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Department of Physiology

The University of British Columbia
Vancouver, Canada

Date July 24/03
ABSTRACT

The ubiquitous serine protease dipeptidyl peptidase IV (DP IV) plays a number of physiological roles including hormone inactivation and immune costimulation. Recent attention has been brought to the molecule because of its ability to cleave and inactivate the potent insulin secretagogues (incretins) glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Use of specific DP IV-inhibitors has been shown to protect the active form of these hormones in the circulation thus enhancing their effects at target tissues. In the following study we show first, that the counterregulatory hormone glucagon serves as a physiologically relevant substrate for DP IV. Also, using a model of obesity-related diabetes (fa/za Zucker rat) and a combination of techniques (oral glucose tolerance testing, pancreas perfusion, immunohistochemistry, and euglycemic-hyperinsulinemic clamp), we show that long-term DP IV-inhibitor (P32/98) therapy ameliorates the type-2 diabetic syndrome through enhancement of both insulin sensitivity and β-cell function. Further, we reveal the applicability of long-term DP IV-inhibitor treatment towards type-1 diabetes. In a model of insulin insufficiency and post-traumatic islet plasticity (streptozotocin-induced diabetic rat), marked improvements in disease severity are shown, associated with enhancement of β-cell survival and islet neogenesis. An in vitro analysis of the anti-apoptotic potential of GIP and GLP-1 implicate the two hormones mechanistically in the in vivo findings. Also, in a model of the autoimmune progression of type-1 diabetes (the BioBreeding rat) we show both a delay and partial prevention of the disease as well as improved glucose homeostasis prior to disease onset, supporting our hypothesis of combined immunosuppression and incretin enhancement and establishing for the first time the potential for DP IV-inhibition in the treatment of type-1 diabetes. In addition to a review of the literature surrounding DP IV and glucose regulation, the findings of the present study are discussed in the context of glucose counterregulation, diabetes and autoimmunity.

In summary, the work presented here identifies a novel regulatory mechanism for the effects of the counterregulatory hormone glucagon, demonstrates the benefits of long-term incretin enhancement as a therapy for type-2 diabetes, and establishes DP IV-inhibition as a unique therapeutic strategy in the treatment of type-1 diabetes.
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<th>Amino Acid</th>
<th>3 Letter Code</th>
<th>1 Letter Code</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>Serine</td>
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</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Try</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
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- **aa**: Amino Acid
- **AC**: Adenylate Cyclase
- **ACC**: Acetyl CoA Carboxylase
- **ADA**: Adenosine Deaminase
- **BB**: BioBreeding
- **BSA**: Bovine Serum Albumin
- **CAD**: Coronary Artery Disease
- **cAMP**: cyclic Adenosine 3', 5'-Monophosphate
- **CCK**: Cholecystokinin
- **CD26**: Dipeptidyl Peptidase IV (DP IV)
- **CGRP**: Calcitonin Gene-Related Peptide
- **CHO**: Chinese Hamster Ovary
- **CISI**: Composite Insulin Sensitivity Index
- **CRE**: cAMP Response Element
- **CREB**: cAMP Response Element Binding Protein
- **DP-**: Diabetes Prone (BB rat)
- **DP IV**: Dipeptidyl Peptidase IV (CD26)
- **DPP**: Dipeptidyl Peptidase
- **DR-**: Diabetes Resistant (BB rat)
- **ECM**: Extracellular Matrix
- **ERK**: Extracellular Regulated Kinase
- **FAP-α**: Fibroblast Activating Protein-α
- **FFA**: Free Fatty Acid
- **GDR**: Glucose Disposal Rate
- **GIP**: Glucose-Dependent Insulinotropic Polypeptide
- **GIR**: Glucose Infusion Rate
- **GLP-1**: Glucagon-Like Peptide-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>GLP-1a</td>
<td>Active Glucagon-Like Peptide-1</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon-Like Peptide-2</td>
</tr>
<tr>
<td>GRF</td>
<td>Growth Hormone Releasing Factor</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin-Releasing Peptide</td>
</tr>
<tr>
<td>GRPP</td>
<td>Glicentin-Related Polypeptide</td>
</tr>
<tr>
<td>hGlucR</td>
<td>Human Glucagon Receptor</td>
</tr>
<tr>
<td>HGO</td>
<td>Hepatic Glucose Output</td>
</tr>
<tr>
<td>IDGTT</td>
<td>Intraduodenal Glucose Tolerance Test</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal Glucose Tolerance Test</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage-Derived Chemoattractant</td>
</tr>
<tr>
<td>MCR</td>
<td>Metabolic Clearance Rate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral Endopeptidase</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-Obese Diabetic</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OGT TT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary Adenylate Cyclase Activating Polypeptide</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl Glycine α-Adamating Monooxygenase</td>
</tr>
<tr>
<td>PC 1/3</td>
<td>Prohormone Convertase 1/3</td>
</tr>
<tr>
<td>PC 2</td>
<td>Prohormone Convertase 2</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxy-kinase</td>
</tr>
<tr>
<td>PHM</td>
<td>Peptide Histidine Methionine</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein Tyrosine Kinase</td>
</tr>
<tr>
<td>QPP</td>
<td>Quiescent-cell Proline Dipeptidase</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal Cell-Derived Factor-1</td>
</tr>
<tr>
<td>SH</td>
<td>Spontaneously Hypertensive</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell Receptor</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Polypeptide</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventro-Medial Hypothalamus</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Zeta-Associated Protein Tyrosine Kinase of 70 kDa</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

My sincerest thanks go first of all to Ray, for holding my hand the first few years and for having the faith to let it go once my feet were wet. When I started I needed a friend more than a supervisor; and with the gods on my side Ray, I got you, the best of both, a friend and father-figure, a lone crusader in the pursuit of true balance in life. And Margaret, as the saying goes, “Behind every great man……..”.

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PREFACE

Parts of the following work have been published as follows:


INTRODUCTION

Despite large daily fluctuations in energy intake and expenditure, blood glucose levels in a healthy individual rarely fall outside the range of 4 to 8 mM. This tight glucose control results from the combined effects of a multi-layered regulatory network involving neural, endocrine, and substrate mediated pathways that is designed to store glucose in times of excess, and to liberate those stores during times of need. The primary endocrine regulators of blood glucose are glucagon, a catabolic stimulant of glucose release from the liver, and insulin, the anabolic corollary.

GLUCAGON, GLUCAGON-LIKE PEPTIDES AND THE INCRETIN CONCEPT

History

Since its initial isolation as a glucose-elevating contaminant in pancreatic insulin extracts in 1923, glucagon has been intensively studied (Kieffer et al. 2001). Major leaps in our understanding of the hormone came with the development of a crystallization purification scheme (Staub et al. 1953), which set the stage for elucidation of the 29 amino acid sequence of glucagon, and with the development of a glucagon-specific radioimmunoassay* (RIA; Unger et al. 1959) which helped establish the glycogenolytic, lipolytic, and insulin-stimulating properties of the hormone. Further, these advances aided in the elucidation of glucagon’s role in the pathogenesis of diabetes mellitus (Unger et al. 1970, Sakurai et al. 1975). A number of these early studies also pointed to the existence of two larger immunoreactive forms of glucagon that had relatively high intestinal expression levels when compared to glucagon itself, peptides later identified as glicentin and oxyntomodulin.

On the basis of intestinal extract studies dating back as far as 1902 by a number of groups, Unger and Eisentraut coined the term “enteroinsular axis” to describe a proposed regulatory system where gut-derived hormonal factors would influence the endocrine secretion of pancreatic islets of Langerhans (Bayliss and Starling 1902, Moore et al. 1906, Heller 1929, Zunz and La Barre 1929, La Barre and Still 1930, Scow and Cornfield 1954, Unger and Eisentraut 1969). The development of an insulin-specific RIA, prompted further leaps in the field, establishing that the postulated intestinally derived factor(s) “incretin” and/or “duodenin” reduced postprandial hyperglycemia through an insulin-dependent mechanism. This unidentified factor was held responsible for the phenomenon, later termed the “incretin effect”, where orally administered glucose elevates plasma insulin levels to a far greater extent than an equivalent intravenous load. The mediators of the enteroinsular axis, to which neural and

* Circulating hormone concentrations determined by immunoassay (RIA or enzyme-linked immunosorbent assay; ELISA) are most accurately termed immunoreactive (IR) peptides (e.g. IR-glucagon or IR-insulin) as the accuracy of the method is dependent on the specificity of the antibody used. For the sake of brevity, the prefix “IR-“ has been omitted from descriptions of hormone concentration or flux in this text.
substrate components were later ascribed, were estimated to stimulate over 50% of nutrient-induced insulin secretion (Perley and Kipnis 1967, Creutzfeldt 1979).

The first incretin was isolated as a contaminant of a crude porcine intestinal cholecystokinin-pancreozymin preparation on the basis of its inhibitory actions on gastric motility and secretion (Brown and Pederson 1970). Upon demonstration of an additional glucose-dependent insulinotropic activity (Dupre et al. 1973), the peptide, initially termed “gastric inhibitory polypeptide”, was alternately labeled glucose-dependent insulinotropic polypeptide (GIP; Pederson and Brown 1976). Soon, however, it was realized that GIP alone could not account for the entirety of the incretin effect and after a relatively long search, the amino terminally truncated forms of glucagon-like peptide 1 (GLP-1), GLP-17-36amide and GLP-17-37, were found to be potent stimulators of insulin secretion (Holst et al. 1987, Kreymann et al. 1987, Mojsov et al. 1987). The incretin effect is now believed to be the result of the combined actions of GIP and the truncated GLP-1 fragments.

The Glucagon Superfamily of Polypeptides

Glucagon, GIP and GLP-1 belong to a family of hormones, the glucagon superfamily of polypeptides, classified on the basis of their sequence homology to glucagon. The mammalian members include, in order of degree of homology, GLP-1, GIP, secretin, peptide histidine methionine (PHM; PHI, peptide histidine isoleucine in rodents), GLP-2, pituitary adenylate cyclase activating polypeptide (PACAP)-38, PACAP-27, PACAP-related peptide, growth hormone releasing factor (GRF), and vasoactive intestinal polypeptide (VIP). They are produced in a peptide specific manner throughout the GI-tract, the endocrine pancreas, and in the central and peripheral nervous systems. Extending through the cardiovascular, endocrine, and immune systems, their physiological actions are diverse though collectively they effect regulation of nutrient flux and cell development and fate (Sherwood et al. 2000). Glucagon and GLP-1 in particular, have been highly conserved throughout evolution.

