (PRK2), termed PDK1-interacting fragment (PIF), converts PDK1 to an enzyme capable of phosphorylating both Thr\textsuperscript{308} and Ser\textsuperscript{473} residues in PKB \textit{in vitro}, indicates that PDK1 might be responsible for phosphorylation of these residues in vivo [106, 116]. In addition to PKB, PDK1 also phosphorylates and activates other proteins including PKA, a range of PKC isoforms, p70\textsuperscript{S6K} and serum- and glucocorticoid-induced protein kinase (SGK) [96, 119].

The current opinion is that PI3-K generates PIP\textsubscript{3} which recruits PDK1, PDK2, as well as PKB to the cell membrane via the PH-domain in these proteins [30, 45, 103]. PIP\textsubscript{3} also activates PDK1 and PDK2 to phosphorylate Thr\textsuperscript{308} and Ser\textsuperscript{473} respectively, on PKB to fully activate this kinase. Furthermore, interaction of PIP\textsubscript{3} with the PH-domain of PKB results in a conformational change, which removes the auto-inhibitory effect of the PH-domain on Thr\textsuperscript{308} and exposes this site to PDK1 leading to its phosphorylation [103, 110, 120].

One of the important downstream enzymes of PKB in mediating the metabolic effects of insulin is glycogen synthase kinase-3 (GSK-3) [102]. However, this enzyme is also subject to phosphorylation by several other enzymes such as PKC, PKA, p70\textsuperscript{S6K} and p90\textsuperscript{RSK} [102, 121, 122]. Insulin stimulation leads to inhibition of both GSK-3 isoforms by inducing the phosphorylation of GSK-3α on Ser\textsuperscript{21} and GSK-
PKB-induced phosphorylation (inactivation) of GSK-3β leads to decreased phosphorylation and consequently activation of glycogen synthase (GS), the rate-limiting enzyme in glycogen synthesis [47, 49, 124]. Glycogen synthase is activated by insulin via dephosphorylation of at least four regulatory serine residues in both the N- and C-termini, while inhibition by GSK-3 cannot account for insulin-mediated dephosphorylation of the N-terminal sites (2 and 2a) [47]. Also, inactivation of GSK-3 alone, is not enough to completely activate GS, revealing that additional mechanism(s) are involved in the regulation of GS via its dephosphorylation. Protein phosphatase-1 (PP-1) is a serine/threonine phosphatase, which has been suggested to contribute to the dephosphorylation of this enzyme [47, 124]. It has been shown that insulin activates PP1G, the glycogen-bound form of type 1 protein phosphatase, in both L6 rat skeletal muscle cells and adipocytes by phosphorylation of its regulatory subunit. Dephosphorylation catalyzed by PP-1 activates glycogen synthase and simultaneously inactivates phosphorylase a and phosphorylase kinase promoting glycogen synthesis [125]. Indeed the importance of PP-1 as a regulatory mechanism for GS varies with cell type [45, 125]. Also, PP1G itself is regulated by different pathways including activation by its phosphorylation via insulin-stimulated protein kinase (ISPK), c-Jun NH₂-terminal kinase (JNK) and PI3-K and inhibition by cAMP agonists and TNF-α [47, 125].

In addition to GSK-3, several other physiological substrates have been identified for PKB, which are also important targets for insulin indicating an important role for PKB in insulin signaling. These substrates include 6-phosphofructo-2-kinase.
(PFK-2) [126] and, Bcl2/BclXₐ-associated dead factor (BAD) [103], glucose transporter GLUT4 [127], 4E-binding protein-1 (4E-BP1) [59] and phosphodiesterase 3B (PDE3B) [57]. The phosphorylation of BAD, a pro-apoptotic Bcl-2 family member, by PKB promotes its dissociation from Bcl2/BclXₐ, which prevents activation of the caspase protease cascade resulting in the protection of the cell from apoptosis [116]. Recently it was reported that PKB could regulate the level of nitric oxide (NO) through direct phosphorylation and activation of endothelial NO synthase (eNOS) [128]. P70 ribosomal S6 kinase (p70^{S6K}), a 70 kDa protein kinase that phosphorylates ribosomal protein S6 is another enzyme suggested to lie downstream of PKB. This enzyme undergoes multi-site phosphorylations [45]. Although expression of constitutively active forms of both PI3-K and PKB have been shown to increase p70^{S6K} activity [129, 130], direct phosphorylation of this enzyme with PKB has not yet been reported, and it has been proposed that mTOR, which is thought to lie downstream of PKB is involved in activation of p70^{S6K} [45, 96]. However, PDK1 is shown to directly phosphorylate p70^{S6K} at the residue in the kinase catalytic domain equivalent to T⁳⁰₈ of PKBα, which results in activation of this enzyme. Therefore, PDK-1 appears to play a central role in mediating insulin-stimulated activation of this enzyme [45, 105, 131].

1.4.2.5. Regulation of Protein Kinase B (PKB)

Many studies have shown that insulin activates PKB via a PI3-K-dependent pathway both in vitro [49, 132] and in vivo [105]. However, PKB is also shown to be activated via several PI3-K-independent pathways including cellular stress,
cAMP/PKA system and activation of G protein-coupled receptors, indicating that in vivo regulation of this kinase may be far more complex [133-136].

It has been reported that PKB is activated by cellular stresses such as heat shock and hyperosmolarity through association with HSP27 [133, 134]. Furthermore, heat shock induces association of PKCδ with the PH-domain of PKB, although the significance of this finding is not clear [103, 133]. Another PI3-K-independent pathway involved in the activation of PKB is through the cAMP/PKA system [135, 137]. The cAMP-elevating agents such as forskolin, chlorphenylthio-cAMP, prostaglandin E1, and 8-bromo-cAMP have been shown to activate PKB through PKA but to a lesser extent than insulin [116]. The PH-domain of PKB is not necessary for this activation, although the phosphorylation of Thr^{308} (but not Ser^{473}) is required [116, 137]. The mechanism by which PKA activates PKB is not clear but is unlikely to involve direct phosphorylation, as it is not dependent on the putative PKA site in PKB [135, 137]. Furthermore, isoproterenol, a β-adrenergic agonist, was shown to activate PKB via a PI3-K-independent pathway [138]. Although activation of MAPK-activated protein (MAPKAP) kinase-2 by cellular stress, IL-1 or TNF-α also results in phosphorylation of PKB on Ser^{473} in vitro, this kinase does not appear to be a physiologically relevant Ser^{473} kinase, since inhibition of MAPKAP kinase-2 does not prevent activation of PKBα by insulin [104]. Recently another mechanism was reported for activation of PKB, which involves compounds acting through G protein-coupled receptors [136, 139, 140]. Regulation of PKB by G-proteins is complex and poorly understood. It has been suggested that G-proteins regulate PKB by two distinct and potentially opposing mechanisms: activation by Gβγ
heterodimers in a PI3-K-dependent manner and inhibition mediated by the G\(\alpha_q\) subunit [140]. The finding that class 1B PI3-K can be stimulated by the \(\beta\gamma\) subunit of G-proteins indicates a role for PI3-K in G-protein regulation of PKB [136, 140]. Finally, it has been reported that in vitro, Ca\(^{2+}\)/calmodulin-dependent kinase activates PKB directly through phosphorylation of T\(^{308}\) [116]. Although a very recent study has demonstrated that over expression of full-length protein kinase C-related kinase-1 (PRK1/PKN) or PRK2 inhibit PDK1-mediated PKB phosphorylation in vitro, the significance of this finding in vivo remains unclear [106].

Similar to its activation, several mechanisms have been proposed for inactivation of PKB, involving both direct and indirect effects of protein phosphatases. Protein phosphatase 2A (PP2A) is a serine/threonine protein phosphatase, which has been shown to dephosphorylate and therefore, inactivate PKB and hence may be a key enzyme in the regulation of PKB both in vitro and in vivo [116, 141]. In vitro studies have demonstrated that insulin inactivates PP2A by its phosphorylation via a wortmannin/rapamycin sensitive pathway indicating that both PI3-K and MAPK pathways are involved in the regulation of this phosphatase [142].

Recently a tumor suppressor with sequence homology to protein tyrosine phosphatases referred to as PTEN was identified and suggested to contribute to the negative regulation of PKB [143]. The PTEN gene encodes a 403-amino acid polypeptide with high degree homology to other protein tyrosine phosphatases and tensin [143, 144]. PTEN dephosphorylates position 3 of PtdIns(3,4,5)P\(_3\) and hence negatively regulates intracellular levels of PIP\(_3\) [145]. In vitro studies have demonstrated that both phosphorylation and activation of PKB is increased in PTEN
deficient cells indicating a role for PTEN in the inactivation of PKB [144]. SH2-containing inositol 5-phosphatase (SHIP) is another negative regulator of PKB. SHIP converts PtdIns(3,4,5)P$_3$ and PtdIns(1,3,4,5)P$_4$ to PtdIns(3,4)P$_2$ and PtdIns(1,3,4)P$_3$, respectively [96, 146]. Two well-studied isoforms of SHIP have different tissue distributions; SHIP1 is only expressed in hematopoietic cells, while the recently cloned SHIP2 is expressed in insulin-responsive tissues [116]. Studies with SHIP deficient mice have shown that SHIP is a critical negative regulator of growth factor-mediated activation of PKB [146].

1.4.3. Links between PKB and Metabolic Effects of Insulin

There is a growing body of evidence from in vitro studies showing that the regulatory effects of insulin on several metabolic steps including stimulation of glucose uptake, glycogen and protein synthesis, and inhibition of lipolysis and gluconeogenesis require PKB activation [30, 45]. Glucose uptake, the rate-limiting step in glucose utilization, is stimulated by insulin via enhancing the membrane translocation of glucose transporter GLUT4 through a PI3-K-dependent pathway [127, 147-151]. Many studies have shown an important role for both PKB$_\alpha$ and PKB$_\beta$ in insulin-induced GLUT4 translocation to the plasma membrane [127, 147-152]. These findings have been supported by a recent study on mice lacking PKB$_\beta$ that has provided in vivo evidence for a critical role of PKB$_\beta$ in the muscle glucose uptake [153].

It is well documented that glycogen synthesis, the main pathway of glucose disposal in peripheral tissues, is promoted by insulin through the PI3-K/PKB/GSK-3 axis [48, 49, 132]. Furthermore, in the heart the stimulatory effect of insulin on PFK-
2, a key glycolytic enzyme, has been shown to be mediated via a PI3-K-dependent pathway involving PKB [53, 126]. In addition, evidence from in vitro studies indicate that the inhibitory effects of insulin on PEPCK and G6Pase are at least partially transmitted by PKB [154, 155], although these inhibitory effects of PKB have not yet been investigated in vivo.

Recent studies have indicated that PKB is involved in mediating the anti-lipolytic effects of insulin via activation of the insulin sensitive phosphodiesterase - 3B (PDE3B) leading to the inhibition of hormone sensitive lipase (HSL) [56, 57]. Many studies support an important role for PKB in insulin-stimulated protein synthesis [156, 157]. It has been suggested that PKB and mTOR may work in concert to mediate insulin-induced phosphorylation of 4E-BP1 which results in its dissociation from eIF-4E and hence initiates protein translation by promoting the formation of cap binding complex [59].

This thesis has focused on the two key gluconeogenic enzymes, PEPCK and G6Pase, as potential in vivo substrates for PKB.

1.4.4. Phosphoenolpyruvate Carboxykinase (PEPCK)

Phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (PEP), plays a crucial role in gluconeogenesis. PEPCK has no known allosteric modifiers and its activity is principally controlled at the level of gene expression [55]. This enzyme is expressed primarily in the liver, kidney cortex, small intestine, and adipose tissue, although low levels of the enzyme have been detected in other tissues [55, 158]. The two known isoforms of PEPCK, namely mitochondrial (PEPCK-M) and cytosolic (PEPCK-C) are
immunochemically distinct but have similar molecular masses (~ 69 kDa) and kinetic properties [55, 159]. The mitochondrial isoform (PEPCK-M) is constitutively expressed, while the cytosolic form (PEPCK-C) is tightly regulated by hormonal and dietary factors, and is the form of this enzyme that is elevated in diabetes [55, 160]. In the liver, PEPCK expression is enhanced by glucagon (acting via cAMP), glucocorticoids, thyroid hormone, and fasting, while insulin and high-carbohydrate diet decrease PEPCK-C synthesis [55, 161]. In the kidney, transcription of the PEPCK-C gene is stimulated by glucocorticoids, fasting and metabolic acidosis [55, 161, 162]. Physiological suppressors of basal renal PEPCK expression have not yet been described and insulin, the main regulator of PEPCK in the liver, has been reported to have no effect on PEPCK in the kidney [161, 163]. PEPCK enzyme activity and mRNA levels are elevated in the liver and kidney of most animal models of diabetes and insulin resistance [161-166].

1.4.5. Glucose-6-Phosphatase (G6Pase)

Glucose-6-phosphatase (G6Pase) (EC 3.1.3.9) catalyzes the hydrolysis of glucose-6-phosphate to glucose, the last step of both hepatic gluconeogenesis and glycogenolysis and hence plays a critical role in endogenous glucose production. Furthermore, this enzyme is capable of catalyzing the hydrolysis of inorganic pyrophosphate (PPI) as well as the synthesis of glucose-6-phosphate (G-6-P) via phosphotransferase activity [54]. It is mainly expressed in the liver, kidney, small intestine and pancreas [54, 167]. G6Pase is thought to be a multicomponent protein complex, which is composed of a set of discrete proteins residing in the endoplasmic reticulum (ER), including a G-6-P translocase that transports G-6-P into the lumen of
the ER, a phosphohydrolase catalytic subunit (P36) residing in the lumen, and putative glucose and inorganic phosphate transporters that allow exit of the products of the reaction [54, 167]. Currently, there are two proposed concepts of structure-function relationship for G6Pase. The first model termed "substrate transport-catalytic unit" model was suggested [168] and developed [169] by Arion and co-workers in 1975. According to this model, G6Pase consists of a fairly nonspecific phosphohydrolase/phosphotransferase catalytic unit with its active site located on the luminal side of the ER and at least four transmembrane spanning translocases. Associated translocases (T1, T2β, T2α and T3) confer specificity to this system by allowing selective substrates/products access to or egress from the sequestered catalytic unit [54]. The second model termed "combined conformational flexibility-substrate transport" model was first introduced by Schulze and co-workers [170] in 1986 and modified by van de Werve and co-workers [171] in 1995. This model depicts G6Pase as a multifunctional enzyme embedded deep within the ER possessing both catalytic and substrate/product transport activities [54]. Although both models have received support over the years, neither fully explains all of the characteristics of G6Pase. G6Pase activity is regulated by various hormones mainly at the transcriptional level. Activity and mRNA levels of the catalytic subunit of G6Pase, are increased by glucose, fatty acids, fasting, glucocorticoids and glucagon (via cAMP) [54, 167, 172-174], whereas insulin strongly inhibits both basal and glucocorticoid-induced G6Pase gene expression [54, 167, 175, 176]. In several animal models of diabetes G6Pase mRNA and activity are elevated in the liver and kidney [166, 172, 177, 178].
1.5. VANADIUM

1.5.1. Chemical and Biological Characteristics

Vanadium is a group 5 transition metal which has several oxidation states. Under physiological conditions vanadium predominantly exists in either an anionic form [vanadate (VO$_3^-$): oxidation state, +5] or a cationic form [vanadyl (VO$_2^{+}$): oxidation state, +4] [179, 180]. The plasma concentration of vanadium in humans is about 20 nM, and the total body pool is estimated to be about 100-200 μg [179]. In animals fed laboratory chow, the plasma vanadium is in the range of 0.1-1.0 μM [181]. The predominant form of vanadium in plasma is vanadate and about 90% of plasma vanadium is bound to proteins mainly albumin and transferrin [180, 181]. Vanadium ions appear to enter cells via the anion transport system in a similar manner to phosphate [180]. In the cytosol, vanadate is reduced to vanadyl via a non-enzymatic reaction by glutathione. Most of the intracellular vanadium is probably bound to proteins and/or peptides especially glutathione, which prevents its oxidation (<1% free vanadium) [180, 181]. Therefore, it is likely that, in vivo, vanadyl, the predominant intracellular form of vanadium plays an important role in mediating its metabolic effects [181, 182]. The physiological intracellular concentration of vanadium in mammalian cells is about 20 nM [183].

Three general classes of vanadium-containing compounds are of interest for their ability as insulin-mimetic/enhancing agents including: (1) inorganic vanadium salts (vanadate and vanadyl), (2) peroxovanadium complexes and (3) organic vanadium compounds [180]. Effective glucose lowering doses of sodium orthovanadate and to a lesser extent vanadyl sulfate, may produce gastrointestinal
discomfort such as diarrhea and dehydration [184-186]. Hence, various organic compounds were designed to enhance the bioavailability and reduce the side effects of inorganic compounds. Peroxovanadium complexes are of less interest, due to their toxicity related to production of free radicals, which may increase oxidative stress in the cell [187]. Bis(maltolato)oxovanadium (IV) (BMOV), an organic vanadium compound that has been used in these studies, was developed in collaboration with Dr. Chris Orvig [188]. The advantages of BMOV over the inorganic vanadium compounds include greater potency, lower toxicity and improved tolerance [189, 190]. Previous studies in this laboratory have shown that the initial effective dose and the maintenance dose of BMOV are 0.45 and 0.18 mmol/kg/day, respectively [191].

1.5.2. Insulin-Mimetic/Enhancing Effects of Vanadium In Vitro

Several in vitro studies have demonstrated that vanadium mimics most of the metabolic actions of insulin including stimulation of hexose transport [192, 193], glycolysis [192, 194], glucose oxidation [182, 192], glycogen synthesis [192, 195, 196], lipogenesis [195, 197-199], inhibition of lipolysis [198, 200] and gluconeogenesis [187, 201].

Stimulatory effects of vanadium on glycolysis are mediated by increasing the expression of (L-type) pyruvate kinase and inhibition of fructose 2,6-bisphosphatase activity [187, 194]. Stimulatory effects of vanadium on glycogen synthesis have been reported in both hepatocytes and muscle strips [192, 195]. Finally, inhibitory effects of vanadium on gluconeogenesis in the hepatocytes are associated with
suppression of the key gluconeogenic enzymes PEPCK [202], G6Pase [203] and fructose-2,6-bisphosphatase [194].

Several *in vitro* studies have shown that vanadium inhibits lipolysis and stimulates lipogenesis in rat adipocytes. In both adipocytes and hepatocytes the lipogenic effects of vanadium are observed at higher concentrations (0.5-1 mM) than that required for its anti-lipolytic effects (~100 μM) [197-199]. However, it has been reported that low concentrations of vanadium (10-100 μM) are able to stimulate lipogenesis in the presence of glucose (2 mM) in the starved rat adipocytes [199].