All members of the superfamily mediate their actions through interaction with cognate seven transmembrane domain G-protein coupled receptors at the target cell surface. Signaling molecules common to all tested members include Gαs and the AC/cAMP/PKA, ERK1/2 and CREB cascades. Receptor-level structure-function studies typically indicate multiple bioactive domains within each peptide with a common N-terminal sequence important for both the affinity and specificity of peptide-receptor binding. Interestingly, the N-terminal dipeptide exhibits significant homology across family members, containing exclusively His or Tyr residues at position 1, and either Ser or Ala at position 2. Not surprisingly, the N-terminal dipeptide has been shown to be requisite for proper receptor activation (Sherwood et al. 2000).
Biosynthesis and Tissue Distribution of Glucagon, GIP and GLP-1

Gene Structure
In keeping with the high degree of homology between glucagon superfamily members, the genes encoding proGIP and proglucagon share several structural features. For example, both span ~10 kb and contain 6 exons and 5 introns. In addition, the 5' and 3' untranslated regions of both peptide precursors are encoded by exons 1 and 6 respectively, while the signal peptides are encoded by exon 2 (Bell et al. 1983, Heinrich et al. 1984, Takeda et al. 1987, Inagaki et al. 1989). Despite these similarities, the GIP and proglucagon genes are distinct. The human GIP gene resides on chromosome 17, proglucagon on the long arm of chromosome 2 (2q36-7). Also, whereas the sequence encoding mature GIP is shared between exons 2, 3, and 4 (majority in exon 3), the proglucagon coding regions for each of glucagon, GLP-1 and GLP-2 (glucagon-like peptide-2) are contained within individual exons (3, 4 and 5 respectively). Until very recently, little was known regarding the mechanisms surrounding the very discrete, cell-specific expression patterns of GIP and proglucagon. Inagaki et al. showed that the promoter of the human GIP gene contained PKA and PKC regulatory sites in AP-1 and AP-2 consensus sequences in addition to 2 cAMP response element (CRE)-like sequences (Inagaki et al. 1989, Someya et al. 1993). The latter are as of yet functionally undefined. Partial definition of the rat GIP promoter showed a similar regulatory sequence pattern with the addition of a distal, likely tissue-specificity dictating, GATA sequence (Higashimoto and Liddle 1993, Boylan et al. 1997). The 5' promoter of the proglucagon gene has been analyzed in somewhat more detail, revealing at least 7 important upstream transcriptional control elements designated G1 through G4, CRE, CES (CCAAT enhancer-binding protein enhancer site), and ISE (intestinal specific element) (Jin and Drucker 1995, Philippe et al. 1988, and reviewed by Kieffer and Habener 1999). The functional binding partners identified so far for these elements are thought to confer tissue specificity and endocrine regulation of gene expression.

Tissue Distribution
Considering the known functional similarities between the incretin hormones, there is a significant difference in their respective expression patterns indicating a defined divergence in their physiological roles. GIP expression is restricted to the upper small intestine (Buchan et al. 1978), to the submandibular gland (in rats; Tseng et al. 1993), and to the stomach (Yeung et al. 1999), supporting a possible role in priming of the gluco-regulatory system. Conversely, proglucagon expression is localized to more distal regions of the GI-tract, the pancreatic α-cell, and specific regions of the brain stem. Posttranslational modification of proglucagon yields a glucagon dominant phenotype for the α-cell and a GLP-1 dominant phenotype for the remaining known sites of proglucagon gene expression (Drucker and Asa 1988, Eissele et al. 1992). The distal expression pattern of GLP-1 in the GI-tract suggests a preferential role in handling of large nutrient loads as opposed to system priming. Reports of GLP-1 expression in the brainstem and of GLP-1-induced satiation are consistent with this idea (Shimizu et al. 1987, Turton et al. 1996).
Posttranslational Modification

As mentioned, proGIP is processed to a single biologically active peptide while expression of preproglucagon has the potential to yield up to six distinct biologically active products. Though alternative exon splicing of the gene has been reported, tissue specificity of expression is believed to occur downstream of transcription through alternative posttranslational processing (Fehmann et al. 1995). The process is mediated primarily by the prohormone-convertases PC 1/3 and PC 2, the same proteases as those involved in posttranslational maturation of proinsulin to insulin (Rouille et al. 1995). PC 1/3 hydrolysis alone is capable of processing proglucagon to the intestine and brain specific products glicentin, glicentin-related polypeptide (GRPP), oxyntomodulin, GLP-1 and GLP-2, while the formation of mature glucagon, though not fully defined, appears to involve the combined activity of PC2 and the carboxypeptidases E and H (Dhanvantari et al. 1996). Further processing of GLP-17-37 to GLP-17-36amide is thought to require the activity of peptidyl glycine α-amidating monooxygenase (PAM).

Regulation of Glucagon, GIP and GLP-1 Secretion

The factors regulating glucagon and incretin secretion are intricate, including a number of nutrient substrates, hormones and neural pathways. Integration of these metabolic and autonomic signals at the level of the α-, K- and L- cell through equally complex networks of intracellular signals allows distinct secretory profiles for glucagon, GIP and GLP-1 under a variety of physiological circumstances.

Regulation of Glucagon Secretion

Substrate Regulation. Considering that the primary physiological role of glucagon is prevention of hypoglycemia, it is not surprising that the α-cell is finely tuned to respond to changes in extracellular glucose concentrations. Elevation of plasma glucose suppresses glucagon release from the α-cell through a relatively poorly understood mechanism limited, at least in part, by the transport and metabolism of glucose (Buchanan et al. 1969, Ohneda et al. 1969, Gorus et al. 1984, Weir et al. 1989). Similar to glucose, elevation of plasma free fatty acid (FFA) levels in vivo, suppresses glucagon release and vice versa. The exact physiological role of this regulatory mechanism, one that is superceded by glucose-regulation of glucagon secretion, is not clearly understood (Gerich et al. 1974c, Andrews et al. 1975, Quabbe et al. 1977). Some have postulated that elevation of plasma FFA either during feeding or starvation may serve to blunt the glucagon response thereby restricting hepatic glycogenolysis and gluconeogenesis and ensuring preservation of vital gluconeogenic substrates (i.e. protein; the absence of a storage form for amino acids dictates that catabolism of protein always results in loss of function). It therefore follows that in the context of glucagon secretion the α-cell is also responsive to the extracellular concentration of amino acids (Gerich et al. 1974a, Pagliara et al. 1974). Small increases in positively charged amino acid levels, within the physiological range, stimulate biphasic release of both insulin and glucagon, the latter providing both i. a mechanism to prevent insulin-induced hypoglycemia
during absorption of a protein rich meal and \( ii. \) a mechanism to enhance direction of ingested amino acids towards gluconeogenesis during starvation (Muller et al. 1971).

**Endocrine Regulation.** Nutrients aside, several studies have examined the effect of exogenous hormone administration on glucagon release and action. These have revealed a number of important endocrine regulators of glucagon secretion including GIP, GLP-1, insulin and somatostatin as well as stress-factors such as catecholamines, endorphins, and growth-hormone (Kieffer et al. 2001). Of the metabolic hormones, only GIP has been shown to be glucagonotropic; GLP-1, insulin, and somatostatin all show inhibitory effects on glucagon release during pancreas perfusion. Glucagon release by GIP has been shown to be inversely glucose-dependent, stimulating secretion at glucose concentrations less than 5.5 mM (Taminato et al. 1977, Pederson and Brown 1978). The physiological relevance of this system is not clear though it may represent an additional counterregulatory mechanism relevant during absorption of protein and/or fat rich meals in which GIP and insulin levels are highly elevated in the absence of an appreciable rise in blood glucose. In contrast to GIP, insulin and GLP-1 have both been shown to be glucagonostatic. However, stemming in part from a failure to show detectable levels of insulin or GLP-1 receptor mRNA or protein in the \( \alpha \)-cell (Bullock et al. 1996, Moens et al. 1996), debate still exists as to whether these effects are direct or indirect. A postulated indirect mediator of the static effects of these hormones is somatostatin which has been shown in numerous studies to be glucagonostatic. The effects of somatostatin on the \( \alpha \)-cell are receptor-mediated and are believed to impact both release of intracellular glucagon stores and proglucagon promoter activity (Kendall et al. 1995). In addition to the regulational interplay of the incretins, insulin and glucagon, numerous studies have shown a stimulatory effect of glucocorticoids (possibly indirect; Marco et al. 1976), endorphins (Kanter et al. 1980), and growth hormone (Tai and Pek 1976) on glucagon secretion.

**Neural regulation.** Early studies using adrenergic and cholinergic blockade of neural efferents extending from the ventro-medial hypothalamus (VMH) and impacting the pancreatic islet, implicated peptidergic neurotransmitters in the release of both insulin and glucagon. In addition to the classical neurotransmitters (nor)epinephrine and acetylcholine, VIP, gastrin-releasing peptide (GRP), neuropeptide Y (NPY), PACAP, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK) and galanin have all been shown to regulate pancreatic endocrine secretion, including that of glucagon (reviewed by Ahren 2000). Glucopenic stress stimulates the sympathetic and parasympathetic pancreatic nerves, as well as adrenal medullary epinephrine release. Though dissection of the relative contribution of each of these factors (classic and peptidergic) remains a challenge (Ahren 2000), the majority of studies indicate that neural regulation of glucagon secretion is primarily stimulatory, consistent with its role as the principal counterregulatory hormone.
Regulation of GIP and GLP-1 Secretion

Substrate Regulation. By definition, the incretins GIP and GLP-1 potentiate glucose-induced insulin secretion. It follows, therefore, that incretin secretion is stimulated by nutrient ingestion. Interestingly, it is the absorption (and likely metabolism), rather than the mere presence, of luminal nutrients that triggers GIP and GLP-1 release by K- and L- cells, respectively. While absorption of fats has consistently been demonstrated to cause release of both peptides, reports of amino acid and sugar stimulation of secretion are more variable. Sykes et al., in a characterization of mono- and disaccharide stimulation of GIP release, concluded that glucose, sucrose, and maltose were effective stimulators of GIP release (a process dependent on cell entry), while fructose, mannose and lactose were ineffective (Sykes et al. 1980). The same pattern was eventually shown for GLP-1 release from L-cells, suggesting similar stimulus-secretion coupling mechanisms for the two peptides (Shima et al. 1990). Several studies, surprisingly, have failed to demonstrate direct glucose stimulation of GLP-1 secretion (Roberge and Brubaker 1991, Damholt et al. 1998).

Less controversial are studies examining the efficacy of fat in releasing GIP and GLP-1 into the circulation. FFAs have been shown to trigger rapid, robust, and more prolonged incretin secretory responses than glucose. Several groups have shown, in patients with defective lipid metabolism and in pancreatectomized dogs, that it is in fact the products of triglyceride (TG) hydrolysis rather than the TGs themselves, which mediate the secretory response (Ross and Shaffer 1981, Ohneda et al. 1983). Studies examining the modulation of incretin secretion by amino acids have shown varied results (reviewed by Fehmann et al. 1995). Though controversial, the idea of amino acid dependent incretin secretion fits with the concept that substrate entry into metabolism is required for stimulus-secretion coupling, as is hypothesized for glucose-stimulated release.

Endocrine and Neural Regulation. Similar to the secretory regulation of glucagon, the incretin hormones have been shown to be regulated in part at the endocrine level. Insulin and somatostatin have been shown in numerous models to inhibit GLP-1 release while GIP has been shown to augment it, the latter forming the postulated proximal-distal loop (Matsuyama et al. 1975, Bloom and Polak 1982, Brubaker 1991, Damholt et al. 1998). At physiological concentrations, this proposed regulatory loop is believed to be vagally (or myenterically) mediated, a conclusion based upon administration of bombesin (a GRH antagonist) during fat loading as well as selective hepatic branch vagotomy (Roberge et al. 1996, Rocca and Brubaker 1999). The exact mediators of this process are not known, though they are likely to involve acetylcholine, norepinephrine, and gastrin-releasing peptide, all of which have been shown to stimulate GLP-1 release at physiological levels (Bruzzone et al. 1983, Ohneda et al. 1989, Dumoulin et al. 1995, Roberge et al. 1996). Much less is known specifically about the neural and endocrine regulation of GIP release. Studies in a K-cell enriched intestinally-derived cell line suggest direct suppression of GIP release by somatostatin (Kieffer et al. 1995a).
Metabolism of GIP and the Proglucagon-derived Peptides

The circulating concentration of a given peptide hormone is determined by both the rate of entry into and the rate of exit from the plasma compartment (the secretory and metabolic clearance rates (MCR) respectively). Measurement of these parameters is typically made using immunoassays in which the primary (peptide specific) antibody may or may not cross-react with biologically inactive, often truncated, metabolites of the molecule. Thus, reported circulating peptide half-lives often overestimate active concentrations and true biological half-life.

The MCRs for glucagon, GIP and GLP-1 correspond to estimated circulating plasma half-lives of approximately 5 minutes, a relatively consistent figure when compared in humans (Alford et al. 1976, Jorde et al. 1981, Orskov et al. 1993), dogs (Lefebvre and Luyckx 1976, Jaspan et al. 1981), and pigs (Van Hoorn et al. 1978, Deacon et al. 1996, 2001). For all three peptides this relatively rapid MCR results from renal filtration, hepatic clearance and postsecretory metabolism in the circulation, processes dependent in large part on substrate cleavage by a number of proteolytic enzymes. With molecular weights ≤ 5 kDa glucagon, GIP and GLP-1 are freely filtered at the glomerulus. On average renal clearance accounts for ~30% of glucagon and incretin clearance, a figure not well represented by a urinary clearance of near-zero. The latter though, is a physiological consequence of the extremely high density of both specific and non-specific proteases found at the proximal tubule brush border (Peterson et al. 1982, Rovira et al. 1982). A similarly significant extraction rate of ~20% is estimated for the liver, the product of rapid proteolytic degradation by both receptor-binding dependent and independent means. Early studies using serine- or cysteine- protease specific inhibitors implicated both classes of peptidase in the circulatory clearance of the glucagon like peptides. More specifically, and common to all three aforementioned sources of clearance, dipeptidyl peptidase IV (DP IV), Cathepsin B, and neutral endopeptidase (NEP) have all been described as playing a significant role in the process.

Physiological Actions of Glucagon and the Incretins

Glucagon

Counterregulation. Glucagon is chief among a number of redundant “counterregulatory” systems, physiological mechanisms responsible for the prevention of hypoglycemia. During fasting, strenuous exercise, or pharmacological interventions such as insulin overdose, neural inputs originating from the VMH sense hypoglycemia and stimulate glucagon secretion. In addition, direct sensing by the α-cell of a waning glucose concentration in the internal milieu triggers counterregulatory glucagon secretion. Through interaction with its cognate receptor on the surface of hepatocytes, glucagon stimulates glycogenolysis, gluconeogenesis, and ketogenesis while exerting an inhibitory influence over the opposing biochemical processes. In addition, pathways effecting preferential utilization of FFA over glucose are stimulated (Randle et al. 1965). More specifically, these physiological effects are mediated through PKA-dependent activation of phosphorylase kinase and inhibition of glycogen synthase,
phosphofructokinase-2, and pyruvate kinase (Pilkis et al. 1988, Pilkis and Granner 1992). The cumulative effects include a reduction in glucose utilization by non-glucose requiring tissues and hepatic release of glucose either liberated from glycogen stores or synthesized de novo. In the same manner, adrenergic stimulation of glucagon secretion during "fight-or-flight" circumstances enables liberation of energy stores to satisfy the requirements of sudden, intense physical exertion (Gerich et al. 1974b).

Other. Glucagon and the truncated tryptic glucagon_{19,29} fragment exert a potent dual-inotrophic effect on cardiac myocytes. The truncated metabolite, also known as miniglucagon, potently stimulates accumulation and storage of Ca^{2+} into sarcoplasmic stores (cAMP-independent), which then serve as a target for Ca^{2+}-induced Ca^{2+} release by the parent molecule (cAMP-dependent). These effects are exploited clinically during cardiac emergency requiring pharmacological inotropic intervention (Mery et al. 1990, Pavoine et al. 1991). More recently, miniglucagon has been shown to inhibit secretagogue-induced insulin release from immortalized β-cells through a G-protein coupled mechanism with indirect involvement of voltage-gated L-type calcium channels (Dalle et al. 1999).

GIP and GLP-1

β-cell function and turnover. Considerable overlap exists between the known physiological roles of the incretin hormones, a fact poorly reflected by a disproportionate research focus on GLP-1 in the literature. As mentioned above, both hormones potently stimulate the secretion of insulin from the pancreatic β-cell in a glucose dependent manner (Pederson and Brown 1976, Holst et al. 1987, Kreymann et al. 1987, Mojsov et al. 1987). In addition, GIP and GLP-1 appear to play an important role in the maintenance of proper β-cell function, turnover, and development.

With regards to β-cell function, GIP and GLP-1, together with glucagon, have been shown to stimulate β-cell glucose competence and insulin production. The former is a theoretical measure of the ability of a cell to regulate processes in a glucose-dependent manner, in this case insulin secretion and incretin-stimulation of insulin secretion (Pederson and Brown 1978, Holz et al. 1993, Huypens et al. 2000). Glucose competence could also be considered an optimization of the cross-talk or interdependence between glucose metabolism and the insulin secretory mechanism. Since the latter is in large part cAMP-dependent, a number of stimuli likely share the ability to enhance glucose competence and are likely required in a permissive sense for its occurrence. Also, GIP and GLP-1 have both been shown to stimulate both proinsulin gene transcription and insulin biosynthesis (Drucker et al. 1987, Fehmann and Habener 1992, Fehmann and Goke 1995, Wang et al. 1996). Thus, the incretins, through stimulation of intracellular insulin pools and glucose-dependence of insulin release, indirectly create a positive feedback loop for their own actions at the intracellular level.

In addition to enhancement of insulin secretion, GIP and GLP-1 likely exert a trophic and anti-apoptotic tone on the β-cell. Further, a number of reports suggest that GLP-1 stimulates differentiation
of a ductal β-cell precursor population and possibly an intra-islet precursor population, towards a mature β-cell phenotype. For example, GLP-1 was shown by Susini et al. to synergize with glucose to enhance expression of c-fos, c-jun, junB, zif-268, and nur-77, transcription factors intimately involved in cell proliferation and differentiation (Susini et al. 1998, 2000). Also, a number of in vivo studies have lent support to such claims (Xu et al. 1999, Tourrel et al. 2001, Pospisilik et al. 2003). Interestingly, both GIPR −/− and GLP-1R −/− mice exhibit reductions in islet insulin content both at the protein and mRNA levels. However, partial compensation in the form of β-cell hyperplasia is present only in the GIPR null mouse; islets of the GLP-1R −/− mouse are reduced in size. These findings further suggest divergence in the physiological roles offered by the incretins at the level of β-cell turnover (Scrocchi et al. 1996, Pederson et al. 1998a, Pamir et al. 2003).

Functional GLP-1 and GIP receptors have also been identified on a subset of pancreatic α-cells and δ-cells (Fehmann and Habener 1991, Orskov and Poulsen 1991, Moens et al. 1996). The incretin hormones have both been shown, in islet and perfused pancreas preparations, to increase the secretion of somatostatin (Szecowka et al. 1982, Schmid et al. 1990), and to stimulate the secretion of glucagon (Pederson and Brown 1978, Ding et al. 1997). During investigations in vivo however, GLP-1 and GIP appear to inhibit glucagon secretion. Similar to insulin biosynthesis in the β-cell, it has been speculated that this phenomenon may serve as a potential autocrine/paracrine mechanism producing a stimulatory tone for glucagon biosynthesis in the α-cell (Heller et al. 1997).

**Extra-pancreatic effects.** As mentioned previously, GIP was initially discovered on the basis of its ability to suppress gastric acid secretion and gastric motility (Brown and Pederson 1970), though in man these effects may be limited to supraphysiological doses (Nauck et al. 1992). On the contrary, GLP-1 has been shown to potent suppress gastric function in addition to stimulating the release of the inhibitory peptide somatostatin (Schjoldager et al. 1989, Eissele et al. 1990). Species and dose-response variation, and conflicting data between in vitro and in vivo studies have fueled controversy surrounding the physiological role of these peptides in gastric function, though the prevailing thought is that whereas GIP is relatively inert, GLP-1 functions as a potent ileal brake delaying gastric emptying during large nutrient loads (Nauck 1999). The latter is in agreement with the previously stated hypothesis regarding the role of GLP-1 in acceptance of large nutrient loads. From an evolutionary perspective, an ileal brake mechanism might prevent nutrient loss through the GI-tract, optimizing absorption.

The inhibitory effects of GLP-1 on gastric emptying are dependent on intact vagal innervation (Imeryuz et al. 1997, Wettergren et al. 1997). As such, the upper GI effects of the peptide could involve the cognate receptors that have been shown to be present in the central nervous system. GLP-1 has been shown to be a potent inhibitor of the cephalic phase of gastric acid secretion (Wettergren et al. 1994). Also, the peptide has been reported to stimulate satiety (Shimizu et al. 1987, Turton et al. 1996) through a receptor-specific, hypothalamic mechanism that involves, a reduction in expression of the
orexigenic peptides neuropeptide Y (NPY) and GRH (Turton et al. 1996). These effects have been confirmed in humans, though controversy exists as to the interpretation of such data and the distinction between satiety and food aversion (Flint et al. 1998).