**1.5.3. Effects of Vanadium on Type 1 Diabetes**

*In vivo* studies pointing to an effect of vanadium in diabetes date to as early as 1899 when Lyonnet *et al.* [204] reported a decrease in glucosuria in diabetic patients given sodium vanadate, although this was likely a placebo effect. Many years later, in 1985, this finding was confirmed in our laboratory with a study that demonstrated the *in vivo* glucose lowering effects of orally administered vanadium compounds in STZ-diabetic rats [205]. This study and several subsequent reports showed that chronic treatment of diabetic rats with vanadium salts decreased plasma glucose levels and corrected hyperlipidemia without any significant effect on plasma insulin levels [23, 191, 205-208]. Using the euglycemic hyperinsulinemic clamp technique, it was shown that improvement of metabolic state following vanadium treatment was accompanied by a marked increase in the peripheral glucose utilization and complete normalization of elevated hepatic glucose output [209-211]. The improvement in peripheral glucose uptake was associated with the correction of the reduced levels of GLUT4 mRNA and protein in both skeletal and
cardiac muscle [23, 25] as well as an increase in insulin-induced GLUT4 translocation in the cardiac muscle [212]. Furthermore, an increase in liver glycogen content was reported in STZ-diabetic rats [206, 213] due to complete (or partial) recovery of the activities of the key enzymes in glycogenesis [213]. Effects of vanadium on lipid metabolism in this model include both an improvement in adipose tissue function as assessed by basal and epinephrine-stimulated lipolysis [208] and total (or partial) normalization of low mRNA and activities of lipogenic enzymes in the liver [214, 215]. In addition to its effects on glucose and lipid homeostasis, protective effects of vanadium on the pancreatic β-cells in the diabetic rats may also contribute to its metabolic actions [216-218].

In human studies, it has been reported that chronic treatment with vanadium salts reduces insulin requirements in Type 1 diabetic patients but has no (or modest) effect on glucose disposal, insulin sensitivity or hepatic glucose production [196, 219, 220]. The differences observed between human and animal studies might be explained at least partially by shorter duration of treatment (2-3 weeks) and lower doses (100-125 mg/day) of vanadium used in human studies as compared with that in animal studies.

1.5.4. Effects of Vanadium on Type 2 Diabetes

Vanadium has been shown to decrease plasma insulin levels and improve insulin sensitivity in several animal models of insulin resistance and Type 2 diabetes [221-225]. Studies using the euglycemic hyperinsulinemic clamp technique demonstrated that the improvement in glucose homeostasis in fatty Zucker rats was not due to a greater inhibition of hepatic glucose output but involved an increase in
the insulin sensitivity in the peripheral tissues mainly skeletal muscle [224]. Since
the increase in muscle glucose uptake was not associated with alterations in GLUT4
mRNA or protein expression in this tissue, it was suggested that the stimulatory
effects of vanadium on glucose transport might be due to more efficient translocation
of GLUT4 or an increase in its intrinsic activity [226]. The efficient glucose lowering
effect of chronic vanadium treatment has also been reported in ZDF rats during the
diabetic state [227]. Furthermore, there is evidence that vanadium effectively
preserves pancreatic β-cell function in both animal models [227, 228]. The effects of
vanadium on metabolic homeostasis are associated with the correction of key
enzymes in glycogen metabolism (glycogen synthase a and phosphorylase a) and
lipogenic enzymes (malic enzyme and glucose 6-phosphate dehydrogenase) in
some animal models of insulin resistance [223, 229, 230].

Human studies have shown that oral treatment with vanadium results in
reduced fasting plasma glucose, suppression of hepatic glucose production and
improvement in insulin sensitivity in the skeletal muscle of Type 2 diabetic patients
[231-233]. The latter effect appears to be accounted for by an increase in non-
oxidative glucose disposal [219, 232]. However, a few studies have reported an
improvement in insulin sensitivity without any measurable effect on suppression of
hepatic glucose production [219] or vice versa [234].

1.5.5. Mechanism of Action of Vanadium

Despite numerous studies during the past decade, the molecular mechanism
of vanadium action, in vivo, is still not well understood. There are several reports
that vanadium demonstrates insulin-like effects in correcting the metabolic disorders
associated with diabetes. However, there is evidence suggesting that the glucose-lowering effects of vanadium are dependent on the presence of endogenous insulin [181, 235]. Although most of the actions of insulin on glucose and lipid metabolism are mimicked or enhanced by vanadium both in vitro and in vivo, the effects of insulin on protein synthesis, amino acid uptake and mitogenesis are not mimicked by vanadium [192, 236]. Hence, it appears that vanadium compounds mimic/enhance metabolic rather than mitogenic effects of insulin [236].

Vanadium is a potent inhibitor of cellular protein tyrosine phosphatases (PTPases), especially the cytosolic PTPases [187]. Thus, it has been commonly accepted that vanadium would enhance insulin receptor and/or substrate phosphorylation indirectly by inhibiting the dephosphorylation of these proteins [187, 236]. Furthermore, vanadium is an inhibitor of Na⁺, K⁺, ATPase and inhibits all 'P' type phosphorylated ATPases [236]. However, the intracellular form of vanadium (vanadyl) is not a potent PTPase inhibitor [190]. Also, there is a growing body of evidence from in vitro studies indicating that some of the important metabolic actions of vanadium such as stimulation of glucose uptake [193], lipogenesis [237] and glycogen synthesis [238, 239], are independent of insulin receptor tyrosine kinase. Therefore, two non-receptor protein tyrosine kinases, namely cytosolic [240, 241] and membranous protein tyrosine kinases [242], were suggested to mediate the insulin-like effects of vanadium. However, some of the effects of vanadium including stimulation of glucose uptake and inhibition of lipolysis cannot be explained by activation of cytosolic non-receptor tyrosine kinases [240].
One possible alternative action suggested for vanadium is interference with the levels or actions of cAMP. This might involve G-proteins, adenylyl cyclase [243], cAMP phosphodiesterase (type IV) [244], or cAMP-dependent protein kinase (PKA) [198]. Vanadium has been shown to activate MAPK and PI3-K, the two upstream enzymes in insulin signaling pathways, in vitro [89, 242, 245]. However, unlike insulin, activation of the insulin receptor or these enzymes does not seem to be essential for the metabolic effects of vanadium. Recent studies have demonstrated that vanadium stimulates glucose transport and GLUT4 translocation to the membrane by both a PI3-K-dependent pathway (in adipocytes) [246], and a PI3-K-independent pathway (in L6 myotubes) [247]. Furthermore, anti-lipolytic effects of vanadium in adipocytes have been shown to be independent of PI3-K [200]. It is worthwhile to point out that most of the insulin-mimetic effects of vanadium are demonstrable only in vitro and at high concentrations (0.5-1 mM or higher) of vanadium, while the therapeutic concentrations achieved in animal studies are considerably lower (<30 μM) [181], thus many of the actions of vanadium demonstrated in vitro may not correlate with the therapeutic effects of vanadium in vivo. Hence, to have a better understanding of the therapeutic effects of vanadium it is necessary to confirm that in vitro observations can be shown in vivo in intact animals.

1.6. OTHER INSULIN SENSITIZING DRUGS: COMPARISON WITH VANADIUM

Biguanides are a class of anti-hyperglycemic drugs which are used for the treatment of Type 2 diabetes. Although, like vanadium, the glucoregulatory effects of biguanides are associated with an improvement in the peripheral insulin sensitivity
and inhibition of hepatic glucose output, their metabolic effects are likely mediated by different mechanisms.

1.6.1. History and Pharmacokinetic Properties

The glucose lowering effect of guanidine was first discovered in 1918 [248]. Subsequent to this finding three derivatives of guanidine were identified but due to the related toxicity, biguanides were the only group that became available for medical use [249]. The main clinically used biguanides included: phenethylbiguanide, or phenformin; N1,N1-dimethyl-biguanide, or metformin, and butylbiguanide, or buformin [249, 250]. However, phenformin and buformin were withdrawn because of the risk of lactic acidosis, while metformin (Glucophage) gained worldwide acceptance [249]. Metformin is absorbed in the upper segment of the small intestine via an active, saturable transport system. Metformin does not bind to plasma proteins and is not metabolized in the liver. Hence, it is excreted intact via both filtration and secretion by the proximal tubules [248, 250, 251]. This drug is usually regarded to as an antihyperglycemic agent, since it lowers plasma glucose in Type 2 diabetes without causing hypoglycemia [249, 250, 252].

1.6.2. Anti-hyperglycemic Effects of Metformin

The main glucoregulatory effects of metformin involve suppression of hepatic glucose output, increased peripheral glucose utilization, reduced fatty acid utilization and increased glucose turnover, particularly in the splanchnic bed [248, 249, 252]. There is evidence from in vitro studies indicating that metformin, in addition to its peripheral effects, may have a direct effect on metabolism and function of the β-cells [253, 254]. Metformin appears to require the presence of insulin for its glucose
lowering effect, although the drug does not stimulate insulin secretion [248, 252]. Mild to moderate weight loss observed in Type 2 diabetic patients after metformin treatment seems to be related to its anorectic effects [250, 255, 256].

1.6.2.1. Effects of Metformin on Hepatic Glucose Output

In vitro studies in isolated perfused livers [257, 258] and hepatocytes [259, 260] as well as in vivo studies in Type 2 diabetic patients [261-263] and animals [264] have shown that metformin suppresses hepatic glucose production by reduction of gluconeogenesis and inhibition of hepatic glycogenolysis. The inhibitory effect of metformin on hepatic glucose production is dose-dependent and is synergistic with that of insulin at therapeutic doses. At higher concentrations, in vitro, metformin can exert several non-insulin-dependent effects that contribute to the reduction of hepatic glucose output [252]. These include inhibition of hepatic lactate uptake [258], a decrease in cellular ATP concentration which results in an increase in pyruvate kinase flux [259], and inhibition of pyruvate carboxylase/phosphoenolpyruvate carboxykinase [257]. Furthermore, metformin has been shown to suppress the glucagon-induced gluconeogenesis in diabetic rat hepatocytes, which may be associated with its ability to restore the capacity of insulin to inhibit hepatic adenylate cyclase [265] and inhibition of G6Pase activity [266].

1.6.2.2. Effects of Metformin on Peripheral Glucose Utilization

Several studies have shown that insulin-stimulated glucose uptake into skeletal muscle is enhanced by metformin both in vitro [267-270] and in vivo [262, 271, 272]. This effect of metformin has been attributed to increased translocation to
and activity of glucose transporters in the plasma membrane [268, 269, 273-275], and enhanced non-oxidative glucose metabolism [251, 272, 276]. Most studies have shown that the stimulatory effects of metformin on glucose uptake are associated with an increase in glycogen synthesis in the muscle [271, 277]. However, the activity of glycogen synthase, the rate-limiting enzyme in glycogen synthesis, was not changed with metformin treatment in the muscle of Type 2 diabetic patients [272]. Although pharmacological concentrations of metformin have been shown to potentiate insulin-stimulated glucose oxidation [278, 279], this effect was not seen with physiological doses or in the absence of insulin [279].

1.6.2.3. Effects of Metformin on Lipid Metabolism

Other mechanisms involved in the blood glucose lowering effects of metformin include an insulin-independent suppression of fatty acid oxidation and a reduction in hyperlipidemia [252]. These effects reduce the energy supply for gluconeogenesis and therefore, serve to balance the glucose-fatty acid (Randle) cycle. Thus, the glucoregulatory effects of metformin may be mediated indirectly by its effects on the plasma lipids and reducing FFA oxidation [280, 281]. Most studies have shown that metformin decreases circulating free fatty acids (FFA), triglycerides and cholesterol levels in both human and animal models of Type 2 diabetes which is associated with a decline in the plasma VLDL and LDL levels and an increase in plasma HDL levels [281-283]. Studies have shown that therapeutic concentrations of metformin enhance insulin-stimulated lipogenesis in human adipocytes and insulin-resistant hepatocytes [284, 285] in vitro, and inhibit catecholamine-induced lipolysis in adipose tissue from Type 2 diabetic patients in vivo [286]. Therefore, it
has been suggested that metformin reduces plasma FFA levels via increased re-esterification rather than by decreased lipolysis.

1.6.2.4. Effects of Metformin on the Insulin Signaling Pathway

Studies on the effects of metformin on insulin binding have shown conflicting data. While some studies have reported that high concentrations of metformin increase insulin receptor number and/or binding [278, 287, 288], most in vitro and in vivo studies have shown no effect at therapeutic doses [273, 274, 277]. Furthermore, the increase in insulin binding observed in some cell types by metformin does not correlate with the subsequent effects of the drug [278, 287, 288]. However, there is convincing evidence indicating that metformin increases insulin-mediated insulin receptor tyrosine kinase activity, which stimulates post-receptor pathways [271, 275, 289]. It has been reported that metformin may increase glucose uptake by restoring the impaired levels of insulin-induced PI3-K and PKB activities in cells exposed to high concentrations of glucose and insulin [275]. However, these effects seem to be secondary to the activation of insulin receptor rather than to a direct effect of the drug on these enzymes [275].

Effects of metformin on glucose transport in insulin-sensitive tissues (muscle and adipose tissue) are mediated by increasing translocation and intrinsic activity of GLUT4 and GLUT1 [273, 274, 290], without any effect on their mRNA level or protein expression [267, 273, 274, 291, 292]. Recent evidence indicates that the effects of metformin on GLUT4 translocation are mediated via reducing the increased levels of endocytosis induced by chronic exposure to hyperglycemia and
hyperinsulinemia [275]. Effects of metformin on glucose transporters require the presence of insulin [252].

1.6.2.5. How Does Vanadium Differ From Metformin?

Although vanadium and metformin both improve the metabolic state by enhancing peripheral glucose utilization and inhibition of hepatic glucose output (HGO), it seems that the molecular mechanisms by which they mediate at least some of their effects are different. Increased peripheral glucose utilization by both compounds is associated with an increase in GLUT4 and GLUT1 translocation [212, 247, 273, 274] as well as an increase in non-oxidative glucose metabolism [219, 232, 271, 277]. Also, like vanadium [187], inhibition of HGO with metformin is mediated by a reduction in glycogenolysis and inhibition of gluconeogenesis [252]. However, the insulin-like regulatory effects of vanadium observed on the gene expression (i.e. expression of GLUT4 and PEPCK) [23, 202] have not been reported with metformin. Furthermore, unlike metformin, some of the important effects of vanadium compounds (i.e. peroxovanadium) on the insulin signaling cascade are related to their action as potent phosphatase inhibitors [236]. There is convincing evidence from in vivo studies that vanadium (BMOV) has protective effects on the β-cells of the pancreas [208, 217]. Although it has been reported that metformin may have a direct effect on the pancreas [253, 254], such effects have not been observed in vivo. Furthermore, it appears that the effects of metformin, at therapeutic doses, require the presence of insulin and hence metformin is usually referred to as an anti-hyperglycemic drug rather than a hypoglycemic drug [250].
while vanadium treatment can result in hypoglycemia. Finally, unlike vanadium [180], metformin does not bind to plasma proteins [250].

1.7. EXPERIMENTAL MODELS

1.7.1. The Streptozotocin (STZ) Diabetic Rat

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosourea)1-d-glucopyranose)(α+β) is an antibiotic which was first isolated from Streptomyces achromogenes in 1959. This agent is composed of a cytotoxic moiety, 1-methyl 1-nitrosourea, attached to carbon-2 of D-glucose [293]. The diabetogenic action of streptozotocin results from its selective cytotoxic effect on the β-cells of the islets of Langerhans [294, 295]. The basis for the specificity of the attack on β-cells by this agent is not entirely clear. It has been reported that streptozotocin has a preferential cytotoxic effect on insulinoma cells over-expressing GLUT2, the glucose transporter isoform expressed in the pancreatic β-cells and hepatocytes, indicating a possible role for GLUT2 in the transport of this agent [296].

Different models have been proposed by which streptozotocin may cause β-cell damage. The widely accepted model is that streptozotocin induces β-cell damage by initiating DNA strand breaks, likely via an increase in oxygen free radicals, carbonium ions or nitric oxide. As part of the repair of these lesions poly (ADP-ribose) polymerase (PARP) is activated to form poly (ADP-ribose) utilizing NAD⁺ as a substrate, which leads to a critical depletion of NAD⁺ resulting in functional impairment and cell death [297-299]. The importance of PARP activation in mediating the cytotoxic effects of streptozotocin has been reported by several studies. It was shown that administration of PARP inhibitors before STZ-injection
prevented the inhibition of proinsulin synthesis caused by streptozotocin [300]. Furthermore, a study using islet cells from PARP-deficient mice reported that mutant islet cells did not show NAD\(^+\) depletion after exposure to DNA-damaging radicals [301]. Many studies have reported that streptozotocin causes DNA strand breaks by inducing the generation of free radicals [302]. It has also been suggested that streptozotocin might exert its initial biochemical effects by the generation of highly reactive carbonium ions (\(\text{CH}_3^+\)), which can cause DNA strand breaks by alkylating DNA bases at various positions [303]. Finally, streptozotocin has a nitrose moiety, which can liberate nitric oxide. There is evidence that generation of nitric oxide may be a final common pathway for \(\beta\)-cell damage [304]. Nitric oxide has been reported to activate heme-containing enzymes such as guanylyl cyclase, which results in accumulation of cGMP, and inhibit iron-sulfur enzymes such as mitochondrial aconitase, a key enzyme in the Krebs cycle, which results in impaired glucose oxidation in the \(\beta\)-cells [305].

Administration of streptozotocin via intravenous or intraperitoneal routes at doses exceeding 40 mg/kg (50-70 mg/kg) induces stable diabetes in rats [306]. Streptozotocin injection produces an initial rise in plasma glucose (2-4 h post-injection), which is due to inhibition of insulin release, followed by hypoglycemia (6-12 h post-injection) and finally permanent hyperglycemia 24 hours after STZ-injection [307]. STZ-diabetes is associated with an increase in plasma glucagon levels, which has been suggested to contribute to the development of hyperglycemia [295]. This model resembles many features of Type 1 diabetes in humans, and therefore is widely used for studying the pathology of Type 1 diabetes.
1.7.2. The Fatty Zucker Rat

The \((fa/fa)\) Zucker rat, first described by Zucker in 1965 [308], is a result of crossbreeding between the Merck Stock M and Sherman rats. The genetic obesity in Zucker rats is inherited as an autosomal recessive gene \((fa)\), which is identified as a single point mutation in the extracellular domain of the leptin receptor [309, 310]. This animal model resembles most features of the pre-diabetic state of Type 2 diabetes in humans and is characterized by obesity, hyperinsulinemia, insulin resistance, hyperlipidemia, normoglycemia (or mild hyperglycemia) and glucose intolerance [311]. It has been proposed that a hypothalamic defect due to expression of a mutated leptin receptor results in obesity in fatty Zucker rats [311, 312]. Although hyperphagia and hyperinsulinemia contribute to, they do not totally account for, the obesity in these rats, since restriction of food intake and correction of hyperinsulinemia are shown to decrease, but not normalize, body weight [312]. Adipocyte hypertrophy represents one of the very earliest phenotypic alterations in this model therefore, it has been suggested that a selective up-regulation in adipocyte fatty acid uptake, prior to the development of obesity, may be involved in evolution of the obese phenotype observed in these animals [313].

It is believed that both insulin resistance and a \(\beta\)-cell secretory defect in the fatty Zucker rats contribute to hyperinsulinemia [314, 315]. Pancreatic islets from these animals are enlarged and yield an exaggerated response to a glucose stimulus [316]. An increase in parasympathetic input to the endocrine pancreas observed in fatty Zucker rats, may also contribute to the increased levels of insulin [317]. Hyperinsulinemia, which is detectable as early as 3-4 weeks of age, is
accompanied by both progressive insulin resistance in the peripheral tissues associated with an impaired insulin-stimulated glucose disposal [318] and abnormal suppression of hepatic glucose production after glucose digestion [319, 320]. The latter may be exaggerated by impaired regulation of glucagon secretion [315].

Lean littermates (Fa/?) have normal plasma insulin and glucose levels and do not develop insulin resistance [321, 322]. However, heterozygous lean rats (Fa/fa) have been reported to show some degree of impaired glucose and fat metabolism [323].