Pancreatic and gastric effects aside, there is a degree of uncertainty as to effects of the incretins at the major peripheral insulin-sensitive tissues, namely liver, adipose and skeletal muscle. Despite the failure of several studies to demonstrate expression of the receptors to GIP or GLP-1 in the liver (Usdin et al. 1993, Bullock et al. 1996), both peptides have been reported to stimulate glucagon-opposing actions in hepatocytes. Dupre and colleagues, followed by several other groups, described GIP-induced reduction of glucagon-stimulated hepatic glucose production (Dupre et al. 1976, Andersen et al. 1980, Hartmann et al. 1986) while Valverde and colleagues first reported the glycogenic potential of GLP-1 (Valverde et al. 1994). In adipose tissue, the presence of GIP- and GLP-1 binding sites has been demonstrated. Also, GIP has been shown to enhance lipoprotein lipase activity in vitro, and the disposal of circulating triglycerides in vivo, findings suggestive of an anabolic effect of GIP on fat (Eckel et al. 1979, Wasada et al. 1981, Beck 1989, Ebert et al. 1991). However, a recent study by McIntosh et al. demonstrated a possible lipolytic action of GIP at low insulin levels (such as those experienced during fasting). Thus, it has been hypothesized that GIP may serve to prime β-cells during fasting through maintenance of a circulating FFA pool, and in synergy with insulin, enhance fat deposition during feeding (McIntosh et al. 1999). Recent generation of a GIP receptor null mouse line has provided support for an active role for GIP in fat metabolism. GIPR -/- mice were shown to be resistant towards high-fat diet-induced obesity and insulin resistance and to exhibit an ameliorated phenotype when crossed with ob/ob mice (Miyawaki et al. 1999, 2002). Though studies examining the effects of GLP-1 on adipose tissue, and of both incretins on skeletal muscle exist, their interpretation in terms of mechanistic and functional relevancy remains unclear. Particularly confounding has been the aforementioned failure in several studies to detect expression of the GIP- or GLP-1 receptors in these tissues (Usdin et al. 1993, Kaplan and Vigna 1994, Bullock et al. 1996).

Other reported extra-insular effects of GIP include: modulation of mesenteric blood flow (Fara and Salazar 1978, Kogire et al. 1988), stimulation of follicle-stimulating hormone and luteinizing hormone (LH) from the anterior pituitary (pharmacological doses; Ottlecz et al. 1985), stimulation of osteoblasts (Bollag et al. 2001), and stimulation of glucocorticoid release (Mazzocchi et al. 1999). Similarly, increased levels of luteinizing hormone releasing factor and LH have been reported in response to GLP-1 in the rat (Beak et al. 1998), as has the stimulation of tachycardia and arterial hypertension (Barragan et al. 1994).
Glucagon, GIP and GLP-1 and the pathogenesis of Diabetes Mellitus

Clinically, diabetes mellitus presents itself in two major forms, type-1 (previously insulin-dependent diabetes mellitus; IDDM) and type-2 (previously non-insulin dependent diabetes mellitus; NIDDM), distinct pathologies with few shared etiological features. Type-1 diabetes is characterized by hyperglycemia in the complete absence of insulin secretory capacity, a consequence of autoimmune mediated destruction of pancreatic β-cells (reviewed by Rizza et al. 2001). Though poorly understood, the process is believed to involve a number of leukocyte subsets including thymus- and bone-marrow derived lymphocytes (T- and B-cells), dendritic cells, macrophages and natural killer (NK-) cells, the infiltration of which into the islet begins well before the triggering of β-cell apoptotic events. Though clinical onset peaks between the ages 10-15 years, the preceding immune process is believed to take several years. Associated with the early phase of disease progression is the appearance of one or more circulating islet autoantibodies. Commonly targeted autoantigens include glutamate decarboxylase, protein tyrosine phosphatase IA-2b, and insulin itself (Lernmark et al. 1978, Ginsberg-Fellner et al. 1985). And though clinically relevant in the early prediction of disease development, the discordance between clinical onset and autoantibody profiles suggests limited etiological involvement. The concordance rate for type-1 diabetes among monozygotic twins is approximately 60-70% which indicates the involvement of both genetic and environmental components in its etiology (Kumar et al. 1993). Indeed, identification of the enigmatic environmental trigger(s) of type-1 diabetes remains a paramount focus in diabetes research.

While equally complex, the etiology of type-2 diabetes shares little overlap with that of its autoimmune counterpart. Type-2 diabetes is a metabolic disorder with distinct genetic (~80% monozygotic concordance) and environmental components, characterized by hyperglycemia, hyperinsulinemia, hyperglucagonemia, and insulin resistance. The two hallmarks of the disease, insulin resistance and β-cell dysfunction, describe abnormalities in the regulated release and peripheral (target) sensitivity to insulin (reviewed by Cusi and DeFronzo 2001). Contributing factors towards the development of type-2 diabetes (analogous to the environmental triggers described for type-1) include obesity, sedentary lifestyle and poor diet. The complexity, therefore, in the etiology of type-2 diabetes, stems from that inherent to the network of metabolic regulatory systems in the body. Approximately 50% of type-2 diabetic patients are obese and suffer from dyslipidemia (typically hyperlipidemia) the culmination of which results in a predisposition towards secondary cardiovascular complications including coronary artery disease (CAD) and hypertension. There is a body of evidence that links both lipid abnormalities and hypertension to insulin resistance (Ginsberg 2000). There is also considerable evidence that reversing the hyperglycemic state of obesity/type-2 diabetes by diet, exercise and/or drugs leads to a decrease in insulin resistance (increased insulin sensitivity) and in turn reduces risk factors for CAD and hypertension.
Insulin aside, the idea that glucagon plays an essential role in the pathogenesis of diabetes is not new. The concept stems from a hypothesis put forward by Unger and Orci in 1975 that diabetes (types-1 and -2) is a bihormonal disorder (Unger and Orci 1975). The authors recognized that while the major consequence of relative or absolute insulin deficiency was glucose underutilization, and while the major consequence of relative or absolute glucagon excess was glucose overproduction, it was the latter that was more consistently identified in various forms of endogenous hyperglycemia. Indeed, diabetic patients display insulin resistance and β-cell impairment and a consistently dysfunctional α-cell secretory response characterized by insensitivity to glucose-inhibition (Unger and Orci 1975).

In addition to dysfunctional insulin and glucagon signaling, patients with type-2 diabetes exhibit a blunted incretin effect. Oral glucose no longer stimulates insulin secretion beyond that of an isoglycemic intravenous infusion. The secretory responses of GIP and GLP-1 to nutrients in type-2 diabetic patients, however, appear normal if not exaggerated, suggesting a post-secretory defect. More detailed studies of this phenomenon in humans have shown a blunted β-cell response to GIP, while that of GLP-1 appears normal (at least at pharmacological doses; Krarup et al. 1987, Nauck et al. 1992, 1993, Elahi et al. 1994), leading to speculation in the field of defective GIP-receptor expression at the level of the β-cell (Holst et al. 1997). More recently, investigations have extended the defect to first-degree relatives of type-2 diabetic patients, implying a significant role in the etiology of the disease (Meier et al. 2001). And most recently, Lynn et al. provided the first causal link between hyperglycemia and reduced GIP receptor expression, suggesting that the blunted incretin effect may be secondary to hyperglycemia (Lynn et al. 2003). This could explain Greenbaum’s recent observation of a blunted incretin response in a subset of type-1 diabetics (likely those that have poorly controlled glycemia) (Greenbaum et al. 2002), and is in agreement with the recent link demonstrated by Laybutt and colleagues between magnitude and duration of hyperglycemia and loss of β-cell phenotype (Laybutt et al. 2003).

**DIPEPTIDYL PEPTIDASE IV**

*Discovery, Structure, and Distribution*

Since the initial characterization of Dipeptidyl Peptidase IV (DP IV; DPPIV; EC 3.4.14.5; CD26) on the basis of an Xaa-Pro cleaving enzymatic activity (initially described as dipeptide naphthylamidase), the protein has been rediscovered several times as a cellular marker and binding protein (Hopsu-Havu and Glenner 1966, Morimoto and Schlossman 1998, Mentlein 1999). DP IV is a highly-glycosylated, multifunctional ectopeptidase, belonging to the SC clan of serine proteases. The cDNA encoding human DP IV predicts a 766 amino acid protein with 9 potential glycosylation sites and a molecular mass of approximately 88 kDa (Darmoul et al. 1992, Misumi et al. 1992). The purified enzyme, however, is found to be homodimeric and richly glycosylated yielding molecular weights in the range
of 200-240 kDa depending on species, age, tissue, and pathophysiology. The enzyme contains six major domains: i. a highly conserved 6 amino acid N-terminal intracellular tail with 3 basic residues and one threonine (potential phosphorylation site), ii. a single hydrophobic transmembrane anchor, iii. a short flexible stalk, iv. a glycosylation-rich region, v. a cysteine-rich region, and vi. a highly-conserved ~200 aa C-terminal catalytic domain. Cleavage of the N-terminus within the flexible stalk region \textit{in vivo}, yields a truncated soluble form of the protein that can be found in a number of body fluids. Two recent crystal structure determinations of DP IV, have served to confirm the majority of predicted structural properties of the molecule as well as to reveal a new “side”-opening to the active site (Engel \textit{et al.} 2003, Rasmussen \textit{et al.} 2003).

In keeping with the pleiotropy ascribed to DP IV, expression of the enzyme has been reported in most tissues of the body. Particularly high expression levels are found on intestinal and renal brush borders and on endothelial cells of the circulatory system. Also varying levels have been shown throughout the haematopoietic system (Schon \textit{et al.} 1984, Hegen \textit{et al.} 1990, Buhling \textit{et al.} 1994, 1995), on the surface of hepatocytes (Hopsu-Havu and Glenner 1966), within pancreatic islets of Langerhans (Mentzel \textit{et al.} 1996), and in particular, co-localized with glucagon within secretory vesicles of the \( \alpha \)-cell (Poulsen \textit{et al.} 1993).

Interestingly, the gene encoding DP IV, referred to as DPP4, is located adjacent to the proglucagon gene on the long-arm of chromosome 2 (2q24.3 region in humans). DPP4 contains 26 small exons, spans ~80 kb, and though it contains neither a TATA nor a CAAT box, the 5' flanking region is G and C rich (~72%) and contains potential binding sites for the transcription factors NFKB, AP-2, and Sp-1. The G and C rich region and lack of TATA or CAAT regulatory elements is a consistent feature of housekeeping proteins. DPP4 encodes two mRNA strands of ~4.2 and 2.8 kb in length which are differentially expressed in various tissues (Abbott \textit{et al.} 1994, Bohm \textit{et al.} 1995).