1.7.3. The Zucker Diabetic Fatty (ZDF) Rat

The Zucker diabetic fatty (ZDF) rat arose from the inbreeding of a sub-strain of fa/fa Zucker rats that exhibited hyperglycemia [324]. In this strain all males develop obesity, insulin resistance and overt diabetes between 7 and 10 weeks of age [227, 325]. Young male ZDF rats (<7 weeks old) are characterized by hyperinsulinemia, insulin resistance and glucose intolerance, a state similar to that in the fatty Zucker rats [227, 325, 326]. This pre-diabetic state is followed by the development of diabetes, which is accompanied by both hyperinsulinemia and hyperglycemia in 7-12 week old rats. At 12 weeks of age plasma insulin levels decline to low normal values with a concurrent exacerbation of the hyperglycemia [227, 325].

At the onset of diabetes, pancreatic islets of ZDF rats (9-12 week old) are markedly hypertrophic and dysmorphic [325]. β-cells exhibit multiple defects including a decrease in glucose stimulated insulin secretion accompanied with low expression of GLUT2 and a depletion of β-cells insulin stores which is due to a
decrease in insulin mRNA levels [325, 327, 328]. Profound under-expression of GLUT2 in ZDF rats and the finding that GLUT2 levels in pancreatic cells dropped as diabetes developed, implicated a causal role for GLUT2 in the development of hyperglycemia [327, 328]. However, subsequent evidence of defects distal to GLUT2 in the β-cells did not support this hypothesis [329]. A few years ago, Unger described the lipotoxicity hypothesis, which linked the elevations in circulating and intracellular free fatty acids and triglycerides to insulin resistance and Type 2 diabetes. Based on this theory, the increased levels of triglycerides found in β-cells results from a lack of islet responsiveness to leptin [323, 330], which leads to lipotoxic β-cell damage, probably due to induction of nitric oxide synthase (iNOS) [323].

The ZDF rat is an ideal model for human Type 2 diabetes, since it develops pre-diabetic and diabetic state, which closely resembles the features found in human subjects. Homozygous (fa/fa) female ZDF rats are obese and insulin resistant but do not become diabetic.

1.8. RESEARCH STRATEGY

1.8.1. Rationale

Insulin resistance is associated with reduced responsiveness to insulin resulting in an increase in hepatic glucose production (HGP) and a decrease in peripheral glucose utilization. Understanding the molecular mechanisms underlying the impaired insulin action will lead to new insights in the treatment of diabetes. A growing body of evidence indicates that most of the metabolic effects of insulin including stimulation of glucose uptake and glycogen synthesis and inhibition of
hepatic glucose output are mediated by protein kinase B (PKB), an intermediate kinase in the insulin signaling pathway, indicating a possible link between PKB activity and insulin resistance in diabetes.

Vanadium is an insulin-mimetic/enhancing agent, which is a candidate for oral therapy in diabetes. Previous studies have shown that improvement of the metabolic state following vanadium treatment is accompanied by an increase in the peripheral glucose utilization and normalization of hepatic glucose output [211, 233]. Evidence indicates that vanadium may have several targets in the insulin signaling cascade as well as in downstream metabolic pathways.

The first aim of this study was to evaluate the role of PKB in the development of insulin resistance in two animal models of diabetes, STZ-diabetic Wistar rats and fatty Zucker rats. The second objective of this study was to examine whether the glucoregulatory effects of vanadium, in vivo, are mediated by correction of PKB activity leading to an increase in response to insulin and the consequent improvement in the insulin sensitivity in fatty Zucker rats or correction of hyperglycemia in STZ-diabetic rats. Finally, in an attempt to find more selective targets of vanadium, in vivo, we examined the possible inhibitory effects of vanadium on two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), which are also proposed to be downstream targets for PKB in the metabolic pathway.

1.8.2. Hypotheses

It was hypothesized that:
1. Insulin-stimulated activation of protein kinase B (PKB) may be altered in the insulin sensitive tissues (skeletal muscle and/or liver) from fatty Zucker rats (but not STZ-diabetic rats), which is associated with the insulin resistant state in these animals.

2. Oral treatment with vanadium, an insulin sensitizing agent, may normalize PKB activity in the insulin sensitive tissues, leading to an improvement in insulin sensitivity in fatty Zucker rats and correction of plasma glucose levels in STZ-diabetic rats.

3. Hypoglycemic effects of vanadium in STZ-diabetic rats may be mediated by its direct and/or indirect regulation of more specific targets, such as PEPCK and G6Pase, two key gluconeogenic enzymes, which are proposed to be downstream metabolic enzymes for PKB.
CHAPTER 2
MATERIALS AND METHODS

2.1. MATERIALS

Regular beef/pork insulin (lletin R) was from Eli Lilly Co. (Indianapolis, IN, USA). Regular/zinc insulin implants were obtained from Linshin Canada Inc. (Scarborough, ON, Canada). Anti-PKBα (C-terminal) antibody and HRP conjugated anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PKBα (PH domain) antibody, Anti-PKBβ antibody, PKBα specific substrate peptide (RPRAATF) and HRP conjugated anti-sheep IgG were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-phospho Ser473 PKBα antibody and HRP conjugated anti-rabbit IgG were from New England Biolabs (Beverly, MA, USA) and CalBiochem (San Diego, CA, USA), respectively. Protein G Sepharose beads were from Amersham Pharmacia Biotech. (Oakville, ON, Canada). Aprotinin, leupeptin, pepstatin A, soyabean trypsin inhibitor, benazamidine, phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), β-methylaspartic acid, β-glycerophosphate, Triton X-100, cAMP-dependent protein kinase inhibitor (PKI), streptozotocin, phosphoenolpyruvate, malate dehydrogenase (484 U/mg protein), β-nicotinamide adenine dinucleotide (β-NADH), 10 x buffer II PCR reagent, diethylpyrocarbonate (DEPC) and the buffers were purchased from Sigma (St. Louis, MO, USA). \( [\gamma^{\text{32P}}] \text{ATP} \) was from Dupont NEN (Boston, MA, USA). RNAZol B reagent was from Tel-Test Inc. (Friendswood, TX, USA). M-MLV reverse transcriptase (200 U/\( \mu \)l), random primers were from Gibco/BRL (Burlington, ON, USA).
2.2. EXPERIMENTAL PROTOCOLS

2.2.1. Studies on Protein kinase B

2.2.1.1. Time Course Studies: STZ-Diabetic Rats and Fatty Zucker Rats

(Study # 1)

At the beginning of the studies, a time course experiment was performed to:
(1) determine the time point of maximum activation of protein kinase B, in vivo, with insulin; and (2) investigate the possible changes in basal or insulin stimulated PKB activity in STZ-diabetic and fatty Zucker rats. Male Wistar rats (190-220 g) were received from Animal Care Centre, The University of British Columbia, and male lean and fatty Zucker rats (14-16 weeks old) were obtained from Department of Physiology, The University of British Columbia, Vancouver, Canada. Wistar rats were randomly divided into two groups: control (C, n=21) and diabetic (D, n=22). Experimental diabetes was induced by a single intravenous injection of streptozotocin (STZ) dissolved in 0.9% saline (60 mg/kg), via the tail vein under halothane anesthesia. Control rats were injected by 0.9% saline. Three days after STZ-injection, rats with a blood glucose level greater than 14 mM were considered diabetic. Wistar rats were terminated four weeks after STZ-injection. Fatty Zucker rats (F, n=14) were sacrificed between 18-20 weeks of age. An age-matched lean
group (L, n=15) was used as control for the fatty Zucker rats. At termination, overnight (16 h) fasted rats were anesthetized with pentobarbital (65 mg/kg, i.p.) and sacrificed without insulin (basal state) or at different time points after tail-vein injection with insulin (5 U/kg, i.v.) (n=3-5 per group). Hind limb skeletal muscles, from both legs, and liver were removed immediately, freeze-clamped in liquid nitrogen and stored at -70°C until assayed.

2.2.1.2. Basal PKB Activity in ZDF Rats: Comparison with Fatty Zucker Rats

(Study #2)

To investigate the relationship between diabetic state and basal PKB activity, we examined the basal PKB activity in two animal models of diabetes: (1) fatty Zucker rat (ZF), an animal model of insulin resistance and early stage Type 2 diabetes; and (2) Zucker diabetic fatty rat (ZDF), an experimental model of Type 2 diabetes. Zucker Diabetic fatty rats were purchased from Genetic Models Inc., Indianapolis, IN, USA. Overnight (16 h) fasted Zucker fatty (18-20 weeks old, n=5) and Zucker diabetic fatty (12 weeks old, n=5) rats were sacrificed under pentobarbital anesthesia (65 mg/kg, i.p.). Appropriate age-matched lean rats (n=5) were used as controls for each group. Hind limb skeletal muscle and liver were freeze-clamped in liquid nitrogen and kept at -70°C for PKB assays.

2.2.1.3. Effects of BMOV on PKB Activity in STZ-Diabetic Rats

Short-Term Study (Study #3)

There is evidence from in vitro studies that inorganic vanadium compounds, like insulin, are able to phosphorylate (activate) protein kinase B [111]. However, there is no evidence as to whether this effect can be reproduced by doses of
vanadium used in *in vivo* studies, which are many times lower than that used in *in vitro* studies. Hence, this experiment was designed to investigate the effects of chronic treatment with BMOV on basal and insulin-induced PKB activity under both normal and diabetic conditions. Male Wistar rats, (190-220 g) were randomly divided into four groups: control (C, n=21), control treated with BMOV (CB, n=21), diabetic (D, n=20) and diabetic treated with BMOV (DB, n=20). Rats were housed, one per cage in the treated groups and two per cage in the control groups, on a 12 h light/12 h dark schedule and given food and fluid ad libitum in all studies. Body weight, food and fluid intakes were measured daily throughout the studies to monitor animals’ health. STZ-diabetic rats (60 mg/kg, i.v.) with a blood glucose level greater than 14 mM were considered diabetic.

Treatment with BMOV was started one week after STZ-injection. Animals in the BMOV-treated groups received BMOV for three weeks at an initial concentration of 0.5 mg/ml in the drinking water, which was gradually increased to a maximum concentration of 1 mg/ml or until the animal reached an euglycemic state. The dose of BMOV administration was calculated based on the solution concentration, body weight, and fluid consumption. The mean BMOV doses during the last week of treatment in the control and diabetic treated groups were 0.26±0.01 mmol/kg/day and 0.41±0.01 mmol/kg/day, respectively. Plasma glucose and insulin levels were monitored weekly during the treatment. At termination, overnight fasted rats were randomly divided into basal and insulin injected subgroups (n=5-7 per group) and anesthetized with pentobarbital (65 mg/kg, i.p.). Animals were sacrificed either
without insulin injection (basal state) or at different time points (2, 5 and 15 min) after tail-vein injection with insulin (5 U/kg, i.v.).

2.2.1.4. Effects of BMOV on PKB Activity in STZ-Diabetic Rats

*(Long-Term Study) (Study # 4)*

Observations from our previous studies indicated that STZ-diabetic rats develop insulin resistance during longer periods of diabetes [331]. Therefore, to investigate the association between the development of insulin resistance and PKB activity in STZ-diabetic rats, the period of diabetes was extended from 4 to 9 weeks. In this experiment male Wistar rats (190-220 g) were randomly divided into four groups: control (C, n=9), control treated with BMOV (CB, n=8), diabetic (D, n=11) and diabetic treated with BMOV (DB, n=8). Animals in the CB and DB groups were treated with BMOV in the drinking water for eight weeks with the same protocol used in the short-term study. The mean BMOV doses in the last three weeks of treatment in the control and diabetic treated groups were 0.19±0.01 mmol/kg/day and 0.30±0.01 mmol/kg/day, respectively. Plasma glucose and insulin levels were monitored weekly during the treatment. Animals were sacrificed 9 weeks after induction of STZ-diabetes in the basal state (no insulin injection) or 5 minutes post insulin injection (n= 3-5 per group).

2.2.1.5. Effects of BMOV on PKB Activity in Zucker Rats (Study # 5)

The results obtained from the time course study (study # 1) indicated that insulin-induced PKB activity was altered in both skeletal muscle and liver from fatty Zucker rats, indicating that there might be a relationship between PKB activity and insulin resistance in fatty Zucker rats. Hence, in this study we evaluated the
hypothesis that the positive effects of BMOV on insulin sensitivity in this animal model may be associated with normalization of insulin-induced PKB activity in the insulin responsive tissues.

Age-matched male lean and fatty Zucker rats (14-16 weeks old) were randomly divided into four groups: lean (L, n=17), lean-treated with BMOV (LB, n=21), fatty (F, n=24), and fatty-treated with BMOV (FB, n=18). BMOV treatment was started at an initial concentration of 0.25 mg/ml in the drinking water and was increased to a maximum concentration of 0.75 mg/ml within the first week of treatment. The mean BMOV dose during the last week of treatment in the lean and fatty treated rats was 0.19±0.01 mmol/kg/day. To assess insulin sensitivity, animals were fasted overnight (16 h) and two oral glucose tolerance tests (OGTT) were performed, one at 15-17 weeks of age, before the beginning of treatment and one a week before termination. Three weeks after treatment with BMOV, rats were fasted overnight and animals in each treatment group (L, LB, F, and FB) were divided into basal and insulin injected subgroups and sacrificed without insulin injection (basal state) or at different time points (5 and 15 min) after insulin injection (5 U/kg, i.v.).

2.2.2. Effects of BMOV on the key gluconeogenic enzymes in STZ-diabetic rats (Study # 6)

Our studies showed that glucose lowering effects of BMOV were more profound in the overnight fasted STZ-diabetic rats, which had active gluconeogenesis, as compared to fed diabetic rats. Since STZ-diabetes is associated with an increase in hepatic glucose output, this study was designed to investigate the effects of BMOV, in vivo, on hepatic glucose
production/gluconeogenesis in STZ-diabetic rats. To compare the effects of BMOV and insulin, one group of diabetic rats received insulin implants. Male Wistar rats (190-220 g) were obtained from Charles River Laboratories, Montreal, Canada. Animals were monitored for their health during the first week after arrival and then were randomly assigned to two groups: control (C) and diabetic (D). Experimental diabetes was induced in rats by a single intravenous injection of streptozotocin under halothane anesthesia as in other studies. Control rats were injected with 0.9% saline. One week after STZ-injection control and diabetic rats were divided into further subgroups: control (C), control treated with BMOV (CB), diabetic (D), diabetic treated with BMOV (DB) and diabetic treated with insulin (DI) (n=5 per group). BMOV treatment was started at an initial concentration of 0.25 mg/ml in the drinking water, which was increased to a maximum concentration of 0.75 mg/ml or until the animal reached an euglycemic state. Animals were treated with BMOV for 4 weeks. The mean BMOV dose in the last week of treatment was 0.24 mmol/kg/day and 0.60 mmol/kg/day in the control and diabetic treated rats, respectively. Body weight, food and fluid intakes were measured daily during the study and plasma glucose and insulin levels were monitored weekly. Blood samples were collected from the tail vein between 1000-1100 h from fed animals. Seven days before termination, rats in the insulin treated group were anesthetized with halothane and insulin implants [4 units per day (14% regular and 0.4% zinc insulin in palmitic acid)] were inserted subcutaneously in the back of the neck. After 4 weeks of treatment with BMOV rats were anesthetized with pentobarbital (65 mg/kg, i.p.) and sacrificed between 0900-1100 h. Blood was collected by cardiac puncture for
measurement of plasma parameters (glucose, triglycerides, insulin and glucagon). Liver and kidney were removed immediately, rinsed with sterile phosphate buffered saline and frozen quickly in liquid nitrogen. Tissues were stored at -70°C for subsequent mRNA extraction and measurement of enzyme activity.

2.3. METHODS

2.3.1. Oral Glucose Tolerance Test

Two oral glucose tolerance tests (OGTT) were performed, one at 15-17 weeks of age before the beginning of treatment and one a week before termination, to assess insulin sensitivity in the Zucker rats. After an overnight fast (16 h), rats were administered a 40% glucose solution via oral gavage (1 g / kg body weight). Blood samples were obtained from the tail vein at times 0, 10, 20, 30, 60 and 90 minutes after glucose administration. An index of insulin sensitivity (ISI) was calculated as described by Matsuda [332] using the following formula: K/SQRT ((fasted plasma glucose × fasted plasma insulin) × (mean plasma glucose × mean plasma insulin)), K=100. Fasting plasma glucose and insulin values were the time 0 values (16 h fasted) and the mean plasma glucose and insulin values were calculated as the mean value of all time points measured in the test. Results derived from this equation provide an index of both hepatic and peripheral tissue sensitivity to insulin and have been shown to correlate highly to those obtained from the euglycemic insulin clamp studies [332].

2.3.2. Tissue Extract Preparation

Liver and muscle tissues were powdered in liquid nitrogen using a mortar and pestle. Approximately 50 mg tissue powder per ml of buffer were homogenized in
ice-cold buffer using a polytron homogenizer (Brinkmann, model: PT 3100) for 2×15 seconds at 6000 rpm. Homogenization buffer contained 25 mM MOPS buffer, 5 mM EGTA, 2 mM EDTA, 75 mM β-glycerophosphate, 5 μM β-methylaspartic acid, 5 μM pepstatin A, 3 mM benzamidine, 10 μM leupeptin, 10 μg/ml aprotinin, 200 μg/ml trypsin inhibitor, 1 mM DTT, 1 mM PMSF, and 0.5% Triton-X100, pH 7.2. Homogenized tissues were centrifuged at 100,000 × g for 1 h at 4°C. Supernatants were filtered through a 53 μm mesh (Nitex Netting nylon monofilament) carefully avoiding fat, and were stored at -70°C until assayed. Protein concentration in the homogenates was measured using the Bradford method [333].

2.3.3. PKB Immunoprecipitation Assay

PKB kinase activity was measured by an immunoprecipitation assay followed by a 32P kinase assay as previously described [334]. Aliquots of protein (2 mg) were diluted with approximately equal volume of 3% NETF buffer containing 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 50 mM NaF, and 3% Nonidet P-40, (pH 8.0) and pre-incubated with 3 μg of anti-PKBα-β domain or anti-PKBβ antibody (from Upstate Biotechnology) for 2 hours at 4°C. Forty μl of protein G Sepharose beads in 3% NETF were then added and incubated for 1 hour at 4°C. Pellets were washed 2 times with 3% NETF buffer and 2 times with K II buffer (1.25 mM β-glycerophosphate, 1.25 mM MOPS (pH 7.2), 0.5 mM EGTA, 2 mM MgCl2, 5 mM sodium fluoride, 25 μM DTT, 2.5 μM β-methylaspartic acid). Beads were incubated in a reaction mixture containing 10 μl of 0.4 mM Akt/PKB specific substrate peptide, 5 μl of 200 mM MgCl2, 3.2 μl of 25 μM cAMP-dependent protein kinase inhibitor peptide (pH 7.4), 16.8 μl assay dilution buffer, and 10 μl of [γ-32P]ATP (specific
activity: ~3000 Ci/mmol) diluted 1:50 in assay dilution buffer (25 mM β-glycerophosphate, 20 mM MOPS (pH 7.2), 5 mM EGTA, 2 mM EDTA, 20 mM MgCl₂, 0.25 mM DTT, 5 mM β-methylaspartic acid) for 30 minutes at 30°C. Supernatant (25 μl) was spotted onto 2-cm² P81 phosphocellulose papers which were washed 5 times in 1% phosphoric acid, dried and counted for radioactivity. Specific activity of [γ⁻³²P]ATP ranged from 1760 to 2443 cpm/pmol in different experiments. Specific activity of PKBα in the basal state ranged from 0.81 to 2.73 and 0.47 to 1.91 pmol/min/mg of total protein in the muscle and liver, respectively. The maximum insulin-induced activation of PKBα ranged from 13.10 to 27.61 and 6.74 to 11.89 pmol/min/mg in the muscle and liver of different animal models, respectively.

2.3.4. Electrophoresis and Immunoblotting

Immunoprecipitated samples or aliquots of protein (100 μg) were boiled with 20 μl of 5× Laemmli's sample digestion buffer for 5 minutes and subjected to 10% SDS-PAGE (16 hrs, 12 mA/gel) following which the proteins were transferred to nitrocellulose membrane (3 hrs, 0.3 A). Membranes were blocked with 5% non-fat dry milk in TBS (250 mM NaCl, 25 mM Trizma base, 0.02 N HCl, pH 7.5) for 1.5 hours at room temperature, washed with TBS containing 0.5% Tween 20 (TBS-T) and then incubated with anti-PKBα PH-domain (or anti-PKBβ) antibody (1 μg/ml) or anti-PKBα C-terminal antibody (0.4 μg/ml) in TBS-T for 2 hours at room temperature. The membranes were washed with TBS-T and incubated for 1 hour at room temperature with HRP conjugated anti-sheep IgG antibody (for anti-PKBα PH-domain and anti-PKBβ antibody) or anti-goat IgG antibody (for anti-PKBα C-terminal
antibody) diluted 1:10,000 and 1:2,000 in TBS-T, respectively. For phosphorylated PKBα, membranes were incubated overnight at 4°C with anti-phospho Ser\textsuperscript{473} PKBα antibody (1 μg/ml) in TBS and then with anti-rabbit IgG antibody (1:10,000) for one hour at room temperature. Washed membranes were placed in a 1:1 solution of chemiluminescence reagents (from Amersham Pharmacia) and exposed to high performance chemiluminescence films. Films were developed and bands were quantified by densitometry.

2.3.5. PEPCK Enzyme Assay

PEPCK activity in the liver and kidney was determined as described by Dakshinamurti [335] with a few modifications. Briefly, kidney (cortex) or liver tissues (~500 mg) were homogenized in 4 volumes of ice-cold buffer using a polytron homogenizer (Brinkmann, model: PT 3100) for 2 x 15 seconds at 6000 rpm. The homogenates were centrifuged at 10,000 x g for 30 minutes and the supernatants were filtered (through a 53 μm mesh Nitex Netting nylon monofilament) and stored at -70°C until assayed. Homogenization buffer contained 10 mM Tris-HCl, pH: 7.2, 1 mM EDTA, 0.25 M sucrose and 50 mM KCl. Protein concentration in the homogenates was measured using the Bradford method [333]. For PEPCK enzyme assay aliquots of kidney or liver cytosols (0.3 mg of protein) were added to the reaction mixture containing 50 mM Tris-HCl, pH:7.2, 2 mM MnCl\textsubscript{2}, 2.5 mM phosphoenolpyruvate, 10 mM NaHCO\textsubscript{3} (freshly prepared), 5 U malate dehydrogenase, 0.15 mM NADH, in a final volume of 1 ml. The reaction was initiated by adding 0.4 mM dGDP (final concentration) and the decrease in absorbance was monitored at 340 nm, 25°C for 3 minutes. A reaction mixture
without dGDP was used as control for each sample. Reaction rates were proportional to protein concentration and linear for at least 5 minutes. One enzyme unit converts 1 μmole NADH to NAD per minute.

2.3.6. RNA Extraction

Total RNA was extracted from excised kidney (cortex) or liver tissues; using RNAZol reagent (TelTest Inc.), according to the manufacturer's instructions. Briefly, about 100 mg of tissues were homogenized in 2 ml ice-cold RNAZol B reagent using a Polytron homogenizer (BioSpec Products Inc. model 398). RNA was extracted from the homogenates by adding chloroform (1 vol. Homogenate + 0.1 vol. chloroform). After 5 min (4°C) the suspensions were centrifuged at 12,000 x g (4°C, 15 min). The RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol. Samples were incubated (4°C, 15 min) and centrifuged at 12,000 x g (4°C, 15 min). The supernatant was removed and the RNA was washed once with 75% ethanol and centrifuged at 7,500 x g (4°C, 8 min). After removing the supernatant, the RNA pellet was dried briefly and dissolved in 50 μl diethylpyrocarbonate (DEPC)-treated distilled water. RNA was quantified by measuring absorbance spectrophotometrically at 260 nm and its integrity was assessed after electrophoresis on nondenaturating 1% agarose gel stained with ethidium bromide (5 μg/ml).

2.3.7. Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Reverse transcription (RT) of 5 μg total RNA was performed in 60 μl reaction volume containing 200 units of M-MLV reverse transcriptase (Gibco/BRL), 20 U RNase inhibitor, 3 mM MgCl₂, 1 x buffer II from Sigma, 0.3 μg random primers
(Gibco/BRL) and 1 mM dNTP for 50 min at 42 °C. Contaminating genomic DNA present in the RNA preparations was removed by digesting the reaction with 5 U of DNase I for 45 minutes at 37°C prior to the addition of reverse transcriptase. The PCR amplifications were performed in 100 µl reaction mixture. The PCR reaction mixture contained 250 µM dNTP, 2 mM MgCl₂, 0.5 U HotStar Taq DNA polymerase (Qiagen), 1 µl of sense and anti-sense primers, 5 µl of the RT product and 1 x Qiagen buffer. The reaction mixtures were subjected to 32 cycles of PCR amplification consisting of denaturation for 60 s at 94°C, annealing for 60 s at 55°C and elongation for 60 s at 72°C. The final extension was completed at 72°C for 7 min. The oligonucleotide primers (5'-AGCCTCGACAGCCTGCCAGG-3' sense and 5'-CCAGTTGTTGACCAAAGGCTTTT-3' anti-sense) for PEPCK were designed from published reports [158] and the amplified product was a 575-bp cDNA. The primers (5'-TAAGTGGATTCTTTTTGGACA-3' sense and 5'-GAAGAGGCTGGCAAAGGGTGT-3' anti-sense) for G6Pase [336] amplified a 562-bp cDNA. PEPCK and G6Pase mRNA expression levels were normalized to 18S ribosomal RNA (rRNA) expression (Ambion, Austin, TX). Ten µl of 6 x loading buffer (containing 0.25% bromothymol blue, 0.25% xylene cyanol FF and 15% Ficoll type 400, Pharmacia, in DEPC-treated distilled water) was added to the PCR samples. Twenty µl of PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and gels were photographed under UV light. The intensity of mRNA bands was analyzed by densitometry and the amplified products of PCR were purified with a Qiagen PCR purification kit (QIAquick) and sequenced. To determine the optimal number of cycles for the enzymes, PCR assay was performed
on the liver and kidney cDNA samples using different number of cycles from 28 to 34 for amplification of PEPCK and G6Pase. The relationship between optical density (amplification of cDNA) and the number of cycles was linear from 30 to 34 cycles for both enzymes (Fig 2.1).

2.3.8. Plasma Parameters

Blood samples were collected from the tail vein following a 5 h fast during the study, except at termination when the blood samples were obtained by cardiac puncture. Samples were centrifuged (17,500 × g, 20 min, 4°C) and plasma was stored at -20°C until assayed. Plasma glucose and triglyceride levels were measured using a Beckman Glucose Analyzer 2 and a kit from Boehringer Mennheim, respectively. Plasma insulin and glucagon levels were determined with a double antibody radioimmunoassay by using a kit from Linco Research Inc. (St. Charles, MO, USA).

2.4. STATISTICAL ANALYSES

Values are expressed as the mean ± standard error of the mean (SEM) and “n” indicates the number of rats in each group. Statistical analyses were performed using a general linear model (GLM) of ANOVA followed by a Newman-Keuls test for studies with multiple time points, including PKB activity, plasma parameters, body weight, food and fluid intake. PEPCK activity values were examined with a one-way ANOVA. PKβ activities (Fig 3.4) and insulin sensitivity indices (ISI) (Fig 3.10) were analyzed using unpaired t-test. Values were considered significantly different with \( P<0.05 \). All statistical analyses were performed using the Number Cruncher Statistical System (NCSS) software package.
OPTIMIZATION OF PCR ASSAY

PCR assay was performed on the cDNA samples from liver (A, B) and kidney (C, D) using different number of cycles to determine the optimal number of cycles for amplification of PEPCK and G6Pase. The relationship between optimal density (amplification of cDNA) and the number of cycles was linear from 30 to 34 cycles for both enzymes.
CHAPTER 3
RESULTS

3.1. STUDIES ON PROTEIN KINASE B

3.1.1. Time Course Studies: STZ-Diabetic Rats and Fatty Zucker Rats

(Study #1)

In this study control and STZ-diabetic Wistar rats (4-week diabetic), as well as lean and fatty Zucker rats (18-20 weeks old) were injected with 5 U/kg insulin and sacrificed at different time points after insulin injection. Results showed that PKBα was activated by insulin in the skeletal muscle as early as 1 minute, with a maximum activation occurring at 5 minutes post insulin injection (15 minutes in lean rats) (Figs 3.1A and 3.2A). There was no significant difference in insulin-induced PKBα activity in the skeletal muscle between control and STZ-diabetic rats (C: 12-fold vs. D: 10.5-fold). Furthermore, STZ-diabetes did not shift the time point of maximum activation of PKBα (Fig 3.1A). However, insulin-induced activation of PKBα was markedly lower in the skeletal muscle of fatty Zucker rats as compared to lean rats (F:~7-fold vs. L:~14-fold), indicating a decrease in response to insulin in the muscle of fatty Zucker rats (Fig 3.2A). There was no detectable difference in basal PKBα activity in the skeletal muscle between control and STZ-diabetic rats or lean and fatty Zucker rats.

Similar to the results seen in the muscle, in the liver of both control and diabetic Wistar rats, insulin activated PKBα after 1 minute and the enzyme activity remained high up to 15 minutes. As with muscle, there was no significant difference
in PKBα activity between control and STZ-diabetic rats (C: 13.7-fold vs. D: 15.7-fold), suggesting that insulin signaling is normal at the level of PKB in 4-week STZ-diabetic rats (Fig 3.1B). Interestingly, insulin-induced activation of PKBα was markedly increased in the liver of fatty Zucker rats as compared to lean littermates (F: 15.7-fold vs. L: 7.6-fold), indicating that response to insulin is increased in the liver and that changes in PKBα activity in fatty Zucker rats are tissue specific. Although basal PKBα activity in the liver of fatty Zucker rats was higher than lean rats, this difference did not reach the level of significance due to variability in basal PKBα activity among fatty Zucker rats (Fig 3.2B). Insulin stimulation of PKBα activity was accompanied by an increase in its phosphorylation (Figs 3.1 and 3.2) Comparison of basal PKBα activity in the muscle and liver from control Wistar rats and lean and fatty Zucker rats showed that PKBα activity in the skeletal muscle was about 2 times of that in the liver of the same animals (Fig 3.3A). A similar ratio was observed between PKBα activity in the muscle and the liver from control Wistar rats after insulin injection (Fig 3.3B).

Insulin (5 U/kg, i.v.) increased PKBβ activity about 6-fold and 4-fold in the skeletal muscle of Wistar rats and Zucker rats, respectively. As with muscle, in the liver of both animal models, insulin increased PKBβ activity about 3-fold. Furthermore, these results did not detect any significant difference in basal or insulin-induced activation of PKBβ between lean and fatty Zucker rats in either the muscle or the liver (Fig 3.4).
FIGURE 3.1.

TIME COURSE STUDIES: STUDY # 1

PKBα activity in basal state or at different time points after insulin injection (5 U/kg, i.v.) in the skeletal muscle (A) and liver (B) of 16 h fasted control and STZ-diabetic Wistar rats. PKBα was immunoprecipitated using anti-PKBα-PH antibody and assayed for kinase activity as described in methods. Results are presented as the means±SEM for assays performed in triplicate for each muscle or liver homogenate (n=3-5 per group). PKBα activity in the muscle or liver homogenates from control basal rats was taken as 100% in each experiment. The lower panels are representative Western blots of phosphorylated form of PKBα, detected by a specific anti-phospho Ser⁴⁷³ PKBα antibody in the crude muscle or liver homogenates in basal state or after insulin injection. *Different from corresponding insulin injected groups (P<0.05, ANOVA). C: Control, D: Diabetic.
TIME COURSE STUDIES: STUDY # 1

PKBα activity in basal state or at different time points after insulin injection (5 U/kg, i.v.) in the skeletal muscle (A) and liver (B) of 16 h fasted lean and fatty Zucker rats. PKBα was immunoprecipitated using anti-PKBα-PH antibody and assayed for kinase activity as described in methods. Results are presented as the means±SEM for assays performed in triplicate for each muscle or liver homogenate (n=3-5 per group). PKBα activity in the muscle or liver homogenates from lean basal rats was taken as 100% in each experiment. The lower panels are representative Western blots of phosphorylated form of PKBα, detected by a specific anti-phospho Ser473 PKBα antibody in the crude muscle or liver homogenates in basal state or after insulin injection. *Different from corresponding insulin injected groups, #different from lean group at the same time point, @lean (15 min) different from lean (5 min) (P<0.05, ANOVA). L: Lean, F: Fatty.
A) Muscle

Time After Insulin Injection

<table>
<thead>
<tr>
<th>Basal</th>
<th>2 min</th>
<th>5 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) Liver

Time After Insulin Injection

<table>
<thead>
<tr>
<th>Basal</th>
<th>2 min</th>
<th>5 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TIME COURSE STUDIES: STUDY # 1

Comparison of PKBα activity in the skeletal muscle and liver in basal state (A) or after insulin injection (B). Basal PKBα activity was measured in the skeletal muscle and liver of Wistar, lean and fatty Zucker rats using immunoprecipitation assay. Figure B shows insulin-stimulated PKBα activity at the time point of maximum activation (muscle: 5 min, liver: 15 min) in control Wistar rats. Immunoprecipitation assays were performed in triplicate for each animal and the results are given as means±SEM (n=5 per group). PKBα activity in a pooled liver homogenate obtained from control Wistar rats was taken as 100% in each experiment.
TIME COURSE STUDIES: STUDY # 1

Basal and insulin-induced PKBβ activity in the skeletal muscle and liver of control Wistar rats (A, B) or lean and fatty Zucker rats (C, D) at 5 min (muscle) or 15 min (liver) after insulin injection. Immunoprecipitation assays were performed using anti-PKBβ antibody. Results are given as the means±SEM (n=3-5 per group). PKBβ activity in a pooled muscle or liver homogenate from control Wistar rats (Figs A and B) or lean Zucker rats (Figs C and D) was taken as 100%. The upper panels are representative Western blots of the immunoprecipitated PKBβ in the basal state (-) or after insulin injection (+). *Different from basal state (P<0.05, ANOVA or Student's t-test as appropriate).
3.1.2. Basal PKBα Activity in ZDF Rats: Comparison with Fatty Zucker Rats

(Study # 2)

Our earlier study indicated that there was no detectable difference in basal PKBα activity between control and STZ-diabetic rats. In this experiment basal PKBα activity was compared in the skeletal muscle and liver from fatty Zucker rats (18-20 weeks old), an animal model of insulin resistance and a pre-diabetic state, and Zucker diabetic fatty rats (12 weeks old), an animal model of overt type 2 diabetes. At the beginning of the study (9 weeks old) plasma glucose levels were significantly higher in ZDF rats as compared to lean rats (ZDF: 27.5±0.7 vs. L: 7.9±0.1 mmol/l, 5 h fasted). Plasma insulin levels in 9-week old lean and ZDF rats were 1.0±0.1 and 6.0±0.5 ng/ml, respectively. At the end of study (12 weeks old), plasma glucose levels were markedly higher in ZDF rats than lean rats (ZDF: 29.9±0.4 vs. L: 7.9±0.1 mmol/l, 16 h fasted). Termination plasma insulin levels in lean and ZDF rats were 0.9±0.1 and 1.4±0.2 ng/ml, respectively.

Results of this study showed that basal PKBα activity was significantly lower in the skeletal muscle from fatty Zucker rats as compared with control Wistar rats, with no difference in PKBα activity between ZDF and control Wistar rats (Fig 3.5A). In the liver, basal PKBα activity in ZDF rats was lower than control Wistar rats, but there was no significant difference between Wistar and fatty Zucker rats (Fig 3.5B). When compared with lean controls, there was no significant difference in basal PKBα activity between lean and fatty Zucker rats or lean (control for ZDF) and ZDF rats in either tissue.
Basal PKBα activity was measured in the skeletal muscle (A) and liver (B) of control Wistar rats, Zucker fatty rats (21 weeks old) and Zucker diabetic fatty rats (12 weeks old). Appropriate age-matched lean rats were taken as control groups for Zucker fatty and Zucker diabetic fatty rats. Immunoprecipitation assays were performed in triplicate on muscle or liver extracts and results are given as means±SEM (n=5 per group). PKBα activity in a pooled muscle or liver homogenate obtained from control Wistar rats was taken as 100% in each experiment. *Different from control Wistar rats (P<0.05, ANOVA).
3.1.3. Effects of BMOV on PKB Activity in the STZ-Diabetic Rats

Short-Term Study (Study # 3)

Three weeks treatment with BMOV in the diabetic rats normalized food and fluid intakes but did not restore growth retardation. In the control rats BMOV did not have any effect on body weight or food intake but caused a slight decrease in fluid intake (Table 3.1). Streptozotocin injection resulted in an increase in plasma glucose concentrations (C: 8.5±0.1 vs. D: 21.3±0.8 mmol/l, 5 h fasted) (Fig 3.6A) and a decrease in plasma insulin levels (C: 2.3±0.4 vs. D: 1.0±0.1 ng/ml) (Table 3.2). BMOV restored plasma glucose levels in the diabetic rats (D: 25.6±4.4 vs. DB: 7.8±0.4 mmol/l, 16 h fasted), without any effect on plasma glucose levels in the control rats (C: 7.6±0.4 vs. CB: 7.6±0.7 mmol/l, 16 h fasted) (Fig 3.6B). There was no detectable difference in plasma insulin levels between BMOV-treated and untreated diabetic rats at the end of treatment (DB: 0.5±0.2 vs. CB: 0.5±0.2 ng/ml), indicating that BMOV did not have any effect on plasma insulin levels in the STZ-diabetic rats (Table 3.2).

Fifteen minutes following insulin injection (5 U/kg, i.v.), plasma glucose levels were significantly lower in the control and BMOV-treated control rats as compared to control animals in the basal state (C basal: 7.6±0.4 vs. C15 min: 2.8±0.2 mmol/l and CB basal: 7.6±0.7 vs. CB 15 min: 2.7±0.2 mmol/l). In the BMOV-treated diabetic rats a glucose lowering effect of insulin was observed at 15 minutes (DB basal: 7.8±0.4 vs. DB 15 min: 2.8±0.1 mmol/l), while plasma glucose levels remained high in the untreated-diabetic rats up to 15 minutes after insulin injection (D basal:
25.6±4.4 vs. D 15 min: 17.0±3.6 mmol/l), indicating insulin resistance in these animals (Fig 3.6B).