Studies over the last decade have reported a number of DP IV-like enzymes, proteases possessing either structural homology to native DP IV or having a post-proline substrate specificity at position two from the N-terminus (P\(_1\)), an enzymatic property long-believed to be unique to DP IV (Sedo and Malik 2001). Included are the proteins attractin (Duke-Cohan \textit{et al.} 1995, Gunn \textit{et al.} 1999), DP II (Rawlings and Barrett 1996), DP IV-\( \beta \) (Jacotot \textit{et al.} 1996), DPP8 (Abbott \textit{et al.} 2000), DPP9 (Olsen and Wagtmann 2002), DPPX (also DPP6; Hough \textit{et al.} 1998, Kin \textit{et al.} 2001, Nadal \textit{et al.} 2003), fibroblast activating protein-\( \alpha \) (FAP-\( \alpha \); seprase; Rettig \textit{et al.} 1994), and quiescent cell proline dipeptidase (QPP; (Chiravuri \textit{et al.} 1999, Underwood \textit{et al.} 1999). As the sole naturally occurring amino-acid with a cyclic side-chain structure, proline exerts numerous structural and functional influences on the peptide molecules of which it is a part. Perhaps paramount among them is protection from non-specific enzyme degradation. It follows first, that presence within a peptide of an evolutionarily conserved proline residue indicates a probable proteolytic-processing regulatory element and second, that proline-specific
proteases represent an important functional regulatory mechanism for the activity of proline containing peptides (Yaron and Naider 1993, Cunningham and O'Connor 1997). The DP IV-like peptidases mentioned above vary in tissue and sub-cellular expression patterns, in pH optima, and in precise substrate specificity indicating distinct physiological roles; none share as profound and ubiquitous an expression pattern as DP IV as far as accessibility to circulating hormones are concerned.

Role of DP IV in Regulatory Peptide Metabolism

DP IV has been classified as a non-classical serine-protease in that the linear arrangement of the catalytic triad (Ser\textsuperscript{630}, Asp\textsuperscript{709}, His\textsuperscript{741}) is inverted with respect to the trypsin-like serine-proteases (His-Asp-Ser). The consensus sequence proposed for serine proteases (G-X-S-X-G), however, is retained around the catalytic S residue (GW\textsuperscript{SYG}). The inverted triad arrangement likely contributes to the relatively unique substrate specificity of the enzyme. Mammalian DP IV preferentially accepts prolyl, alanyl or seryl residues at the P\textsubscript{1} position of polypeptides less than 70-80 amino acids in length, though longer peptides and those with alternate P\textsubscript{1} residues have been shown to serve as lower rate substrates (Pro > Ala > Ser/Gly >> Val/Leu). Recently, this generally accepted substrate selectivity, whose definition is based on cleavage of short synthetic para-nitroanilide-containing peptides, has been shown to be less stringent when dealing with natural peptide substrates, with a number of seryl containing peptides displaying more favourable cleavage kinetics than the alanyl containing glucagon-like peptides -1 and -2 (Pospisilik et al. 2001, Lambeir et al. 2002). Bulky, hydrophobic or basic amino acids are preferred at P\textsubscript{2} while proline residues are not accepted at P\textsubscript{1}'. Though seemingly restrictive, the substrate specificity for DP IV includes a large number of neuropeptides, metabolic hormones, and cytokines/chemokines. A summary of these is provided in Table I and an overview of the functional relevance of DP IV-mediated degradation of a selected number of these is provided below. A recent review by Mentlein provides a detailed comprehensive review of the role of DP IV in regulatory peptide inactivation (Mentlein 1999).

Vital to the determination of physiological relevance of DP IV-mediated substrate cleavage for peptide hormones has been the existence of a Fischer 344 rat substrain lacking functional DP IV expression (DP IV \textasciitilde\textasciitilde rat). This naturally occurring rat model, first described by Watanabe and colleagues in 1987, exhibits apparently normal DP IV mRNA expression (Thompson et al. 1991). However, a single point mutation in the catalytic site of the molecule (G-X-S-X-G\textsubscript{633}\rightarrow G-X-S-X-R\textsubscript{633}) results in an inactive protein product that is retained and degraded in the endoplasmic reticulum (Tsuji et al. 1992). Demonstration of enhanced peptide stability and functional potency in the DP IV \textasciitilde\textasciitilde rat (and in normal animals following DP IV-inhibitor administration) represents strong evidence in support of a physiological role for the regulation of a given endocrine substrate (Drucker 2003). The more recent generation of a CD26-null mouse model has provided the field with an equivalent murine model to the DP IV \textasciitilde\textasciitilde rat (Marguet et al. 2000).
Table I. Mammalian Regulatory Peptide Substrates of DP IV (adapted from Mentlein 1999)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>N-terminus</th>
<th>Length</th>
<th>Cleavage</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuropeptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>RP-KP-Q...</td>
<td>11</td>
<td>++</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td>β-casomorphin</td>
<td>YP-F...</td>
<td>7</td>
<td>+++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>YP-F...</td>
<td>4</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Enterostatin</td>
<td>VP-DP-R</td>
<td>5</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>GRP</td>
<td>VP-LP-A...</td>
<td>27</td>
<td>+++++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>YP-S...</td>
<td>36</td>
<td>+++</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td>Peptide YY</td>
<td>YP-I...</td>
<td>36</td>
<td>+++</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td><strong>Regulatory Peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHM</td>
<td>HA-E...</td>
<td>27</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>GRF</td>
<td>YA-D...</td>
<td>29/44</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Glucagon</td>
<td>HS-E...</td>
<td>29</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>GLP-1</td>
<td>HA-E...</td>
<td>30</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>GLP-2</td>
<td>HA-E...</td>
<td>34</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td>GIP</td>
<td>YA-E...</td>
<td>42</td>
<td>++</td>
<td>Inactivation</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>SP-Y...</td>
<td>68</td>
<td>+</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td>SDF-1</td>
<td>KP-V...</td>
<td>68</td>
<td>+++</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>GP-A...</td>
<td>74</td>
<td>+</td>
<td>Shifted Receptor Selectivity</td>
</tr>
<tr>
<td>MCP-2</td>
<td>QP-D...</td>
<td>76</td>
<td>+</td>
<td>Inactivation</td>
</tr>
<tr>
<td>MDC</td>
<td>GP-YG-A...</td>
<td>69</td>
<td>+++++</td>
<td>Shifted Receptor Selectivity</td>
</tr>
</tbody>
</table>

* Cleavage rates estimated from literature based on $K_{cat}$ and $K_M$ values.

GRP, gastrin-releasing peptide; PHM, peptide histidine methionine; GRF, growth hormone releasing factor; GLP-1/2, glucagon-like peptide-1/2; GIP, glucose-dependent insulinotropic polypeptide; RANTES, regulated on activation normal T-cell expressed and secreted; SDF-1, stromal cell-derived factor-1; MCP-2, monocyte chemotactic protein-2; MDC, macrophage-derived chemokine.

Neuropeptides
A number of neuropeptides fall under the umbrella of theoretical DP IV substrate specificity. Substance P (SP), for instance, a well-studied 11 amino acid transmitter of noxious stimuli, stimulator of smooth muscle contraction, and immune regulator, is sequentially degraded to the SP$_{5-11}$ form. This fragment does not lose all biological activity, rather, receptor selectivity for the molecule is shifted among tachykinin receptors and a number of immunoregulatory functions are abolished (Ahmad et al. 1992). The endorphins -1 and -2, endogenous tetrapeptide agonists of the μ-opioid receptor, are cleaved and inactivated in vivo. Manipulation of this pathway using either DP IV-resistant endomorphin analogues or a DP IV-inhibitor was shown to elicit lasting potentiation of analgesia (Shane et al. 1999). Similar to SP, the receptor selectivity of NPY and PYY (neuropeptide Y and Peptide YY) are shifted to favour the Y$_2$ and Y$_5$ receptors and to limit activation of Y$_1$. NPY is among the most rapidly cleaved endogenous substrates of DP IV, with a second order rate constant one order of magnitude higher than any member of the glucagon superfamily (Mentlein et al. 1993a), a fact in keeping with the kinetics of neurotransmission at the synaptic level. Though complex, several physiological roles have been postulated for the shift in receptor specificity of NPY and PYY resulting from DP IV-mediated cleavage including regulation of vascular contraction and angiogenesis (Zukowska-Grojec et al. 1998).
Peptide Hormones

With a highly conserved His-Ala, Tyr-Ala, or His-Ser N-terminal homology, all members of the glucagon superfamily of polypeptides satisfy the requirements of substrate specificity for DP IV. Whereas the physiological relevance of DP IV activity in the context of neuropeptides remains unclear, it is accepted that DP IV-mediated processing of several glucagon superfamily members constitutes a crucial determinant of their activity. In 1993, Mentlein and colleagues described GIP and GLP-1 as circulating substrates for DP IV (Mentlein et al. 1993b), an observation that would spark interest in the enzyme as a physiological regulator of metabolic hormone action and provide the basis for a new therapeutic paradigm for type-2 diabetes (reviewed by Drucker 2003). The incretins are potent insulin secretagogues with an N-terminal requirement for receptor activation. Thus, rapid N-terminal truncation by DP IV represents a significant clearance pathway for active (full length) GIP and GLP-1. In fact, DP IV-mediated metabolism of these peptides reduces their clearance half-lives from ~5-6 minutes (largely hepatic and renal clearance) to ~1-2 minutes, a process abrogated by addition of specific DP IV-inhibitors (reviewed by Drucker 2003, and in detail below).

Highly homologous to GLP-1 and also a member of the glucagon superfamily, the intestinotrophic peptide GLP-2 is also readily degraded by DP IV (Drucker et al. 1997). Administration of GLP-2 to DP IV -/- rats or administration of DP IV-resistant GLP-2 analogues to normal rats has been shown to increase villus height and small bowel mass, indicating a potential role for DP IV inhibitors and GLP-2 analogues in the therapeutic improvement of mucosal regeneration. In addition, physiologically relevant degradation of growth-hormone releasing factor (GRF) by DP IV has been reported. Use of degradation resistant GRF-analogues was shown to elicit prolonged elevation in serum growth hormone levels after i.v. injection in cattle (Kubiak and Friedman 1998).