Consistent with the results obtained from the first study (study # 1), there was no significant difference in basal PKBα activity in the skeletal muscle between control and STZ-diabetic rats (Fig 3.7). Insulin (5 U/kg) increased PKBα activity with a maximum activation ranging from 12-fold to 15-fold in various groups at 5 minutes after insulin injection. Three weeks treatment with BMOV (0.75-1 mg/ml) in the drinking water did not affect basal or insulin-stimulated PKBα activity in the skeletal muscle of control and diabetic rats. Furthermore, BMOV treatment did not shift the time point of maximum activation of PKBα. There was no detectable difference in PKBα protein expression between BMOV-treated and untreated control and diabetic rats as judged from the Western blots (Fig 3.7).

3.1.4. Effects of BMOV on PKB Activity in the STZ-Diabetic Rats

**Long-Term Study (Study # 4)**

In this study the period of diabetes was extended from 4 to 9 weeks to investigate the association between the development of insulin-resistance in Type 1 diabetes and PKBα activity. PKBα activity was examined in the skeletal muscle at 5 minutes post insulin injection, the time point of maximum activation of the enzyme. Eight weeks treatment with BMOV in the diabetic rats normalized food and fluid intakes, but not body weight. As with the short-term study (4-week study) BMOV did not affect food and fluid intake in the control rats but caused a slight decrease in the body weight (Table 3.3). Consistent with the results from the short-term study, one week following STZ-injection plasma glucose levels were significantly higher in the
diabetic rats than control rats (D: 20.7±1.5 vs. C: 7.3±0.1 mmol/l, 5 h fasted) (Fig 3.8A). Plasma insulin levels were markedly lower in the diabetic rats as compared with the controls during the study. At termination, there was no difference in plasma insulin levels between BMOV-treated and untreated diabetic rats (D: 0.2±0.1 vs. DB: 0.4±0.1 ng/ml) (Table 3.4). Following 5 U/kg insulin, PKBα activity was increased about 14-fold in various experimental groups (C, CB, D and DB) with no significant difference between BMOV-treated and untreated control or diabetic rats (Fig 3.9).
**TABLE 3.1.**

*Wistar Rats (Short-Term Study) (Study # 3)*

General characteristics of animals at the beginning and the end of study

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Fluid intake (ml/day)</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Termination</td>
<td>Beginning</td>
</tr>
<tr>
<td>Control</td>
<td>299±5</td>
<td>396±5</td>
<td>54±2</td>
</tr>
<tr>
<td>C+BMOV</td>
<td>301±5</td>
<td>365±5</td>
<td>43±2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>278±4</td>
<td>311±5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>206±5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D+BMOV</td>
<td>280±4</td>
<td>316±10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>225±9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are shown as means±SEM and represent 5 h fasted levels at the beginning vs. 16 h fasted levels at the end of study. Control: n=21, C+BMOV: n=21, diabetic: n=20, D+BMOV: n=20. Significant changes are indicated by superscripts: <sup>a</sup>n Diabetic vs. corresponding control, <sup>b</sup>n BMOV-treated vs. corresponding untreated group (P<0.05, ANOVA).
**TABLE 3.2.**

Wistar Rats (Short-Term Study) (*Study # 3*)

Plasma insulin levels (ng/ml) during the study

<table>
<thead>
<tr>
<th></th>
<th>Beginning</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3 (Termination)</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td>2.3±0.4</td>
<td>1.9±0.2</td>
<td>1.9±0.1</td>
<td>0.6±0.2</td>
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<tr>
<td><strong>C+BMOV</strong></td>
<td>2.4±0.2</td>
<td>1.3±0.1</td>
<td>1.0±0.1</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td>1.0±0.1(^a)</td>
<td>0.7±0.1(^a)</td>
<td>0.6±0.1(^a)</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td><strong>D+BMOV</strong></td>
<td>1.5±0.1(^a)</td>
<td>1.1±0.1</td>
<td>0.9±0.1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td></td>
<td></td>
<td></td>
<td>2131±242</td>
</tr>
<tr>
<td><strong>2 min</strong></td>
<td></td>
<td></td>
<td></td>
<td>801±40</td>
</tr>
<tr>
<td><strong>5 min</strong></td>
<td></td>
<td></td>
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<td>311±19</td>
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<tr>
<td><strong>15 min</strong></td>
<td></td>
<td></td>
<td></td>
<td>279±34</td>
</tr>
<tr>
<td><strong>200±49</strong></td>
<td></td>
<td></td>
<td></td>
<td>749±78</td>
</tr>
</tbody>
</table>

Values are shown as means±SEM. Termination values represent 16 h fasted levels vs. 5 h fasted levels in the previous weeks. Rats in each group were sacrificed in basal state or at different time points (2, 5 and 15 min) after insulin injection (5 U/kg, i.v.). Control: n=21, C+BMOV: n=21, diabetic: n=20 and D+BMOV: n=20. Significant changes are indicated by superscripts: "\(^a\)" Diabetic vs. corresponding control (P<0.05, ANOVA).
FIGURE 3.6.

WISTAR RATS (SHORT-TERM STUDY): STUDY # 3

Plasma glucose levels in different treatment groups at the beginning (A) and the end (B) of study. Results are presented as means±SEM and values represent 5 h fasted levels at the beginning of study vs. 12 h fasted levels at the end of study. C, CB, D and DB denote control, BMOV-treated control, diabetic and BMOV-treated diabetic rats, respectively. @Different from control group at basal state, *different from corresponding group at basal state, #different from corresponding non-treated group (P<0.05, ANOVA).
FIGURE 3.7.

WISTAR RATS (SHORT-TERM STUDY): STUDY # 3

The effect of BMOV treatment on basal and insulin-induced PKBα activity in the skeletal muscle from 16 h fasted control and STZ-diabetic Wistar rats after three weeks of treatment with BMOV. PKBα activity in a pooled muscle homogenate from control Wistar rats was taken as 100%. Results are presented as the means±SEM of one experiment performed in triplicate for each muscle homogenate, n=5-7 per group. The lower panel is a representative Western blot of PKBα immunoprecipitates (IP), showing the equal amount of immunoprecipitated protein in different groups. C: Control, D: STZ-diabetic, CB: BMOV-treated control, DB: BMOV-treated diabetic. *Different from corresponding insulin injected groups (P<0.05, ANOVA).
Time After Insulin Injection

PKB\(\alpha\) Activity (% of Control)

- Basal
- 2 min
- 5 min
- 15 min

IP: PKB\(\alpha\)-PH
Blot: PKB\(\alpha\)-PH

- Insulin
+ Insulin

p60 PKB\(\alpha\)
### TABLE 3.3.

**Wistar Rats (Long-Term Study) (Study #4)**

General characteristics of animals at the beginning and the end of study

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Fluid intake (ml/day)</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Termination</td>
<td>Beginning</td>
</tr>
<tr>
<td>Control</td>
<td>407±8</td>
<td>486±12</td>
<td>57±1</td>
</tr>
<tr>
<td>C+BMOV</td>
<td>364±9</td>
<td>416±12</td>
<td>54±1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>343±8</td>
<td>342±5</td>
<td>277±1</td>
</tr>
<tr>
<td>D+BMOV</td>
<td>340±10</td>
<td>390±17</td>
<td>262±6</td>
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</table>

Values are shown as means±SEM and represent 5 h fasted levels at the beginning vs. 16 h fasted levels at the end of study. Control: n=9, C+BMOV: n=8, diabetic: n=11, D+BMOV: n=8. Significant changes are indicated by superscripts: "a" Diabetic vs. corresponding control, "b" BMOV-treated vs. corresponding untreated group (P<0.05, ANOVA).
### TABLE 3.4.

**Wistar Rats (Long-Term Study) (Study # 4)**

Plasma insulin levels (ng/ml) during the study

<table>
<thead>
<tr>
<th></th>
<th>Beginning</th>
<th>Week 4</th>
<th>Week 9 (Termination)</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>1.6±0.2</td>
<td>1.1±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>C+BMOV</td>
<td>1.7±0.1</td>
<td>0.9±0.2</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.6±0.2</td>
<td>0.6±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>D+BMOV</td>
<td>1.7±0.2</td>
<td>0.6±0.1</td>
<td>0.4±0.1</td>
</tr>
</tbody>
</table>

Values are shown as means±SEM. Termination values represent 16 h fasted levels vs. 5 h fasted levels in the previous weeks. Rats in each group were sacrificed in basal state or 5 minutes after insulin injection (5 U/kg, i.v.). Control: n=9, C+BMOV: n=8, diabetic: n=11 and D+BMOV: n=8. Significant changes are indicated by superscripts: "a" Diabetic vs. control, (P<0.05, ANOVA).
FIGURE 3.8.

WISTAR RATS (LONG-TERM STUDY): STUDY # 4

Plasma glucose levels in different treatment groups at the beginning (A) and the end (B) of study. Results are presented as means±SEM and values represent 5 h fasted levels at the beginning of study vs. 12 h fasted levels at the end of study. C, CB, D and DB denote control, BMOV-treated control, diabetic and BMOV-treated diabetic rats, respectively. *Different from control group at basal state, #different from corresponding non-treated group (P<0.05, ANOVA).
A) Beginning

Plasma Glucose (mmol/l)

- C
- CB
- D
- DB

B) Termination

Plasma Glucose (mmol/l)

- C
- CB
- D
- DB

Basal
0 U/kg
5 U/kg

5 min

Page 91
FIGURE 3.9.

WISTAR RATS (LONG-TERM STUDY): STUDY #4

The effect of BMOV treatment on basal and insulin-induced PKBα activity in the skeletal muscle from 16 h fasted control and STZ-diabetic Wistar rats after eight weeks of treatment with BMOV. PKBα activity in a pooled muscle homogenate from control Wistar rats was taken as 100%. Insulin-induced PKBα activity was measured at the time point of maximum activation of the enzyme in the skeletal muscle (5 min). Results are presented as the means±SEM of one experiment performed in triplicate for each muscle homogenate, n=3-5 per group. The lower panel is a representative Western blot of PKBα immunoprecipitates (IP), showing the equal amount of immunoprecipitated protein in different groups. C: Control, D: STZ-diabetic, CB: BMOV-treated control, DB: BMOV-treated diabetic. *Different from corresponding insulin injected groups (P<0.05, ANOVA).
3.1.5. Effects of BMOV on PKB Activity in Zucker Rats (Study # 5)

In this study the effects of BMOV treatment on altered levels of insulin-induced PKBα activity, which were observed in the skeletal muscle and liver of fatty Zucker rats, were investigated. General characteristics of animals were monitored during the study on a daily (body weight, food and fluid intakes) or weekly (plasma glucose and insulin levels) basis. Body weights were significantly higher in fatty Zucker rats than lean rats. Treatment with BMOV in the drinking water (0.75-1 mg/ml) for three weeks resulted in a modest decrease in body weight and fluid intake in fatty Zucker rats, without any effect on food intake. In lean rats BMOV treatment did not affect body weight or food intake but caused a slight decrease in fluid intake (Table 3.5).

At the beginning of the study (14-16 weeks old) there was no marked difference in plasma glucose levels between lean and fatty Zucker rats (L: 7.3±0.4 vs. F: 8.8±0.4 mmol/l, 5 h fasted) (Fig 3.1A). However, plasma insulin levels were significantly higher in the fatty rats (L: 1.1±0.1 vs. F: 12.8±1.1 ng/ml, 5 h fasted) (Table 3.6). Plasma glucose levels in 18-20 weeks old Zucker rats ranged from 7.3±0.6 to 9.5±0.5 mmol/l, 16 h fasted), without any marked difference between lean and fatty Zucker rats (Fig 3.1B). At the end of study, plasma insulin levels were significantly higher in fatty Zucker rats as compared to their lean controls (F: 5.1±0.8 vs. L: 2.5±0.4 ng/ml, (16 h fasted) (Table 3.6). Insulin injection (5 U/kg) lowered plasma glucose levels after 15 minutes in lean Zucker rats (L basal: 8.2±0.4 vs. L15 min: 3.8±0.2 mmol/l) but did not decrease plasma glucose levels in fatty Zucker rats.
(F basal: 9.5±0.5 vs. F15 min: 9.3±0.7 mmol/l), indicating a resistance to the glucoregulatory effects of insulin (Fig 3.10B).

After three weeks of treatment with BMOV plasma glucose levels remained unchanged in the BMOV-treated lean and fatty Zucker rats as compared to untreated animals (L: 8.2±0.4 vs. LB: 7.3±0.6, F: 9.5±0.5 vs. FB: 8.7±0.7 mmol/l, 16 h fasted). However, plasma insulin levels in BMOV-treated fatty Zucker rats were reduced to lean values (F: 5.1±0.8 vs. FB: 3.3±0.7 ng/ml, P<0.05), indicating an improvement in insulin sensitivity following BMOV treatment. In addition, insulin sensitivity indices (ISI) calculated from the results of OGTT, showed a 33% increase in insulin sensitivity in BMOV-treated fatty Zucker rats (F: 0.8±0.1 vs. FB: 1.2±0.1, P<0.05) (Fig 3.10C). There was no significant difference in insulin sensitivity index between BMOV-treated and untreated lean rats (L: 10.9±0.8 vs. LB: 13.0±0.8, P>0.05). However, there was a marked difference in insulin sensitivity index between lean and fatty Zucker rats due to the severe insulin resistance in fatty Zucker rats.

Insulin activated PKBα in the skeletal muscle of both lean and fatty Zucker rats (Figs 3.11). Maximum activation of PKBα was observed at 5 and 15 minutes in fatty and lean Zucker rats, respectively (F: 6.4-fold vs. L: 16-fold). Although BMOV reduced plasma insulin and increased insulin sensitivity in the fatty Zucker rats, it did not restore the reduced levels of insulin-induced PKBα activity in the skeletal muscle (Fig 3.11). Furthermore, BMOV treatment was unable to normalize the elevated levels of insulin-induced PKBα activity in the liver of fatty Zucker rats (Fig 3.12). Densitometry, performed on immunoprecipitated PKBα bands, showed that there
was no detectable difference in PKBα protein expression between lean and fatty Zucker rats. This finding indicated that changes in PKBα activity in the skeletal muscle and liver of fatty Zucker rats were not associated with alterations in the protein expression. There was not any significant difference in basal or insulin-stimulated PKBβ activity in the skeletal muscle between BMOV-treated and untreated lean and fatty Zucker rats (Fig 3.13).
### TABLE 3.5.

**Zucker Rats (4-Week Study) (Study # 5)**

General characteristics of animals at the beginning and the end of study

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Fluid intake (ml/day)</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Termination</td>
<td>Beginning</td>
</tr>
<tr>
<td>Lean</td>
<td>360±6</td>
<td>356±7</td>
<td>53±2</td>
</tr>
<tr>
<td>L+BMVO</td>
<td>371±7</td>
<td>335±14</td>
<td>45±3</td>
</tr>
<tr>
<td>Fatty</td>
<td>538±11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>537±9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47±3</td>
</tr>
<tr>
<td>F+BMVO</td>
<td>513±9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>482±13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>50±3</td>
</tr>
</tbody>
</table>

Values are shown as means±SEM and represent 5 h fasted levels at the beginning vs. 16 h fasted levels at the end of study. Lean: n=17, L+BMVO: n=21, Fatty: n=24, F+BMVO: n=18. Significant changes are indicated by superscripts: <sup>a</sup>Fatty vs. corresponding lean, <sup>b</sup>BMVO-treated vs. corresponding untreated group (P<0.05, ANOVA).
TABLE 3.6.

Zucker Rats (4-Week Study) *(Study # 5)*

Plasma insulin levels (ng/ml) at the beginning and the end of study

<table>
<thead>
<tr>
<th></th>
<th>Beginning</th>
<th>Basal</th>
<th>5 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.1±0.1</td>
<td>2.5±0.4</td>
<td>750±175</td>
<td>502±44</td>
</tr>
<tr>
<td>L+BMOV</td>
<td>1.5±0.2</td>
<td>1.9±0.4</td>
<td>966±266</td>
<td>655±286</td>
</tr>
<tr>
<td>Fatty</td>
<td>12.8±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2267±724</td>
<td>861±137</td>
</tr>
<tr>
<td>F+BMOV</td>
<td>11.9±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1708±590</td>
<td>1086±137</td>
</tr>
</tbody>
</table>

Values are shown as means±SEM. Termination values represent 16 h fasted levels vs. 5 h fasted levels in the previous weeks. Rats in each group were sacrificed in basal state or at different time points (5 and 15 min) after insulin injection (5 U/kg, i.v.). Lean: n=17, L+BMOV: n=21, fatty: n=24 and F+BMOV: n=18. Significant changes are indicated by superscripts: "<sup>a</sup>" Fatty vs. corresponding lean, "<sup>b</sup>" F+BMOV vs. fatty (P<0.05, ANOVA).
Plasma glucose levels in different treatment groups at the beginning (A) and the end (B) of study. Results are presented as means±SEM and values represent 5 h fasted levels at the beginning of study vs. 12 h fasted levels at the end of study. Figure C shows insulin sensitivity indices (ISI) in fatty Zucker rats pre-treatment and post-treatment with BMOV. ISI was calculated using the following formula: K/SQRT ((fasted plasma glucose × fasted plasma insulin) × (mean plasma glucose × mean plasma insulin)), K=100. Fasting plasma glucose and insulin values were the time 0 values (16 h fasted) and the mean plasma glucose and insulin values were calculated as the mean value of all time points measured in the test. L, LB, F and FB denote lean, BMOV-treated lean, fatty and BMOV-treated fatty rats, respectively. *Different from corresponding group at basal state (P<0.05, ANOVA), @different from corresponding non-treated group (P<0.05, t-test).
ZUCKER STUDY (4-WEEK STUDY): STUDY # 5

The effect of BMOV on basal and insulin-induced (5 U/kg, i.v.) PKBα activity in the skeletal muscle of 16 h fasted Zucker rats, 5 and 15 minutes after insulin injection. Rats were treated with BMOV in the drinking water for three weeks. PKBα activity in a pooled muscle homogenate obtained from control lean rats was taken as 100%. Results are presented as means±SEM of one assay performed in triplicate for each animal, n=7-10 per group (basal and 5 min), n=3-4 per group (15 min). The lower panel is a representative Western blot of PKBα immunoprecipitates (IP), showing the equal amount of precipitated protein in different groups. L: Lean, LB: BMOV-treated lean, F: Fatty and FB: BMOV-treated fatty. *Different from corresponding group at basal state, # different from corresponding lean group (P<0.05, ANOVA).
Basal 5 min 15 min

Time After Insulin Injection

PKBα Activity (% of Control)

IP: PKBα-PH
Blot: PKBα-CT

- Insulin + Insulin
FIGURE 3.12.