Chemokines

After examination of the criteria mentioned above, it comes as no surprise that a number of chemokines are suitable substrates for DP IV. Chemokines are a group of small secretory proteins that, through interaction with members of two G-protein coupled receptor subtypes (CXC and CC), stimulate chemotaxis in a cell selective manner (De Meester et al. 1999). CXC subfamily members predominantly target neutrophils, and lymphocytes to a lesser extent, while CC members attract primarily monocytes, but also lymphocytes, basophils, eosinophils, dendritic cells, and macrophages. Of the CC-type chemokine family members, RANTES (regulated on activation normal T-cell expressed and secreted), eotaxin and macrophage-derived chemokine (MDC) have been shown to be rapidly cleaved by DP IV. Cleavage of RANTES1-68 to RANTES3-68 has been shown to shift CXC receptor selectivity to favour chemotaxis of macrophages (CCR5 receptor) and to suppress that of monocytes (Oravecz et al. 1997). A chemokine involved in allergic inflammation, eotaxin is cleaved by DP IV to an inactive form at the CCR3 receptor, thus, DP IV limits eotaxin-mediated inflammatory responses.
Similarly the CXC chemokine stromal cell-derived factor-1 (SDF-1), a chemoattractant for resting T-lymphocytes and monocytes loses biological activity at lymphocytes after N-terminal truncation by DP IV (Shioda et al. 1998). In summary, N-terminal truncation of chemokines can either activate or inactivate chemokines or shift receptor selectivity (as with NPY and PYY). Thus, DP IV-mediated cleavage may provide a mechanism for differential cell recruitment. That chemokines represent a physiologically relevant substrate class for DP IV is certain. A defined DP IV expression pattern in the haematopoetic system and the presence of detectable amounts of truncated chemokines in plasma support this contention. What remains to be elucidated is the complex regulatory network provided by DP IV-mediated chemokine truncation.

**Role of DP IV in Immune Function – Signal Transduction and Protein Binding**

DP IV is highly expressed on activated T- and B- cells, and at lower levels on quiescent T- and B-cells, on NK cells, and on macrophages. The majority of immunological studies on the protease have focused on its role in the functional modulation of T-cells and their responses to activation. DP IV, also known as CD26, costimulates CD3 and CD2 dependent T-cell activation, in part through activation of T-cell receptor (TCR) / CD3 tyrosine phosphorylation pathways. The complexity of functional interactions in which the enzyme is involved is vast, including roles both as a binding partner and a serine protease with potentially disparate roles for the soluble vs. the membrane bound forms. Indeed, circulating levels of soluble CD26 have been shown to fluctuate markedly in a number of disease states. Increases have been reported in rheumatoid arthritis, multiple sclerosis, Graves’ disease, Hashimoto’s thyroiditis, sarcoidosis, and some cancers while decreases have been witnessed in AIDS, Down’s syndrome, anorexia, pregnancy, hypogamma-globulinemia, and lupus (reviewed in Drucker 2003). The functional significance of these changes, in most cases, is unknown.

Each of the three chief functions of CD26, that is ADA binding, dipeptidase activity, and extracellular matrix (ECM) interaction, can modulate T-cell proliferation and chemotaxis (Dang et al. 1990b). These effects, mediated by both the soluble and membrane bound forms of the enzyme appear to act via interaction with CD2 and the TCR/CD3-complex signaling pathways (Dang et al. 1990a, Tanaka et al. 1994, Hegen 1997). Studies suggest that while CD26 signaling requires expression of the TCR and the CD3 ζ-chain and shares a number of substrates, the association is not direct nor is it bilaterally interdependent (Fleischer 1987, Dang et al. 1990a). Cross-linking of surface CD26 into tetrameric structures or as heteromeric structures with the T-cell receptor stimulates the phosphorylation of the protein tyrosine kinases (PTK) p56(Lck), p59(Fyn), and zeta associated PTK of 70 kDa (ZAP-70), as well as downstream activation of MAP kinase, Cbl, and phospholipase Cy (Hegen 1997, Kahne et al. 1998). Despite the overlap, it is the TCR/CD3 complex that is the predominant trigger. Signaling through CD26 is TCR/CD3 dependent, a statement in accordance with data showing that specific
modulation of CD3 can lead to a refractory state unresponsive to CD26 modulation. The converse is not true (Dang et al. 1990b).

The first of the three functions of CD26, ADA binding, has been shown to enhance CD26-induced T-cell proliferation even in the presence of ADA enzyme inhibitors indicating an extra-enzymatic, costimulatory effect of ADA (Martin et al. 1995). Further, exogenously applied soluble ADA has been shown to elicit a similar though reduced costimulatory effect (Franco et al. 1998). Second, the peptidase activity of CD26 in the context of chemokine cleavage (reviewed above) has been shown to regulate chemotaxis and the cell-specificity of several chemokines including MDC, eotaxin, RANTES, and SDF-1, with a definite role in T-cell and monocyte migration (Iwata et al. 1999). Similarly, interaction of the protease with specific inhibitors has been shown to impair DNA synthesis, IgG production and secretion and production of a number of Th1-type cytokines including IL-2, IFN-γ, IL-6, and IL-1β (Reinhold et al. 1993). Also, DP IV-inhibitors have been shown to impair DNA synthesis in B cells and downregulate proliferation, though not cytotoxicity, of NK cells (Buhling et al. 1994, 1995). Interestingly, the dogma regarding the DP IV-activity dependence of CD26-mediated T-cell costimulation has recently been challenged through studies using numerous CD26 molecules containing loss of function mutations within the DP IV active site (Tanaka et al. 1997, von Bonin et al. 1998).

Third, ECM interactions are very likely to be involved in the recently reported ability of both soluble and membrane bound CD26 to augment T-cell migration across endothelial barriers (Ikushima et al. 2002). In the same study Ikushima et al. clearly showed a reduced efficacy of DP IV-inactive CD26 mutants to augment migration. These data raise the intriguing possibility that DP IV-inhibitors might interfere with T-cell migration and thus tissue infiltration, raising CD26-assisted tissue infiltration as a therapeutic target with obvious implications for autoimmunity. The functional nuances of collagen-, fibronectin-, and integrin-binding by DP IV require clarification.

DP IV INHIBITION IN THE TREATMENT OF DISEASE

DP IV-Inhibition and Type-2 Diabetes

It was Mentein and colleagues who first demonstrated the potential of DP IV to cleave the potent insulin secretagogues GIP and GLP-1, an idea postulated over ten years earlier after the purification of GIP3-42 from intestinal extracts (Brown et al. 1981, Jörnvall et al. 1981). In 1995, Kieffer and colleagues demonstrated GIP and GLP-1 to be substrates of DP IV in vivo, and showed the rapidity of the process, yielding a circulating half-life of 1-2 minutes for the parent peptides (Kieffer et al. 1995b). Considering that the N-terminally truncated products GIP3-42 and GLP-19-36amide were previously shown to be inactive at the receptor level and thus non-insulinotropic (Schmidt et al. 1986, Suzuki et al. 1989, Hinke et al. 2002), it was theorized that DP IV-mediated truncation of the incretins served as the primary mechanism for GIP and GLP-1 inactivation (Pauly et al. 1996a). Several groups confirmed this
hypothesis and went on to demonstrate the therapeutic potential of DP IV-inhibitors in augmentating circulating incretin levels (Kieffer 1994, Pauly et al. 1996b, Hansen et al. 1999, Deacon et al. 2000). Considering their insulinotropy, their shared abilities to inhibit gastric motility and secretion (Brown and Pederson 1970, Schjoldager et al. 1989), to promote β-cell glucose competence (Huypens et al. 2000), insulin gene transcription and biosynthesis (Drucker et al. 1987, Fehmann and Habener 1992), differentiation and growth (Hui et al. 2001), as well as to restore islet-cell glucose responsiveness (Zawalich et al. 1993), DP IV-inhibitor mediated incretin enhancement proved to be a unique pleiotropic approach to the treatment of type-2 diabetes. Studies in rats using the specific, reversible DP IV-inhibitor P32/98 showed enhancement of insulin secretion and glucose tolerance after a single intraduodenal glucose bolus (Pauly et al. 1996a). Further examination by Pederson et al. during oral glucose tolerance testing in the obese Zucker rat model of type-2 diabetes showed that these improvements were much more profound in diabetic, “fatty” animals than in their lean littermates (Pederson et al. 1998b). Balkan et al. confirmed these findings using the DP IV inhibitor NVP-DPP728, and went on to provide direct evidence for the previously postulated stabilization of, and rise in, plasma active-GLP-17,36 (GLP-1a) after inhibitor treatment (Balkan et al. 1999).

**DP IV Inhibition in Immune and Inflammatory Diseases**

Recently, a therapeutic strategy has been postulated for use of DP IV-inhibitors in the suppression of T-cell lymphoid malignancies on the basis of a distinct correlation between DP IV expression and neoplastic cell behaviour. Unfortunately this phenomenon is cell-specific; reports have surfaced suggesting DP IV expression pattern correlates with both increased and decreased tumorigenicity, cell-growth, migration and invasion (Sato and Dang 2003). Thus, a deeper understanding of the complexity of these relationships will be required before any therapeutic potential of DP IV-activity modulators can be realized. In fact, this work may identify a serious potential side effect of therapeutic DP IV-inhibitor use, particularly in patients prone to the development of specific tumors. Our present knowledge regarding CD26 function on T-cells certainly points towards a role for DP IV manipulation in the treatment of T-cell lymphoid malignancies in particular (Sato and Dang 2003).

The relevance of DP IV activity and the potential therapeutic use of DP IV-inhibitors in experimental inflammatory disorders has also been addressed. Reinhold et al. recently showed an attenuation of disease severity and partial prevention of experimentally-induced autoimmune encephalitis (EAE) in mice after treatment with the DP IV-inhibitor Lys[Z(N02)]-pyr, a promising finding for such inhibitors in the treatment of multiple sclerosis (Steinbrecher et al. 2001). Similar results have been obtained after DP IV-inhibition in rats treated with collagen- and alkyldiamine-induced arthritis, also T-cell dependent inflammatory disorders (Tanaka et al. 1997). Using a slightly different approach towards the same end, Shinosaki et al. employed a DP IV-specific monoclonal antibody to elicit suppression of experimental nephritis in rats (Shinosaki et al. 2002), an effect associated with a reduction in complement activation.
ANIMAL MODELS OF DIABETES

Vancouver Diabetic Fatty Rat

The Vancouver Diabetic Fatty rat (VDF) is a substrain of the fa/fa Zucker (Zucker fatty; obese Zucker) rat that has been maintained at the University of British Columbia, Department of Physiology for ~ 10 years. The fatty (fa;Lepr^b) mutation, a Gln_266Pro substitution in the extracellular domain of the leptin receptor, arose spontaneously in an outbred stock in the Zucker laboratory in 1961 (Zucker and Zucker 1961). Homozygous recessive littermates, devoid of a functional leptin receptor (reduced binding and signaling) (Yamashita et al. 1997), exhibit reduced production and secretion of satiety-inducing hormones (e.g. CRH) and an associated increase in orexigenic peptides such as NPY. The resultant defect in central autonomic regulation is believed to be the major causative factor for the metabolic abnormalities seen in the fa/fa Zucker rat. Reduced sympathetic responsiveness appears to contribute to a reduction in energy metabolism (particularly in brown adipose tissue) while enhanced parasympathetic signaling to the endocrine pancreas lead to hyperinsulinemia and hyperglucagonemia. Phenotypically, VDF rats are hyperphagic and severely obese. Significant increases in body weight over lean littermates can be seen as early as weaning (21 days of age), at which point hyperinsulinemia and hyperlipidemia are also evident. Relatively profound insulin resistance is partially compensated for, and likely exacerbated by, β-cell hyperplasia and an exaggerated insulin secretory response (left shifted and increased in magnitude). The glucose-dependence of numerous secretagogues has similarly been reported to be left-shifted. Extensive literature exists on the Zucker fatty rat (reviewed by McIntosh and Pederson 1999) including detailed examinations of insulin resistance by means of euglycemic-hyperinsulinemic clamp. This technique, considered the gold-standard of insulin sensitivity measurement, may be performed in conscious animals and allows dissection of both hepatic and peripheral contributions towards whole body insulin sensitivity (Steele 1959). In one such study, Terrettaz and associates tested hepatic glucose output and peripheral glucose disposal rates in obese Zucker rats over a wide range of insulin concentrations allowing a comprehensive evaluation of both responsiveness (the efficacy or magnitude of the response to insulin) and sensitivity (the potency or half-maximal concentration of the response to insulin) to the hormone (Terrettaz et al. 1986). The authors concluded that the obese animals demonstrate marked hepatic insulin resistance (characterized by a fully responsive yet right-shifted hepatic glucose output) and severely impaired glucose disposal (characterized by a total lack of responsiveness over a wide range of insulin concentrations) when compared to their lean littermates.