ZUCKER STUDY (4-WEEK STUDY): STUDY #5

The effect of BMOV on basal and insulin-induced (5 U/kg, i.v.) PKBα activity in the liver of 16 h fasted Zucker rats, 5 and 15 minutes after insulin injection. Rats were treated with BMOV in the drinking water for three weeks. PKBα activity in a pooled liver homogenate obtained from lean Zucker rats was taken as 100%. Results are presented as means±SEM of one assay performed in triplicate for each animal, n=7-10 per group (basal and 5 min), n=3-4 per group (15 min). The lower panel is a representative Western blot of PKBα immunoprecipitates (IP), showing the equal amount of precipitated protein in different groups. L: Lean, LB: BMOV-treated lean, F: Fatty and FB: BMOV-treated fatty. *Different from corresponding group at basal state, # different from corresponding lean group (P<0.05, ANOVA).
Basal 5 min 15 min

Time After Insulin Injection

PKBα Activity (% Control)

- Insulin  + Insulin

IP: PKBα-PH
Blot: PKBα-CT

Page 104
ZUCKER STUDY (4-WEEK STUDY): STUDY # 5

The effect of BMOV on basal and insulin-induced (5 U/kg, i.v.) PKBβ activity in the skeletal muscle of 16 h fasted Zucker rats, 5 minutes after insulin injection. Rats were treated with BMOV in the drinking water for three weeks. PKBα activity in a pooled muscle homogenate obtained from control lean rats was taken as 100%. Results are presented as means±SEM of one assay performed in triplicate for each animal, n=7-10 per group. The lower panel is a representative Western blot of PKBβ immunoprecipitates (IP), showing the equal amount of precipitated protein in different groups. The band shift observed following insulin injection indicates phosphorylation of PKBβ. L: Lean, LB: BMOV-treated lean, F: Fatty and FB: BMOV-treated fatty. *Different from corresponding group at basal state (P<0.05, ANOVA).
Basal 5 min

Time After Insulin Injection

PKBβ Activity (% of Control)

0 100 200 300 400 500 600

L
LB
F
FB

* * *

Band shift

IP: Anti-PKBβ
Blot: Anti-PKβ

- Insulin + Insulin

Page 106
3.2. EFFECTS OF BMOV ON THE KEY GLUCONEOGENIC ENZYMES IN STZ-DIABETIC RATS (Study # 6)

General characteristics and plasma parameters of the animals in different treatment groups are summarized in Tables 3.7-3.10. At the end of study, body weights were significantly lower in the diabetic and BMOV-treated diabetic groups as compared to control group (Table 3.7). Treatment with BMOV did not improve the growth retardation in diabetic rats, while one week after receiving insulin there was a significant difference in body weight between insulin-treated and untreated diabetic rats, showing the anabolic effects of insulin as compared to BMOV. BMOV treatment in control rats showed a slight but significant decrease in body weight gain compared with untreated control rats.

The attenuation of hyperglycemia in BMOV-treated diabetic rats was accompanied by normalization of food and fluid intakes (Table 3.7). There was no significant difference in food or fluid intakes between control and BMOV-treated diabetic rats at the end of study. Insulin treatment of diabetic rats for one week resulted in a significant decrease in the fluid intake, but not food intake.

Plasma glucose and insulin levels were measured weekly by sampling blood from the tail between 1000-1100 h in fed rats (Table 3.8). Streptozotocin injection resulted in a profound increase in the plasma glucose concentration and a significant decrease in plasma insulin levels in Wistar rats. Two weeks after initiation of BMOV treatment, plasma glucose levels were significantly lower in the BMOV-treated diabetic rats as compared to untreated diabetic rats [DB (wk 2): 18.2±4.5 vs. D (wk 2): 26.9±1.9 mmol/l, P<0.05]. The effects of BMOV on plasma glucose were even
more evident after 4 weeks of treatment [DB (wk 4): 16.3±2.7 vs. D (wk 4): 29.2±1.9 mmol/l, \( P<0.05 \)]. There was no significant difference in the plasma insulin levels between BMOV-treated and untreated diabetic rats, indicating that treatment with BMOV did not have any significant effect on plasma insulin levels (Table 3.9). Consistent with our previous studies, BMOV treatment had no effect on plasma glucose levels in the control rats (Table 3.8). However, plasma insulin levels were slightly lower in BMOV-treated control rats as compared with untreated rats (Table 3.9). One week after treatment of diabetic rats with insulin, plasma glucose levels dropped from 24.5±1.2 to 5.7±1.8 mmol/l. Plasma glucagon levels were about 2-fold higher in the diabetic rats as compared to control rats and treatment with either BMOV or insulin markedly lowered plasma glucagon levels (Table 3.10). There was no significant difference in the glucagon levels between control and BMOV-treated diabetic rats (CB: 60.5±4.8 vs. DB: 71.9±1.0 pg/ml, \( P>0.05 \)). Elevated levels of plasma triglycerides were normalized after treatment with both BMOV and insulin (Table 3.10).

3.2.1 PEPCK Activity and mRNA Expression (Study # 6)

Measurement of enzyme activity in the fed rats showed that PEPCK activity was significantly higher in the liver of diabetic rats as compared to control animals (D: 52.3±2.5 vs. C: 31.8±1.9 mU/mg protein, \( P<0.05 \)) (Fig 3.14A). Four weeks of treatment with BMOV completely normalized PEPCK activity in the diabetic rats (DB: 32.8±2.7 vs. C: 31.8±1.9 mU/mg protein, \( P>0.05 \)). Results from an RT-PCR assay indicated, that in parallel with the elevated enzyme activity, PEPCK mRNA levels were significantly increased in the diabetic rats and were restored to normal levels,
following BMOV treatment (Fig 3.15A). As with liver, renal PEPCK activity was significantly higher in the diabetic rats compared with controls (D: 63.4±3.0 vs. C: 40.2±4.9 mU/mg protein, P<0.05) (Fig 3.14B). This increase in enzyme activity was accompanied with an increase in mRNA levels in the kidney of diabetic rats (Fig 3.15B). Both PEPCK mRNA expression and activity in the kidney of diabetic rats were normalized by BMOV treatment (C: 40.2±4.9 vs. DB: 43.9±2.7 mU/mg protein, P>0.05). BMOV had no apparent effect on PEPCK activity or mRNA in the liver or kidney of control rats. Treatment with insulin for one week restored PEPCK mRNA and activity in both tissues of diabetic rats (Figs 3.14 and 3.15).

3.2.2 G6Pase mRNA Expression (Study # 6)

STZ-diabetes resulted in a profound increase in G6Pase mRNA levels in the liver of diabetic rats and to the same extent in the kidney, as compared with control animals (Fig 3.16). The increase in G6Pase mRNA (2 to 3-fold) was more marked than that of PEPCK mRNA (somewhat less than 2-fold) in the STZ-diabetic rats, after 5 weeks of diabetes. Treatment with BMOV completely normalized the elevated levels of G6Pase in the liver (Fig 3.16A) and kidney (Fig 3.16B) of diabetic animals, while it did not have any effect on G6Pase mRNA expression in the control rats. Treatment with insulin for one week decreased the diabetes-induced increase in G6Pase mRNA to the same extent that was seen with BMOV.
TABLE 3.7.

**Wistar Rats (Study #6)**

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Fluid intake (ml/day)</th>
<th>Food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Termination</td>
<td>Beginning</td>
</tr>
<tr>
<td>Control</td>
<td>319±8</td>
<td>462±15</td>
<td>43±1</td>
</tr>
<tr>
<td>C+BMOV</td>
<td>324±5</td>
<td>408±8</td>
<td>44±2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>292±11</td>
<td>317±21</td>
<td>210±6</td>
</tr>
<tr>
<td>D+BMOV</td>
<td>306±7</td>
<td>323±35</td>
<td>250±14</td>
</tr>
<tr>
<td>D+Insulin</td>
<td>298±10</td>
<td>404±19</td>
<td>190±12</td>
</tr>
</tbody>
</table>

Values are presented as means±SEM. Measurements were performed in the fed rats (between 1000-1100 h) at the beginning and the termination of study (n=5 per group). Significant changes are indicated by superscripts: *a* Diabetic vs. corresponding control, *b* Diabetic + insulin vs. control, *c* BMV-treated vs. corresponding untreated group (P<0.05, ANOVA).
TABLE 3.8.

Wistar Rats (Study # 6)

Plasma glucose levels (mmol/l) during the study

<table>
<thead>
<tr>
<th></th>
<th>Beginning</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.3±0.1</td>
<td>7.7±0.1</td>
<td>7.7±0.4</td>
<td>7.4±0.2</td>
<td>9.9±0.5</td>
</tr>
<tr>
<td>Control + BMOV</td>
<td>8.8±0.2</td>
<td>7.7±2.2</td>
<td>6.9±0.1</td>
<td>7.0±0.1</td>
<td>10.0±0.7</td>
</tr>
<tr>
<td>Diabetic</td>
<td>27.9±1.1^a</td>
<td>29.5±1.7^a</td>
<td>26.9±1.9^a</td>
<td>26.5±0.9^a</td>
<td>29.2±1.9^a</td>
</tr>
<tr>
<td>Diabetic + BMOV</td>
<td>28.8±0.9^a</td>
<td>24.1±4.3^ab</td>
<td>18.2±4.5^ab</td>
<td>17.0±2.4^ab</td>
<td>16.3±2.7^ab</td>
</tr>
<tr>
<td>Diabetic + Insulin*</td>
<td>30.8±1.2^a</td>
<td>29.0±1.5^a</td>
<td>28.8±1.0^a</td>
<td>24.5±1.2^a</td>
<td>5.7±1.8^cd</td>
</tr>
</tbody>
</table>

Values are presented as means±SEM. Measurements were performed in the fed rats every week (between 1000-1100 h).
* Rats in the "diabetic + insulin" group received insulin implants at the end of week three (one week before the termination). Significant changes are indicated by superscripts: "^a" Diabetic vs. corresponding control, "^b" BMOV-treated vs. corresponding untreated group, "^c" Diabetic + insulin vs. control, "^d" Diabetic + insulin vs. Diabetic + BMOV (P<0.05, ANOVA).
<table>
<thead>
<tr>
<th></th>
<th>Beginning</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2±0.4</td>
<td>2.7±0.2</td>
<td>2.4±0.2</td>
<td>3.0±0.3</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>Control + BMOV</td>
<td>2.4±0.5</td>
<td>1.3±0.3b</td>
<td>1.1±0.1b</td>
<td>1.2±0.2b</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.7±0.1a</td>
<td>0.6±0.1a</td>
<td>0.4±0.1a</td>
<td>0.3±0.1a</td>
<td>0.5±0.2a</td>
</tr>
<tr>
<td>Diabetic + BMOV</td>
<td>0.6±0.1a</td>
<td>0.5±0.1a</td>
<td>0.4±0.1a</td>
<td>0.5±0.2a</td>
<td>0.5±0.2a</td>
</tr>
<tr>
<td>Diabetic + Insulin*</td>
<td>0.6±0.1a</td>
<td>0.4±0.1a</td>
<td>0.3±0.1a</td>
<td>0.3±0.1a</td>
<td>7.9±1.8abcd</td>
</tr>
</tbody>
</table>

Values are presented as means±SEM. Measurements were performed in the fed rats every week between 1000-1100 h. * Rats in the "diabetic + insulin" group received insulin implants at the end of week three (one week before the termination). Significant changes are indicated by superscripts: "a" Diabetic vs. corresponding control, "b" BMOV-treated vs. corresponding untreated group, "c" Diabetic + insulin vs. control, "d" Diabetic + insulin vs. diabetic + BMOV (P<0.05, ANOVA).
### Table 3.10

**Wistar Rats (Study # 6)**

Effects of BMOV on plasma glucagon and triglycerides

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucagon (pg/ml)</th>
<th>Triglycerides (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49.1±2.9</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Control + BMOV</td>
<td>60.5±4.8</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>100.0±3.8(^a)</td>
<td>5.6±1.6(^a)</td>
</tr>
<tr>
<td>Diabetic + BMOV</td>
<td>71.9±1.0(^b)</td>
<td>1.0±0.1(^b)</td>
</tr>
<tr>
<td>Diabetic + Insulin</td>
<td>63.6±5.3(^b)</td>
<td>0.3±0.1(^b)</td>
</tr>
</tbody>
</table>

Measurements were performed in the fed rats (between 1000-1100 h) after four weeks of treatment with BMOV in the drinking water (0.75-1 mg/ml) or one week treatment with insulin (4 U/day). Values are presented as the means±SEM. Significant changes are indicated by superscripts: "\(^a\)" Diabetic vs. control, "\(^b\)" Diabetic + BMOV or diabetic + insulin vs. diabetic (P<0.05, ANOVA).
FIGURE 3.14.

STZ-DIABETIC WISTAR RATS: STUDY # 6

Effects of chronic treatment with BMOV and insulin on PEPCK activity in the liver (A) and kidney (cortex) (B) from fed control and STZ-diabetic rats. Four weeks after treatment with BMOV or one week after treatment with insulin (4 U/day) rats were sacrificed in the fed state. PEPCK activity in the liver or kidney homogenates (0.3 mg of protein) was measured as described in Methods. The reaction was initiated by adding dGTP and a reaction mixture without dGTP was used as control for each sample. C, CB, D, DB and DI denote control, BMOV-treated control, diabetic, BMOV-treated diabetic and insulin-treated diabetic, respectively (n=5 per group). Results are presented as means±SEM of PEPCK activity in each group. *Different from all other groups (P<0.05, ANOVA).
FIGURE 3.15.

STZ-DIABETIC WISTAR RATS: STUDY # 6

Effects of treatment with BMOV and insulin on PEPCK mRNA levels in the liver (A) and kidney (cortex) (B) of control and STZ-diabetic rats. Four weeks after treatment with BMOV or one week after treatment with insulin rats were sacrificed in the fed state. RNA was extracted from the liver or kidney tissues and used for RT/PCR assay. PEPCK mRNA abundance was normalized against 18S rRNA levels, which did not vary significantly between samples. Results are presented as means±SEM of the relative optical densities (PEPCK/18S internal control) of scanned images in each group. C, CB, D, DB and DI denote control, BMOV-treated control, diabetic, BMOV-treated diabetic and insulin-treated diabetic, respectively (n=5 per group). *Different from all other groups (P<0.05, ANOVA). The lower panels show representative analysis of PCR products from different treatment groups following electrophoresis on 2% agarose gel.
FIGURE 3.16.

STZ-DIABETIC WISTAR RATS: STUDY # 6

Effects of treatment with BMOV and insulin on G6Pase mRNA levels in the liver (A) and kidney (cortex) (B) of control and STZ-diabetic rats. Four weeks after treatment with BMOV or one week after treatment with insulin rats were sacrificed in the fed state. RNA was extracted from the liver or kidney tissues and used for RT/PCR assay. G6Pase mRNA abundance was normalized against 18S rRNA levels, which did not vary significantly between samples. Results are presented as means±SEM of the relative optical densities (PEPCK/18S internal control) of scanned images in each group. C, CB, D, DB and DI denote control, BMOV-treated control, diabetic, BMOV-treated diabetic and insulin-treated diabetic, respectively (n=5 per group). *Different from all other groups (P<0.05, ANOVA). The lower panels show representative analysis of PCR products from different treatment groups following electrophoresis on 2% agarose gel.
A) Liver

B) Kidney

Treatment Groups

Relative G6Pase mRNA (G6Pase/18S)

G6Pase 562 bp

18S

C  CB  D  DB  DI
CHAPTER 4
DISCUSSION

4.1. Overview

Insulin resistance is a common finding in both Type 1 and Type 2 diabetes, although the underlying mechanism is different. Impairment in insulin action is accompanied by a decrease in glucose uptake in the peripheral tissues as well as an increase in hepatic glucose production, which both contribute to the hyperglycemia in diabetes [18, 20]. Despite numerous studies in the past decade the molecular mechanisms causing insulin resistance are still not well understood. During recent years much attention has been focused on early (i.e. IRS proteins and PI3-K) and intermediate (i.e. PKB) steps in the insulin signaling cascade. However, whether these signaling defects are causative in the development of insulin resistance or secondary to the metabolic disorders associated with diabetes has yet to be investigated. Studies which attempted to find genetic mutations failed to show any changes in the structure of genes encoding the proteins downstream of insulin receptor that could explain impaired insulin action [16, 337, 338], supporting the idea that post-transcriptional changes at the level of protein expression and/or activity of these enzymes rather than genetic defects contribute to the development of insulin resistance.

PKB has been proposed to be an intermediate protein kinase in the insulin signaling pathway by which insulin controls glycogen synthesis [48, 49, 151] and glucose uptake [148, 149, 150] in insulin sensitive tissues as well as hepatic glucose output [154, 155]. Many studies have shown that insulin-induced glucose uptake
and glycogen synthesis, both of which are decreased in diabetes [18, 20, 27, 29], are mediated by PI3-K via activation of PKB [49, 157, 339, 340], suggesting a potential role for PKB in the development of insulin resistance in diabetes. To determine whether impaired PKB activation contributes to insulin resistance, in vivo, we examined the expression, phosphorylation and kinase activity of PKB in the skeletal muscle and liver, two insulin responsive tissues. Basal and insulin-induced PKB activity were investigated in two animal models of diabetes: STZ-diabetic Wistar rat, an animal model of poorly controlled Type 1 diabetes and fatty Zucker rat (fa/fa), an animal model that represents several characteristics of Type 2 diabetes. Furthermore, basal PKB activity was compared in the pre-diabetic (fatty Zucker rats) and diabetic (ZDF rats) state. We chose skeletal muscle since it is known to be the primary site of insulin resistance in Type 2 diabetes [20, 27]. Because of its mass, skeletal muscle is the major target for insulin-induced glucose disposal and accounts for approximately 80% of insulin-stimulated glucose disposal [341, 342]. We also studied PKB activity in the liver, the main site of gluconeogenesis, which contributes to elevated fasting plasma glucose levels in diabetes [20]. While much attention has been focused on investigating alterations in insulin signaling proteins in the skeletal muscle, little is known about the in vivo regulation of PKB activity in the liver during diabetes. Glucose uptake in the liver is not rate-limiting for glucose utilization [18]. However, as with muscle, insulin-induced activation of glycogen synthase in hepatocytes is mediated via the PI3-K/PKB/GSK-3 axis [48], although glucose-6-phosphate also contributes to the regulation of glycogen synthesis in the liver by its allosteric effects on glycogen synthase activity [47].
Many studies from this laboratory and other laboratories have shown that vanadium compounds lower plasma glucose levels in Type 1 diabetes [207, 211] and improve insulin-sensitivity in Type 2 diabetes and animal models of insulin resistance [222, 228, 343-345]. Hence, these compounds are candidates for oral therapy in both Type 1 and Type 2 diabetes. However, the mechanism(s) by which vanadium produces its in vivo anti-diabetic effects are still under investigation. Several potential sites in the insulin signaling cascade, including both receptor and non-receptor pathways have been proposed for the insulin-like effects of vanadium [187, 236, 240, 242]. PKB because of its crucial role in most of the metabolic effects of insulin and its possible association with insulin resistance seems to be a potential target for vanadium action. In the present study, the effects of chronic treatment with BMOV on PKB activity and protein expression were investigated in both STZ-diabetic rats and fatty Zucker rats to examine the possible role of PKB in mediating the glucoregulatory effects of BMOV in vivo. Also, in an attempt to find more selective targets for the glucose lowering effects of vanadium in vivo, PEPCK and G6Pase, two key enzymes in gluconeogenesis, which have also been proposed to be downstream metabolic targets for PKB, were measured in STZ-diabetic rats.

4.2. TIME COURSE STUDIES

Similar to other in vitro [104, 111] and in vivo studies [105, 340], we found that insulin (5 U/kg, i.v.) increased PKBα activity more than 10-fold and PKBβ activity more than 3-fold in the skeletal muscle and liver of both animal models (Figs 3.1-3.4). Insulin-induced activation of PKBα was observed as early as 1 minute with a maximum activation occurring at 5 and 15 minutes after insulin injection in the
skeletal muscle and liver, respectively. Also, the increased activation of PKB by insulin was accompanied by an increase in its phosphorylation (Figs 3.1 and 3.2). In agreement with this study Krook et al. [346] reported that insulin induced a maximal 10-fold increase in PKBα activity in human skeletal muscle. In addition, the time point of PKB activation in this study, correlated with that of PI3-K in vivo [347], further supporting the understanding that PKB is an enzyme downstream of PI3-K in the signaling pathway. Furthermore, the maximum fold increase in PKBα activity was almost the same in liver and muscle, indicating that 5 U/kg insulin was sufficient to achieve maximal stimulation of PKBα in both tissues (Figs 3.1 and 3.2). Another finding in this experiment was that regardless of the rat strain, basal PKBα activity in the skeletal muscle was twice of that in the liver (Fig 3.3A). Interestingly, the same ratio was observed in the maximum insulin-stimulated PKBα activity (Fig 3.3B). Previously, Walker et al. [105] had shown that protein expression of PKBα in the skeletal muscle was about half of that in the hepatocytes. Taken together, these observations indicate that PKBα kinase activity per microgram protein in the muscle is higher than that in the liver. In agreement with other studies, insulin increased PKBβ activity in the skeletal muscle and liver of both animal models, although to a lesser extent than PKBα (3- to 6-fold). Both isoforms were activated by insulin with a similar time course (Figs 3.1-3.4).