The obese Zucker rat is one of the best-accepted animal models of type-2 diabetes due to the strong link between the metabolic abnormalities and obesity exhibited in the animal. The VDF rat in particular, displays a more profound fasting hyperglycemia and glucose intolerance relative to other fa/fa substrains making it especially well-suited to modeling the disorder. Similarly, both the VDF rat
and the human type-2 diabetic patient have been shown to exhibit an abnormally reduced incretin effect (Perley and Kipnis 1967, Nauck et al. 1993, Elahi et al. 1994, Lynn et al. 2001). Lynn et al. recently linked this abnormality, in the VDF rat, to a deficit in islet GIP-receptor expression both at the mRNA and protein levels (Lynn et al. 2001). In a subsequent study, the same group went on to propose a model of glucose-stimulated downregulation of the GIP-receptor, providing the first potential mechanism for the long-recognized defect in the diabetic state (Lynn et al. 2003).

**Streptozotocin Rat**

Streptozotocin (STZ; 2-deoxy-2-(3-methyl-3-nitrosourea) 1-D-glucopyranose] is a broad-spectrum antibiotic isolated from *Streptomyces achromogenes* that causes selective β-cell death after single high-dose administration (Junod et al. 1969). A structural analogue of glucose, STZ contains a highly reactive nitrosourea side chain thought to effect much of its cytotoxicity. Experiments using stereoisomers and alterations in the sugar backbone of STZ suggest a specific recognition site at the β-cell, believed at present to be the glucose transporter GLUT2 (Schnedl et al. 1994). Once inside the cell three mechanisms are thought to promote β-cell death: DNA methylation, free radical generation and nitric oxide production. DNA breaks, secondary to methylation, stimulate the activity of poly(ADP-ribose) synthetase, an intracellular DNA-repair mechanism (Uchigata et al. 1982). The resultant irreversible exhaustion of intracellular NAD⁺ levels, leads to the cessation of NAD⁺-dependent energy and protein metabolism, ultimately causing cell-death (Wilson et al. 1984). Elevated H₂O₂ levels have been shown to increase both in vitro and in vivo after STZ administration. Also, free radical scavengers such as superoxide dismutase have been shown to confer protection against the diabetogenic properties of STZ (Gandy et al. 1982). An integrated hypothesis summarizing these three postulated mechanisms of action suggests that peroxynitrite generated secondary to superoxide could dissociate into NO and hydroxyl radicals which would subsequently lead to DNA damage and apoptosis (Bedoya et al. 1996). The signal that directs STZ towards the β-cell has been postulated to include a high affinity of STZ for the β-cell membrane, a unique SH group that renders the β-cell membrane especially sensitive to oxidative reactions, a low capacity of β-cells to scavenge free radicals, and a low NAD⁺/DNA ratio relative to other cell types.

Within the first 24 hours after STZ injection, rodents undergo a triphasic pattern of blood glucose and insulin changes (Junod et al. 1969). Approximately 1 hour of hyperglycemia is followed by marked hypoglycemia (believed to be the result of large scale β-cell degranulation) lasting about 6 hours. Development of stable hyperglycemia ensues within 24-48 hours at blood glucose levels between 15 mM and 25 mM. Concurrent with the rise in glucose, plasma insulin and total pancreatic insulin levels fall to ~50% and <5% of normal, respectively. β-cell necrosis can be seen within 4 hours on an ultrastructural level and within 24 hour using a light microscope. The process of β-cell destruction is
largely terminated after 4 days. What remains is a largely degranulated population of β-cells with limited capacity for proliferation and regeneration. The majority of such regeneration is believed to originate from islet, rather than ductal or acinar, precursor cells (Hamming and Reynolds 1977, Fernandes et al. 1997, Guz et al. 2001).

In summary, the STZ rat provides researchers with a model of insulin insufficiency, post-traumatic β-cell plasticity, and of complications secondary to severe hyperglycemia and insulin deficiency. More suitable than alloxan due to a greater selectivity, lower mortality rate, and long half-life, the relatively permanent diabetes induced by STZ is suitable for long-term studies making it one of the most-studied models of type-1 diabetes. The major limitation of the model in the study of the disease, however, remains the absence of any significant autoimmune component. The burden in that respect is met by the availability of two autoimmune models of diabetes, the biobreeding (BB) rat and the non-obese diabetic (NOD) mouse.

**BioBreeding Rat**

Spontaneous diabetes was first detected in the BB rat in 1974 at the BioBreeding Laboratories commercial breeding facility in Ottawa, Canada, in a noninbred but closed outbred Wistar rat colony (Nakhooda et al. 1977). “Clinical” onset of diabetes in the BB rat is similar to that of the human condition with the animals exhibiting hyperglycemia, glucosuria, and weight loss concomitant with reduced plasma insulin levels within a period of several days. The BB/Ottawa colony exhibits an approximate incidence rate of 60% with no gender discordance like that of the NOD mouse. Of the diabetes-prone animals ultimately developing diabetes, over 90% display clinical onset between the ages of 60-120 days, another advantage over the NOD mouse which displays clinical onset over a longer 3-4 month period and at a later age. Onset of diabetes in the BB rat, involves a selective and complete autoimmune destruction of pancreatic β-cells, with pancreatic insulin content <0.1% of normal. Other islet endocrine cell populations appear to remain normal. At present there is no clear index of impending diabetes though larger body mass and a short (2-3 day) period of glucose intolerance immediately prior to onset have been reported (Crisa et al. 1992).

Development of diabetes in both the type-1 human patient and the BB rat, is associated with inflammatory lymphocytic infiltration of the islets of Langerhans with T-, B-, NK-, and dendritic-cells as well as macrophages. Biopsies in the BB rat suggest that such infiltration, termed insulitis, begins 2-3 weeks prior to onset; enters a phase characterized by islets that are enlarged, poorly delineated and exhibit infiltration by lymphocytes and debris laden macrophages, followed by multicellular insulitis immediately prior to onset. End-stage islets are devoid of β-cells though α- and δ-cells are preserved. Whether the pancreatic β-cell itself plays an abnormal role in the etiology of the disease remains controversial (Seemayer et al. 1982, Logothetopoulos et al. 1984).
The majority of data from the BB rat suggest that β-cells from diabetes prone (DP) animals are intrinsically normal, however, a few reports of β-cell hyperexpression of class I antigens have been reported (Li et al. 1995). Transplantation studies demonstrating recurrence of the disease in islets from congenic rat strains resistant to diabetes supports an “innocent bystander” role for the β-cell (Prowse et al. 1986). During more recent investigations, some have hypothesized a role for aberrant exposure of β-cell antigens to the immune system during the otherwise normal waves of β-cell apoptosis and neogenesis that mediate pancreatic development. This hypothesis is supported by studies demonstrating a reduction in incidence of diabetes secondary to early insulin intervention, a strategy aimed at reducing the secretory demand at the β-cell and thus reducing β-cell turnover. A similarly limited role has been postulated for autoantibodies. Though autoantibodies may be instigators of β-cell attack it is believed that they represent a secondary symptom of β-cell destruction. This is based in part on the variation in the degree and time-course of autoantibody appearance in the circulation across DP-BB rats and their poor performance as predictors of the disease (Schlosser et al. 1994).

Although extensive literature exists on the BB rat, the precise immune mechanisms responsible for initiating, amplifying, and mediating β-cell destruction remain enigmatic. However, strong evidence supports a T-cell dependent etiology. Much of this evidence stems from studies showing disease transfer by injection of immune cells from newly diabetic animals into naïve recipients (termed adoptive transfer) (Koevary et al. 1983). CD8⁺ and CD4⁺ T-cells are necessary for successful adoptive transfer of the disease. Similarly, immunoneutralization of either of these cell types has been shown to prevent disease in DP-BB animals. Also, and perhaps more specific to the BB rat, a regulatory subset of T-cells (RT6⁺) appear important in disease etiology. BB rats are deficient in this population of T-cells (or demonstrate defective RT6 expression), the restoration of which (through transfusion) prevents diabetes in DP-BB rats provided the graft is accepted (Burstein et al. 1989). This population appears to be somewhat analogous to the CD25⁺ subsets of CD4⁺ and CD8⁺ T-cells described as regulatory T-cells in mice. Indeed, infusion of mature CD4⁺ (w3/25⁺) cells from DR rats to young DP-BB rats has been shown to prevent diabetes (Mordes et al. 1987).
THESIS INVESTIGATION

The actions of DP IV on the entero-insular axis and immune system have numerous implications for both type-1 and type-2 diabetes. Under the general scope of DP IV-modulation of glucose and immune regulation in the context of diabetes, the aims of this thesis were three-fold. Glucagon is the chief endocrine counterregulatory factor and contains an N-terminal sequence consistent with theoretical substrate specificity for DP IV. The first major goal was to characterize DP IV-mediated glucagon metabolism and to demonstrate the physiological relevance, if any, of this regulatory system in vivo. Next, the use of DP IV-inhibitors to enhance insulin secretion had been convincingly demonstrated by the outset of this study. These earlier acute experiments though, failed to exploit the longer-term effects of the incretin hormones such as stimulation of β-cell insulin biosynthesis, glucose competence, proliferation, neogenesis, differentiation, and survival, as well as postulated peripheral effects on glucose uptake. Thus, the second major goal of this work was to shed light on the added potential of DP IV-inhibition borne from long-term therapy. Considering the marked effects of both incretin hormones on β-cell survival, growth and proliferation previously demonstrated in the literature, these studies were extended to include models of type-1 diabetes. First an investigation was made in the STZ rat, a model of insulin insufficiency and islet plasticity, and subsequently in the BB rat, an autoimmune model of the disease. The substance of the latter investigations served also to address, in part, the third major hypothesis of the investigation: that DP IV inhibition might suppress disease progression in type-1 diabetes via combined immunosuppression and incretin enhancement. The following studies have deepened our understanding of glucose counterregulation, set the stage for long-term DP IV-inhibition as a pharmacological tool in man, and opened the field of DP IV-inhibition to encompass its utility in the treatment of type-1 diabetes.
MATERIALS AND METHODS

OVERALL DESIGN

The current thesis is comprised of four independent studies: i. an investigation of glucagon degradation by DP IV, ii. an investigation of long-term DP IV-inhibition in the VDF rat, iii. an investigation of long-term DP IV-inhibition in the STZ rat, and iv. an investigation of long-term DP IV inhibition in the BB rat.