4.3. BASAL PKBα ACTIVITY IN ZDF RATS

To investigate the effects of chronic hyperinsulinemia and hyperglycemia on PKB activity in Type 2 diabetes, basal PKBα activity was measured in two animal models of insulin resistance and diabetes at normoglycemic hyperinsulinemic state.
(fatty Zucker rat) and hyperglycemic normoinsulinemic state (ZDF rats). ZDF rats were sacrificed at 12 weeks of age when they were hyperglycemic and normoinsulinemic. Results demonstrated that although there were some strain-dependent differences in basal PKBα activity among rats, there was no detectable difference in basal PKBα activity in the muscle or liver between fatty Zucker rats or ZDF rats and their appropriate lean controls (Fig 3.5), suggesting that basal PKBα activity is likely to be more genetically dependent and tissue specific rather than being dependent on the diabetic state of the animals. Consistent with this findings, in vivo studies in human subjects [114, 346] and other animal models of Type 2 diabetes [151, 348] have also shown that, despite the changes found in insulin-induced PKB activity, basal enzyme activity remained unchanged during diabetes.

4.4. PKB ACTIVITY IN STZ-DIABETIC RATS

Hypoinsulinemia and associated hyperglycemia in STZ-diabetic rats (Fig 3.6 and Table 3.2) were not accompanied by any detectable changes in basal PKBα activity or its protein expression in either skeletal muscle or liver from STZ-diabetic Wistar rats (Figs 3.1 and 3.7). Furthermore, insulin stimulated PKBα activity in STZ-diabetic rats to a similar extent as in control rats in both skeletal muscle (C: 12-fold vs. D: 10.5-fold) and liver (C: 13.7-fold vs. D: 15.7-fold) (Fig 3.1). These findings indicated that 1) basal PKBα activity was not impaired in STZ-diabetic rats up to 4 weeks of diabetes; 2) response to exogenous insulin was normal at the level of PKB; 3) STZ-induced diabetes did not shift the time point of maximum activation of PKB (Fig 3.7). Previous results from our laboratory have shown that both basal and insulin-induced IRS-1 associated PI3-K activity were also normal in STZ-diabetic
rats [347]. However, studies on glycogen synthase, the rate-limiting enzyme in glycogen synthesis, and its two regulatory enzymes, PP-1 and GSK-3 showed that despite normal basal and insulin-induced inhibition of GSK-3 and activation of glycogen synthase, basal PP-1 activity was elevated in these animals [349]. Hence, it appears that although the PI3-K/PKB/GSK-3/GS axis is intact in STZ-diabetic rats up to 4 weeks of diabetes, alterations in other parts of the pathway (i.e. PP-1) may positively or negatively contribute to the metabolic defects observed in this model. It has been reported that recent-onset STZ-diabetes is accompanied by an increase in the expression of insulin receptor (IR) and insulin-induced phosphorylation of IR and IRS-1, indicating that STZ-diabetes is associated with up-regulation in early steps of insulin signaling cascade which is likely to be time dependent [350, 351].

A previous study showed that insulin-stimulated (but not basal) PKBα activity was slightly elevated in STZ-diabetic Male Lewis rats as compared with control rats and that islet transplantation normalized PKBα activity [93]. One possible explanation for the difference observed between the results of these two studies is that factors such as animal strain, insulin dose and time point at which PKB activity was measured were different. The key observation addressed in our study was that despite the chronic hyperglycemia, insulin signaling remained unchanged in STZ-diabetic rats up to 4 weeks of diabetes. Hence, chronic hyperglycemia (glucotoxicity) may not be a sufficient explanation for causing impairment in the insulin signaling at least in some models of diabetes. Recently, it was reported that, the signaling cascade from insulin receptor to PKB was markedly altered in the skeletal muscle of STZ-diabetic rats, which had received streptozotocin during their
neonatal period (nSTZ). Correction of plasma glucose with T-1095, a Na\(^+\)-glucose co-transporter inhibitor, reversed all the changes, indicating that these effects may be associated with the chronic hyperglycemia [352]. The neonatal STZ-diabetic rat represents an animal model of Type 2 diabetes. Hence, these findings indicate that inducing Type 2 diabetes by giving streptozotocin to young rats results in defects not only in PKB but also in various enzymes downstream of the insulin receptor, while STZ injection to older rats which produces an animal model of Type 1 diabetes has no (this study) or modest [93] effects on PKB activity. Again, this emphasizes that in addition to hyperglycemia other factors associated with Type 2 diabetes, may be involved in the impairment of insulin signaling.

The observations from previous studies in this laboratory [212] and other laboratories [353, 354] have shown that insulin resistance becomes more severe in STZ-diabetic rats during longer periods of diabetes. This finding raised the question that development of insulin resistance in STZ-diabetic Wistar rats might be able to alter PKB activity. To answer this question PKB activity was studied after the development of insulin resistance in STZ-diabetic rats by extending the period of diabetes from 4 to 9 weeks. In this experiment PKB\(\alpha\) activity was measured at the time point of maximal activation by insulin (5 min). Development of insulin resistance in STZ-diabetic rats was accompanied by a decrease in GLUT4 protein expression and its translocation to the plasma membrane in the heart muscle [212] as well as a decrease in insulin-induced glycogen synthase fractional activity and an increase in PP-1 activity in the skeletal muscle [349]. As with the short-term study, insulin stimulated PKB\(\alpha\) activity about 14-fold in various experimental groups (C, CB,
D and DB (Fig 3.9). However, despite the development of insulin resistance, basal and insulin-induced PKB activity and its protein expression remained normal up to 9 weeks of diabetes, indicating that changes in PKBα activity and/or expression are not important in the development of insulin resistance in Type 1 diabetes (Fig 3.9).

4.5. PKB ACTIVITY IN FATTY ZUCKER RATS

4.5.1. Skeletal Muscle

Fatty Zucker rats were characterized by hyperinsulinemia and normoglycemia during the study (Table 3.6, Fig 3.10). Insulin injection (5 U/kg, i.v.) significantly lowered plasma glucose levels in lean rats, but not in fatty rats, indicating the insulin resistant state in these animals. Also, insulin sensitivity indices calculated from the results of an OGTT test were markedly decreased in fatty rats as compared to lean animals (L: 10.9±0.8 vs. F: 0.8±0.1) (Fig 3.10C). Unlike STZ-diabetic rats, insulin-induced activation of PKBα was significantly reduced in the muscle of fatty rats (7-fold) as compared to their lean littermates (12-fold), indicating a decrease in response to insulin in these animals (Figs 3.2 and 3.11). Hence, it seems that PKB activity is differently regulated in hypoinsulinemic STZ-diabetic rats and hyperinsulinemic fatty Zucker rats. Results obtained from Western blots showed that a decrease in PKBα activity in fatty rats was not due to its lower protein expression (Fig 3.11). These findings are in agreement with those obtained from previous studies in this laboratory and others that have shown impairment in kinase activity at the level of PI3-K [32-34, 347] and PKB without any changes in the protein expression [151, 348, 355] in the skeletal muscle of human subjects and animal models of Type 2 diabetes. Decreased levels of insulin-induced PI3-K activity in
fatty Zucker rats have been shown to be associated with reduced IRS-1 and IRS-2 protein levels resulting in decreased tyrosine phosphorylation in response to insulin [32, 33, 350, 355]. Furthermore, it was reported that both protein level and enzyme activity of GSK-3β, a downstream enzyme of PKB, were increased in the muscle of Type 2 diabetic patients [34], which was accompanied by reduced levels of insulin-induced glycogen synthase activity [28, 34]. As with STZ-diabetic rats, basal PP-1 activity was elevated in the skeletal muscle of fatty Zucker rats compared to lean controls [356]. Hence, it appears that insulin signaling from the insulin receptor to glycogen synthase is impaired in fatty Zucker rats and response to insulin is reduced at different levels of the signaling cascade.

Glucose uptake is the rate-limiting step in glucose disposal in the skeletal muscle [30]. Most of the glucose that enters muscle cells in response to insulin is deposited as glycogen [29] and hence, a defect in muscle glucose uptake and glycogen synthesis is suggested to contribute to insulin resistance in diabetes [18, 27, 346, 357]. While most studies have shown that PKB is essential in glucose transport and GLUT4 vesicle translocation [127, 148, 149, 151], others have disputed this claim [156]. However, parallel defects observed in insulin-stimulated PKB activity and glucose transport in diabetic skeletal muscle and adipose tissue in both human subjects and animal models of Type 2 diabetes [151, 348] supports the idea that PKB might have an important role in the development of insulin resistance. Importantly, in the Goto-Kakizaki rat, a non-obese model of NIDDM, reduced levels of insulin-induced PKBα activity and glucose transport were completely restored by normalization of plasma glucose [348] and thus, it was suggested that
hyperglycemia may directly contribute to the development of muscle insulin resistance through alteration in insulin activation of PKB and glucose transport in Type 2 diabetes [348, 358]. This concept was supported by a similar report on restoration of glucose transport following normalization of hyperglycemia with insulin in Type 2 diabetic patients [357]. However, reduced levels of insulin-induced PKBα activity found in the skeletal muscle of the insulin resistant fatty Zucker rats in the absence of hyperglycemia indicates that other factors in addition to hyperglycemia are required to explain the impaired PKB activity in the diabetic skeletal muscle.

Recently, it was reported that insulin-induced activation of PI3-K was impaired in skeletal muscle from Type 2 diabetic patients without any defects in PKBα activity [34]. A possible explanation for this finding is that only a small amount of PI3-K activity is necessary to maximally activate PKB and therefore, severe impairment in the PI3-K activity might be required to affect PKB activity. These observations in human studies, differ from animal studies which have shown a parallel decrease in PKBα and PI3-K activity in different models of Type 2 diabetes [347, 348, 355, 358]. This implies that the impairment in PKBα activity observed with supra physiological doses of insulin in animal studies, may not be seen with lower physiological doses used in human studies.

We also measured PKBβ activity, the other isoform of PKB that has been shown to be activated by insulin to a lesser extent in the skeletal muscle [105], in order to investigate the possibility that changes in the PKBβ activity could compensate for the marked decrease observed in the PKBα activity in the skeletal muscle of fatty rats. Results showed that there was no detectable difference in
basal or maximal insulin-induced PKBβ activity between lean and fatty rats (Fig 3.13). Although it has been reported that insulin-induced PKBβ activity is impaired with higher doses of insulin in the skeletal muscle of fatty rats, this finding may not have physiological significance due to high pharmacological doses of insulin (10 U/kg) used in that study [355].

In summary, present observations taken together with the results from other studies indicate that reduced insulin-induced PKBα activity in the skeletal muscle of fatty Zucker rats is associated with decreased protein levels and/or activity of its upstream signaling proteins including IRS proteins and PI3-K as well as impairment in its downstream enzymes such as GSK-3 and glycogen synthase and GLUT4 transporters, which reflects a reduced response to insulin at different steps in the insulin signaling cascade. The consequent decrease in glucose uptake and glycogen synthesis contribute to the insulin resistance in the skeletal muscle.

4.5.2. Liver

In contrast to muscle, insulin-induced activation of PKBα was significantly higher in the liver of fatty Zucker rats than lean controls, indicating an increased response to insulin and a possible relationship between PKB activity and insulin resistance in the liver of these animals (Figs 3.2, 3.12). Changes in PKBα activity were not associated with increased levels of protein expression (Fig 3.12). Furthermore, there was no significant difference in basal PKBα activity between lean and fatty rats. As with muscle, there was no detectable difference in basal or insulinstimulated PKBβ activity in the liver between lean and fatty Zucker rats (Fig 3.4). The increase in response to insulin observed in the liver of fatty rats was an
interesting and unexpected finding. This observation indicated that 1) the changes in PKB\(\alpha\) activity are tissue specific, a decrease was observed in the skeletal muscle (Fig 3.11) while an increase was seen in the liver (Fig 3.12); 2) other pathways may also be involved in the regulation of PKB in the liver; 3) PKB may have different roles in various tissues.

Hepatic glucose production is known to play a crucial role in the systemic glucose homeostasis. Insulin decreases this by activating glycogen synthesis and glycolysis and by inhibiting gluconeogenesis [359]. Glycogen synthesis in the liver is mainly regulated by glucose input that correlates with plasma glucose levels and activation of the PI3-K/PKB/GSK-3 axis [47, 48, 132]. Hence, PKB plays an important role in hepatic glycogen synthesis. Furthermore, recently PKB has been shown to directly phosphorylate and activate PFK-2, \textit{in vitro}, therefore it might be an important regulator of insulin-dependent glycolysis [53, 126]. In addition, there is evidence that PKB might be involved in insulin-induced suppression of hepatic glucose production via inhibition of PEPCK and G6Pase [154, 155]. These findings support the concept that PKB may contribute to the control of net hepatic glucose production [359]. Therefore, taken together, it can be postulated that an increase in PKB\(\alpha\) activity in the liver of fatty Zucker rats may be due to the activation of a compensatory mechanism, which results in the stimulation of PKB, probably via a PI3-K independent pathway, in order to inhibit the elevated levels of hepatic glucose production by at least one of the above mechanisms. Indeed, there is convincing evidence from recent studies that support an important role for PKB in hepatic glucose homeostasis [153, 359]. A recent study has reported that restoration of
IRS-1 in the liver was sufficient for normalization of systemic insulin resistance in IRS-1 deficient mice, indicating that regulation of hepatic glucose production is pivotal for glucose homeostasis and that PKB activity mediated by IRS-1 might play a crucial role in this regulation [359]. Furthermore, mice lacking PKBβ demonstrated a complete failure in insulin-induced suppression of glucose production in the liver and provided definite in vivo evidence that PKBβ is required for the metabolic actions of insulin in the liver [153].

Another finding in the liver of fatty Zucker rats was the dissociation between activation of PI3-K and PKB in this tissue. It has been shown that IRS-1 associated PI3-K activity is decreased in the liver of fatty rats [33, 355], while at the level of PKB a marked increase in the enzyme activity was found in this study. In support, Kim et al. [355] recently showed that insulin-induced (10 U/kg, i.v.) PKBa activity was elevated in the liver of female fatty Zucker rats. These observations further support the idea that other PI3-K-independent pathways may be involved in the regulation of PKB in the liver, at least in insulin resistant state. There is a growing body of evidence that PKB can be activated via several PI3-K-independent pathways such as cellular stresses and the cAMP/PKA system [133-135, 137], both of which are elevated in diabetes. For example, PKB has been shown to be activated via the cAMP/PKA system [135, 137], which is up regulated in the diabetic liver due to high glucagon levels [178, 213]. However, further studies are required to understand the steps following PKB activation and the key enzymes that may act as substrates for PKB in the liver still remains to be identified.
4.6. EFFECTS OF BMOV TREATMENT ON PKB ACTIVITY

Although several studies have shown that vanadium compounds mimic/enhance most of the metabolic effects of insulin both in vitro and in vivo, the molecular mechanisms by which vanadium mediates its metabolic effects are still not clear. Evidence indicates that regulatory effects of vanadium have a complex pattern and at least some of the in vitro targets of vanadium are not involved in mediating its regulatory actions in vivo. One of the main objectives of this study was to investigate some of the mechanisms underlying the in vivo hypoglycemic effects of vanadium. Results of this study showed a correlation between PKB activity and insulin resistance suggesting that BMOV treatment might be able to restore PKB activity resulting in an improvement in the metabolic state.

Consistent with other studies in our laboratory [191, 205] and other laboratories [23, 206] with vanadium compounds, chronic BMOV treatment completely normalized plasma glucose levels in overnight-fasted STZ-diabetic rats without any detectable effect on plasma insulin levels (Fig 3.6 and Table 3.2). Acute insulin treatment (5 U/kg, i.v.) significantly lowered plasma glucose levels after 15 minutes, in BMOV-treated and untreated control and BMOV-treated diabetic rats but not untreated diabetic rats, indicating insulin resistance in the untreated diabetic rats which was reversed following BMOV treatment (Fig 3.6B). Using the euglycemic hyperinsulinemic clamp technique, it was shown that hypoglycemic effects of vanadium in STZ-diabetic rats were associated with a marked increase in the peripheral glucose utilization and normalization of the elevated hepatic glucose output [209, 210, 211]. Improved insulin sensitivity was largely mediated through
increased non-oxidative glucose disposal (glycogen synthesis) [196, 219]. In addition, vanadium was shown to correct the reduced levels of GLUT4 mRNA and protein in the skeletal and cardiac muscle [23, 25] and enhance its translocation to the plasma membrane [212]. Improvement in insulin sensitivity following vanadium treatment in STZ-diabetic rats could be at least partially attributed to the correction of hyperglycemia. In support, other studies have reported that peripheral insulin resistance is reversed by correction of hyperglycemia with phlorizin [24, 348, 358] or Na⁺-glucose co-transporter inhibitors [352], which decrease glucose levels by inhibiting re-absorption of glucose in renal proximal tubules, or by insulin treatment in Type 1 diabetic patients [18, 22]. This indicates that improvement of insulin sensitivity in STZ-diabetic rats observed with chronic BMOV treatment is at least partially due to the correction of hyperglycemia.

Despite the complete normalization of plasma glucose levels, BMOV did not have any effect on basal or insulin-induced PKB activity and its protein expression in the skeletal muscle of STZ-diabetic rats up to 9 weeks of diabetes (Fig 3.9), suggesting that glucose-lowering effects of BMOV are independent of PKB activity in STZ-diabetic rats. Although, there is evidence from in vitro studies with adipocytes that stimulation with either vanadate (mM) or peroxovanadate (µM) results in activation (phosphorylation) of both isoforms of PKB (PKBα, PKBβ) [107, 111, 113], which is associated with its translocation to the plasma membrane [111, 113]. Also, the effects of vanadium on PKB activity were shown to be additive to that of insulin in vitro [111]. These observations imply that high concentrations of vanadium (mM), in vitro, may activate PKB through mechanism(s) that are not activated by the lower
concentrations of vanadium achieved in vivo. Two possible mechanisms, by which vanadium may mediate its effects on PKB in vitro, include more potent activation of the enzymes upstream of PKB (PI3-K, PDKs) and/or inhibition of phosphatases involved in the inactivation of PKB. Indeed, there is evidence that vanadium inhibits PIP3-5-phosphatases leading to an increase in PIP3 products, which could potentially contribute to the activation of PKB in vitro [90, 360]. Furthermore, vanadium has been shown to inhibit cAMP-induced activation of PP2A, the phosphatase which negatively regulates PKB, by its phosphorylation, in vitro [142].

To examine whether BMOV is able to normalize the impaired levels of PKB in the liver and/or muscle of fatty Zucker rats, animals were treated with BMOV for three weeks. BMOV treatment significantly reduced plasma insulin levels (Table 3.6) and improved insulin sensitivity (Fig 3.10C), without having any effect on either parameters in the lean control rats. The reduction in plasma insulin levels by BMOV is probably not secondary to the reduction in plasma glucose or food intake, since BMOV did not affect plasma glucose (Fig 3.10B) and had modest effects on the body weight and food intake in the animals (Table 3.5). Studies using the euglycemic hyperinsulinemic clamp technique have demonstrated that the improvement in glucose homeostasis following vanadium treatment in fatty Zucker rats was not due to a greater inhibition of hepatic glucose output but involved an increase in the insulin sensitivity in the peripheral tissues mainly skeletal muscle [224]. Similar observations have been reported from human studies with vanadyl sulfate [361].
Despite the improvement of insulin resistance, BMOV did not restore PKBα activity to normal levels in the liver or muscle, neither did it have any effect on PKBβ activity in fatty Zucker rats (Figs 3.11-3.13), indicating that similar to what was observed in the Wistar rats, the glucoregulatory effects of BMOV in fatty Zucker rats are independent of PKB activity. Recently Carvalho et al. [362] reported that insulin-induced PKB activity and GLUT4 translocation were decreased in the adipose tissue of fatty Zucker rats and were restored by vanadium (4 mM). This finding taken together with the results of our study supports the idea that vanadium at high concentrations may demonstrate some metabolic effects that do not contribute to its hypoglycemic effects in vivo, and hence the results of in vitro studies should be interpreted carefully.

Previous studies in this laboratory have shown that BMOV was also unable to normalize the reduced levels of insulin-induced IRS-1 associated PI3-K activity in the skeletal muscle of fatty rats [347]. Furthermore, BMOV had no effect on GSK-3 activity in the skeletal muscle or liver in vivo [356]. These observations indicated that other mechanism(s) rather than the PI3-K/PKB/GSK-3 axis are involved in mediating the in vivo glucoregulatory effects of BMOV. Vanadium is a potent inhibitor of cellular protein tyrosine phosphatases [187]. Hence, one possible explanation would be that specific regulatory phosphatases might be targets for BMOV in the insulin signaling pathway. Studies in this laboratory demonstrated that BMOV restored the elevated levels of both PTP1B [363] and PP-1 [356], indicating that in vivo, BMOV can modulate the phosphorylation of insulin receptor and IRS proteins as well as glycogen synthase activity through its effects on the key
regulatory phosphatases. Furthermore, vanadium can regulate glycogenolysis by normalization of phosphorylase a activity [223].

4.7. EFFектS OF INSULIN AND BMOV ON PEPCK AND G6PASE IN DIABETES

Glucose homeostasis depends largely on the balance between its formation by the liver and its utilization by the three major insulin dependent tissues (liver, muscle and adipose tissue). Results from the present study and other studies in this laboratory demonstrated that chronic treatment of diabetic rats with BMOV did not have any effect on insulin signaling at the level of PI3-K, PKB or GSK-3, three enzymes involved in glucose utilization. Hence, one alternative mechanism by which BMOV may mediate its glucose lowering effects would be via inhibition of hepatic glucose production (HGP). If vanadium exerts its plasma glucose lowering effects by inhibition of HGP, less profound effects should be seen in non-fasted diabetic rats, since endogenous glucose production is reduced under such conditions. While under fasting and diabetic conditions HGP contributes significantly to the glucose homeostasis and therefore vanadium would be expected to normalize plasma glucose levels. In contrast, if the predominant effect of vanadium is on the dephosphorylation of insulin receptor or the activation of its downstream enzymes, then a more profound effect would be expected on the basal plasma glucose levels in fed animals. This hypothesis is supported by the observation that vanadium compounds normalize plasma glucose in the fasted diabetic or glucagon stimulated states, but not in the fed diabetic state [364].

Increase in endogenous glucose production is associated with elevated levels of PEPCK and G6Pase activity and gene expression in several animal models of
diabetes [54, 55]. In this study, we investigated the possibility that vanadium might be able to decrease glucose production and lower plasma glucose levels by its direct and/or indirect inhibitory effects on PEPCK and/or G6Pase gene expression in the liver and kidney, two main sites of gluconeogenesis, in the STZ-diabetic rats.

4.7.1. Effects of Insulin on PEPCK and G6Pase

In agreement with previous reports on animal models of diabetes [161, 365, 366], both PEPCK activity and mRNA levels were significantly higher in the liver of STZ-diabetic rats as compared with control rats (Figs 3.14A and 3.15A). It has been reported that the elevated levels of PEPCK in STZ-diabetic rats are due to hyperglucagonemia (acting through cAMP) and hypoinsulinemia in these animals [161, 213, 365]. As in other studies [161, 335, 365], insulin treatment restored PEPCK enzyme activity and mRNA levels in the liver of diabetic rats, indicating that the signaling pathway is not impaired. Recent in vitro studies have shown that the inhibitory effects of insulin on PEPCK gene expression in the liver are mediated by the effects of phosphatidylinositol 3-kinase (PI3-K) [367-369] on an insulin responsive region in the PEPCK gene promoter [55, 368]. The links between PI3-K and PEPCK promoter are still undefined. Some in vitro studies [154], but not all [367], support a role for protein kinase B (PKB) in the repression of glucocorticoid and cAMP induction of PEPCK. Results from this study cannot rule out a linkage between activation of PKB and inhibition of PEPCK in vivo. However, these results do not support a major role for PKB, since PKB activity was normal in the liver of STZ-diabetic rats (Fig 3.1) despite the elevated levels of PEPCK activity and mRNA in this tissue (Figs 3.14 and 3.15). If insulin inhibition of PEPCK was mediated by
PKB, one would expect a decrease in PKB activity and a consequent increase in PEPCK expression associated with hypoinsulinemia in STZ-diabetic rats. Increased levels of plasma glucagon observed in STZ-diabetic rats by others [213] and by us (Table 3.10), indicate that in vivo, the inhibitory effects of insulin on PEPCK may also be mediated indirectly by the suppression of secretion and/or actions of glucagon.

The kidney is the second major site of endogenous gluconeogenesis. The contribution of kidney to the elevated levels of gluconeogenesis in diabetes is important to consider, in view of the finding that the kidney may account for more than 40% of total endogenous glucose production during prolonged fasting [54]. As in other studies [161, 162, 164], PEPCK activity and mRNA levels were significantly higher in the kidney of diabetic rats than in non-diabetic controls (Figs 3.14B and 3.15B). It is believed that diabetes increases renal PEPCK mRNA levels indirectly by causing acidosis [161-163], since correction of acidosis prevents the increase in PEPCK gene expression in diabetic animals [162]. Treatment with insulin normalized both PEPCK activity and expression in the kidney. In contrast to liver, metabolic acidosis is the primary regulator of renal PEPCK [55, 162, 163], which acts via a transcriptional mechanism in rats [161]. Hence, it appears that the effect of insulin on renal PEPCK in this study was due to an improvement in the metabolic state.

STZ-diabetes markedly increased mRNA levels of the G6Pase catalytic subunit in the liver and to almost the same extent in the kidney, and levels in both tissues were normalized with insulin treatment (Fig 3.16). These results are in agreement with those obtained from other studies, which have shown elevated
levels of G6Pase activity and gene expression in different experimental models of diabetes [166, 172, 177, 178, 370]. Interestingly, the increase in G6Pase mRNA in both tissues was more profound than the increase in PEPCK mRNA, emphasizing the likely significance of G6Pase in control of gluconeogenesis. Insulin plays an important role in the regulation of G6Pase in both liver and kidney in vivo [177], indicating that hypoinsulinemia and unopposed hyperglucagonemia may contribute to the elevated levels of G6Pase mRNA in these tissues. A multi-component insulin responsive sequence identified in the promoter region of the G6Pase gene [371] appears to be regulated via PI3-K and downstream PKB-dependent and independent pathways [155, 336]. However, the finding that correction of hyperglycemia per se is able to suppress the marked diabetes-induced increase in G6Pase mRNA in the liver by acting through both transcriptional and post-transcriptional mechanisms [172, 174], supports a role for hyperglycemia in the up-regulation of G6Pase in STZ-diabetic rats.

4.7.2. Effects of BMOV on PEPCK and G6Pase

Four weeks treatment with BMOV, significantly lowered plasma glucose levels in the fed STZ-diabetic rats (D: 29.2±1.9 vs. DB: 16.3±2.7 mmol/l) (Table 3.8). However, the first part of this study (Wistar rats: 4-week study) had shown a further decrease in plasma glucose levels in the overnight fasted rats (D: 25.6±4.4 vs. DB: 7.8±0.4 mmol/l) (Fig 3.6), implying that BMOV is more efficient in lowering plasma glucose levels in the overnight fasted diabetic rats, which have active gluconeogenesis, as compared with fed animals. There was not any significant difference in body weights between BMOV-treated and -untreated diabetic rats at
the end of study, while body weights were markedly higher in the insulin-treated animals compared to the untreated diabetic rats, indicating that vanadium does not reproduce the effects of insulin replacement on the growth pattern of STZ-diabetic rats (Table 3.7).

Results of this study showed that normalization of plasma glucose levels by treatment of STZ-diabetic rats with BMOV was accompanied by inhibition of hepatic PEPCK and G6Pase mRNA expression (Figs 3.14-3.16). These results are consistent with in vitro studies performed on PEPCK in hepatoma cells [202] and in vivo studies with inorganic vanadium compounds in animal models of diabetes [166, 366, 372]. Although there is no information on the in vivo effects of vanadium on G6Pase mRNA expression in the liver or kidney, it has been reported that vanadium inhibits G6Pase activity both in vitro [203, 373, 374] and in vivo [166, 375, 376]. Vanadium is a potent competitive inhibitor of G6Pase, which acts through the catalytic site of the enzyme [364]. Hence, the effects of vanadium on G6Pase are partially mediated via its direct effects on the enzyme activity. In this study, we showed that in addition to its inhibitory effects on G6Pase activity, vanadium is a negative regulator of G6Pase expression.

As with the effects of insulin, inhibitory effects of BMOV on PEPCK and G6Pase mRNA in the liver could involve direct effects on the gene promoters, and/or indirect effects via alteration in counterregulatory hormones. The first hypothesis is supported by the finding that vanadate inhibits both basal and cAMP-stimulated expression of PEPCK in hepatocytes in vitro in the absence of endogenous hormones and that there is a vanadate response region in the PEPCK gene
promoter [202]. However, the observation that BMOV lowered the elevated levels of plasma glucagon in the diabetic rats indicates that BMOV may also inhibit PEPCK and G6Pase indirectly by restoring plasma glucagon levels. Furthermore, vanadium might act more directly on the cAMP system, enhancing cyclic nucleotide phosphodiesterase [377] or inhibiting cAMP-dependent protein kinase [198]. Finally, because high concentrations of free fatty acids and glucose are known to increase G6Pase expression [172-174], while free fatty acids also enhance PEPCK expression [55], therefore it could be suggested that BMOV may decrease PEPCK and G6Pase mRNA levels by normalizing hyperglycemia and hyperlipidemia. This hypothesis is supported by the finding that treatment of STZ-diabetic rats with phlorizin, a glucose lowering agent which normalizes plasma glucose and restores the normal plasma glucagon levels, results in partial inhibition of mRNA and activity of PEPCK [26]. Taken together, it is likely that the inhibitory effects of BMOV on PEPCK and G6Pase are mediated both by a direct effect on the respective gene promoters and indirect effects via improvement of metabolic state as well as normalization of glucagon levels and the actions of cAMP in vivo.

As with liver, treatment with BMOV normalized PEPCK activity and mRNA expression and restored G6Pase mRNA in the kidney of STZ-diabetic rats. Whether the inhibitory effects of BMOV on PEPCK mRNA in the kidney are primary (via PEPCK promoter) or secondary to a decrease in ketone bodies and improvement of the metabolic acidosis is not clear. It was reported that in diabetic rats vanadate normalized blood ketone body levels and improved metabolic acidosis [378], indicating that the effects of BMOV on PEPCK in the kidney might be related to an
improvement in the metabolic state. In summary, the results of this study showed that chronic treatment of STZ-diabetic rats with BMOV completely restored normal levels of PEPCK mRNA expression and activity, as well as mRNA expression of G6Pase in the liver and kidney. Therefore, inhibition of these enzymes via direct or indirect pathways is one of the mechanisms involved in the glucose lowering effects of BMOV in vivo. The finding that unlike insulin, BMOV was unable to completely normalize plasma glucose levels despite its similar inhibitory effects on PEPCK and G6Pase, indicate that vanadium mimics most, but not all, metabolic actions of insulin.

4.8. SUMMARY

Insulin resistance in STZ-diabetic rats is a secondary phenomenon associated with chronic hyperglycemia and is accompanied by both an increase in the hepatic glucose output and a decrease in the peripheral glucose utilization [209]. Results of this study demonstrated that basal and insulin-induced PKBα activity and protein expression remained normal in the skeletal muscle and liver, two main sites of insulin resistance in the STZ-diabetic rats, indicating that PKB is not involved in the development of insulin resistance in this model. Furthermore, the observation that both PI3-K [347] and GSK-3 [349] activity, upstream and downstream enzymes of PKB in the signaling pathway respectively, were also not changed indicates that response to insulin at the level of PI3K/PKB/GSK-3 axis is normal. However, G6Pase and PEPCK, two key gluconeogenic enzymes were markedly elevated in the liver and kidney of STZ-diabetic rats due to hypoinsulinemia and hyperglucagonemia which may in turn contribute to the development of insulin
resistance via increasing plasma glucose levels. There was no correlation between 
PKB activity and the expression of PEPCK and G6Pase therefore, it is unlikely that 
PKB has a major role in the inhibition of these enzymes. Consistent with a previous 
study with inorganic vanadium compounds [209], chronic treatment with BMOV 
normalized plasma glucose levels and reversed insulin resistance in STZ-diabetic 
rats. These effects of BMOV were associated with complete normalization of 
PEPCK and G6Pase expression without any effects on PKB activity, indicating that 
the glucose lowering effects of BMOV are at least partially mediated by inhibition of 
these two gluconeogenic enzymes via a PKB independent pathway. Results of this 
study suggest that the inhibitory effects of BMOV on PEPCK and G6Pase might be 
mediated via its direct effects on their gene expression as well as its indirect effects 
via normalization of hyperglucagonemia and hyperlipidemia (Table 3.10).

In contrast to STZ-diabetic rats, insulin resistance in fatty Zucker rats is a 
primary defect, although it is also associated with abnormal suppression of HGP 
[320] and insulin-stimulated glucose disposal [318]. Unlike STZ-diabetic rats, insulin 
resistance in this model was associated with marked changes in insulin-induced 
PKBα activity in both skeletal muscle and liver. These changes were tissue specific, 
indicating that PKB is regulated differently in the liver and skeletal muscle. On the 
one hand, reduced levels of insulin-induced PKBα activity observed in the skeletal 
muscle of fatty Zucker rats in this study, together with diminished levels of insulin 
stimulated PI3-K activity [347] and IRS-1 phosphorylation [350] indicate a decrease 
in insulin response at several steps of the signaling cascade and the consequent 
insulin resistance in this tissue. On the other hand, the elevated levels of PKB in the
liver of fatty rats supported the idea that PKB might be regulated differently in this tissue. Since there was a dissociation between PKBα and PI3-K activity, it is likely that a PI3-K-independent pathway is involved in the regulation of PKB in the liver of insulin resistant fatty Zucker rats. Treatment with BMOV lowered plasma insulin and improved insulin sensitivity without any effects on PKBα, PI3-K [347] or GSK-3 [356] activity. Hence, it appears that key enzymes in the PI3-K pathway, the main pathway involved in mediating the metabolic effects of insulin are not in vivo targets for BMOV in the insulin sensitive tissues, although some of the metabolic actions of vanadium can be attributed to its effects on the key regulatory phosphatases [356, 363] in the insulin signaling (i.e. PTP1B and PP-1). The in vivo effects of vanadium on insulin signaling pathway and its relevant in vitro effects are summarized in Scheme 3.
Scheme 3.

In vivo effects of vanadium on PI3-K pathway, the main cascade involved in mediating the metabolic effects of insulin, are shown in the left side of the figure. The right side demonstrates the effects of vanadium, in vitro, which might be relevant to its glucoregulatory effects observed in vivo. Results from studies in this laboratory demonstrated that vanadium had no effect on the activity of PI3-K, PKB and GSK3, in vivo, but inhibited two regulatory phosphatases namely PTP1B and PP1, which dephosphorylate insulin receptor/insulin receptor substrates and glycogen synthase, respectively. Vanadium also stimulated glycogen synthase activity and inhibited PEPCK and G6Pase mRNA expression.
Potential Sites of Vanadium Action

V (-): Negative effect  IR: Insulin receptor
V (+): Positive effect  GPR: G protein-coupled receptor
V (N): No effect
CHAPTER 5
CONCLUSIONS

1. Insulin stimulated PKBa activity more than 10-fold and PKBβ activity more than 3-fold, *in vivo*, with a maximal activation observed at 5 and 15 minutes post insulin injection in the skeletal muscle and liver, respectively. Activation of PKB by insulin in both tissues was accompanied by an increase in its phosphorylation.

2. There was a direct correlation between the time course of glucose lowering effects of insulin and activation of PKB in the skeletal muscle in both control Wistar rats and lean Zucker rats, supporting an important role for PKB in mediating insulin-stimulated glucose disposal.

3. Basal PKBa activity was normal in all three animal models studied including STZ-diabetic rats, Zucker fatty and Zucker diabetic fatty rats, implying that basal PKBa activity is not affected by the diabetic state or that circulating levels of insulin are enough to maintain basal PKBa activity during absolute or relative hypoinsulinemia.

4. Despite the development of insulin resistance, both basal and insulin-induced PKBa activity and its protein expression were normal in the skeletal muscle and liver, up to 9 weeks of STZ-induced diabetes, indicating that the response to insulin is normal and that PKB is unlikely to contribute to the development of insulin resistance in STZ-diabetic rats.
5. In fatty Zucker rats, insulin-induced PKBα activity was significantly reduced in the skeletal muscle, while it was markedly increased in the liver. Impairment in PKBα activity was accompanied by a diminished response to the glucose lowering effects of insulin, indicating an association between impaired levels of PKB activity and insulin resistance in this model.

6. BMOV, at a dose sufficient to normalize fasting plasma glucose levels in STZ-diabetic rats and attenuate plasma insulin levels in fatty Zucker rats, had no detectable effect on basal or insulin-induced PKBα and PKBβ activities, indicating that the glucoregulatory effects of BMOV are not mediated via PKB in vivo.

7. Treatment of STZ-diabetic rats with the same therapeutic dose of BMOV completely restored the elevated mRNA levels of PEPCK and G6Pase, two key gluconeogenic enzymes, in both liver and kidney. Hence, we suggest that the glucose lowering effects of BMOV, in vivo, are at least partially mediated by its direct and/or indirect inhibitory effects on these enzymes via mechanism(s) independent of PKB.

8. The more potent and rapid glucose lowering effects of insulin, as compared to BMOV, despite their similar inhibitory effects on PEPCK and G6Pase in STZ-diabetic rats, indicate that BMOV mimics/enhances some, but not all, of the glucoregulatory effects of insulin in vivo.

9. Normal levels of basal PKB activity observed in the liver of hypoinsulinemic STZ-diabetic rats in the presence of elevated levels of PEPCK and G6Pase indicate that the signaling cascade involved in mediating the inhibitory effects
of insulin on PEPCK and G6Pase is impaired without any defects in PKB activity. These results imply that either PKB is not involved in mediating the insulin-induced suppression of these enzymes or response to PKB is reduced in the diabetic rat liver.
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