A number of overlapping methods were used for the experiments of the latter three studies including those for monitoring of daily/weekly body weight, blood glucose, food and water intake, and plasma analytes, and those for measurement of oral glucose tolerance and pancreatic glucose responsiveness. Unless otherwise specified, the protocols outlined below were applied to all relevant studies.

MATERIALS

Glucagon, synthetic glucagon fragments and analogues, and the DP IV-inhibitor P32/98 (Di-[2S,3S]-2-amino-3-methyl-pentanoic-1,3-thiazolidine fumarate; isoleucine thiazolidide) were a generous gift of Dr. Hans-Ulrich Demuth (Probiodrug, Germany). The glucagon analogues were synthesized using an automated peptide synthesizer SYMPHONY (RAININ), according to an Fmoc-protection protocol (Hinke et al. 2000). All reagents for peptide synthesis were analytical grade and obtained from Novabiochem (Schwalbach, Germany), Roth (Karlsruhe, Germany) or J.T. Baker (Griesheim, Germany). Products were purified by HPLC, and MALDI-TOF mass spectrometry was used to confirm peptide identity and purity (>95%). A detailed description of the synthesis of P32/98 can be found elsewhere (Demuth and Heins 1995).

Purified DP IV was also obtained from Dr. Hans-Ulrich Demuth after purification from porcine kidney according to the method described by Wolf et al. (Wolf et al. 1978). The specific activity of the DP IV used in these studies was 31 units/mg protein.

Unless otherwise specified all other materials were obtained from Sigma-Aldrich Canada Ltd. (Canada). Radioactively labeled compounds were obtained from Amersham (NJ, USA).

ANIMALS

Several different strains of rat were used for the following studies and, unless otherwise stated, all were males 12 ± 1 weeks of age at the outset of each respective experiment. Animals were housed in pairs or in triplicate, save the VDF rats undergoing post-operative recovery (clamp studies; housed singly). Specific housing conditions included a 12 hour light/dark cycle (lights on at 6 am) and access to standard rat chow (Purina 5012) and water ad libitum. DP IV -/- and VDF rats were obtained from colonies maintained in-house in the Department of Physiology at UBC (Pederson et al. 1996; Lynn et al. 2001). Fischer 344 rats, used as controls for studies involving the DP IV -/- animals, were obtained...
from Charles River (Ontario, Canada), as were spontaneously hypertensive (SH) rats used in a follow-up investigation in study II. Wistar rats were obtained from the animal care facility at UBC. All animal work was performed in accordance with the guidelines set out by "Principles of animal laboratory care" (NIH publication No. 85-23, revised 1985). Newly weaned BB rats were obtained from the Health Canada, Ottawa breeding facility and housed under semi-sterile conditions. Cages and bedding were autoclaved and replaced every three days while animals were allowed unlimited access to irradiated diet (LabDiets 5053, PMI Nutritional International, MO) and autoclaved water.

For studies involving streptozotocin-induced diabetes, male Wistar rats were administered streptozotocin (STZ; 50 mg/kg) intravenously (tail vein) while under halothane anesthesia. STZ was dissolved in a 10 mM citrate buffer (pH 4) immediately prior to injection. The control group was administered citrate buffer alone.

**GLUCAGON BIOASSAY**

*Subcutaneous Assay.* Testing of glucagon and N-terminally truncated fragments was carried out using a bioassay monitoring blood glucose concentration. Degradation of glucagon by purified pig kidney DP IV, Wistar or Fischer 344 rat plasma, or DP IV /-/- rat plasma, with or without DP IV inhibitor (P32/98, 50 µM) was assessed. Glucagon Bioactivity was assessed by incubating synthetic peptides with 0.31 units of DP IV in 1.0 mL phosphate buffered saline (PBS, pH 7.4) at 37°C for 3 hours, followed by subcutaneous injection into conscious, non-fasted, unrestrained male Wistar rats. In a similar intravenous bioassay, glucagon was incubated with serum obtained from Fischer, Wistar or DP IV /-/- rats (1.0 mL) under the same conditions (37°C, 3 hours), followed by injection via the jugular vein in anesthetized Wistar rats (65 mg/kg sodium pentobarbital; Somnitol, MTC Pharmaceuticals, Cambridge, Ont.).

Bioactivity of synthetic N-terminally truncated peptides dissolved in saline was tested using analogue doses 10-fold higher than for native glucagon (Doses: 7 nmol/kg glucagon_{1-29}, and 70 nmol/kg for glucagon_{3-29}, pyroglutamyl-glucagon_{3-29} {[pGlu]glucagon_{3-29}} and glucagon_{5-29}). Blood glucose concentration was measured using a handheld SureStep glucose analyzer (LifeScan Canada Ltd., Burnaby, B.C.) on samples obtained from the tail vein.

*Intravenous Assay.* Driven by the hypothesis that the return of the glucagon-stimulated hyperglycemic responses back to basal levels was primarily insulin-dependent (as opposed to simple glucose effectiveness) a more precise bioassay was developed in which endogenous insulin and glucagon secretion were suppressed by continuous somatostatin infusion. In this protocol animals were anaesthetized and jugular and carotid cannulae introduced. After a 15-minute equilibration period, a continuous somatostatin infusion (0.4 µg/kg/min; SS_{1-14}; Bachem, USA) was initiated via the jugular line. Following a further 20-minute equilibration period, a bolus of glucagon (or analogue; 7 pmol/kg)
was introduced into the venous line preceded by either saline or a 10 mg/kg bolus (200 μl) of P32/98. Blood was sampled from the arterial line directly into heparinized blood collecting tubes. Samples were centrifuged (12,000g, 15 min) and the plasma separated and stored at -20°C. A 25 μl fraction of each blood sample was used for glucose determination using the glucose oxidase method (Beckman Glucose Analyzer 2; Beckman Instruments, Palo Alto, USA).

Analysis of plasma glucagon levels was performed using the GL-32K Glucagon RIA kit (Linco Research Inc., MO, USA). The kit has a sensitivity of 6 pM when used according to the manufacturer’s instructions. Analysis of plasma insulin values was made using an in-house insulin RIA (3-day disequilibrium assay; guinea pig α-insulin antibody GP-01 diluted 1:10⁶; charcoal separation)(Pederson and Brown 1976). The detection limit of this assay when using a 100 μl plasma sample is ~37.5 pM.

24-HOUR PROFILES

In order to examine the effects of DP IV-inhibition on glucagon concentration (Study I; Wistar rats) and on glucose metabolism (Study II; VDF rat) over the 24-hour day/night cycle, blood samples for glucose, insulin, glucagon, and/or DP IV determination were obtained every 3 hours (tail vein) for 24 hours. In the VDF study the 24-hour profile was performed 6 weeks into the DP IV-inhibitor dosing regimen. Drug dosing, where appropriate, was continued on schedule. In order to minimize effects on metabolic parameters during the dark phase (1800-0600 hr), blood sampling was performed under the light of a 10W red incandescent bulb.

GLUCOSE TOLERANCE TESTS

Oral Glucose Tolerance Test. Oral glucose tolerance tests were performed after 16-18 hours of fasting and complete drug washout where appropriate. D-glucose (1 g/kg; Abbott Laboratories, QU) was administered by oral gavage at t=0. Blood samples for serum analysis were collected into heparinized tubes via the tail vein, centrifuged at 12,000g and stored at -20°C. Blood samples for determination of active GLP-1 content were collected directly into tubes containing the DP IV inhibitor P32/98 (final concentration 1 mM), and vortexed briefly, and immediately placed on ice. Plasma was separated and stored at -20 °C within one hour of collection. Samples were analyzed for active GLP-1 using a commercially available ELISA-based kit (EGLP-35K, sensitivity ~2 pM; Linco Research Inc., USA), and for insulin using the in house RIA.

Intra-duodenal Glucose Tolerance Test. While the proof-of-concept regarding DP IV-inhibitor induced incretin enhancement had been demonstrated by numerous groups in the Wistar and fa/fa Zucker rat, the same was not true for the STZ model of diabetes. To address this issue, a set of Wistar rats distinct from those used for determination of metabolic parameters was injected with STZ as described above. Six days following STZ-injection, animals were anaesthetized (sodium pentobarbital 65 mg/kg), and subjected to two successive IDGTTs (1 g/kg; 30 guage needle), first in the absence of a
DP IV-inhibitor and second, following an intravenous bolus of P32/98 (10 mg/kg). Glucose was administered directly into the duodenum using a 30g needle via a 1 cm ventral midline incision. A protocol involving anaesthesia and insertion of a carotid cannula was used in order to minimize the blood sampling time and reduce the bias of DP IV-mediated GLP-1 degradation during the control trial. Samples taken at regular intervals from the carotid cannula were collected directly into tubes containing P32/98 (final concentration 1mM), vortexed briefly and immediately placed on ice. Plasma was separated and stored at -20 °C within one hour of collection. Samples were analyzed for active and total GLP-1 using ELISA (EGLP-35K) and RIA (GLP1T-36HK) based kits respectively (Linco Research Inc., USA).

**PANCREAS PERFUSION AND TOTAL INSULIN DETERMINATION**

After induction of anesthesia, pancreata were isolated and perfused with a low-to-high glucose (4.4 mM to 8.8 mM) perfusion protocol as follows: the pancreas was exposed through a mid-line incision on the ventral aspect, isolated, all minor vessels ligated, and a glucose perfusate (modified Krebs-Ringer bicarbonate buffer, 3% dextran, 0.2% BSA, gassed with 5% CO₂ balance O₂) introduced through the celiac artery. Perfusion effluent was collected at 1 minute intervals via the portal vein with a perfusion rate of 4ml/min (Pederson and Brown 1976). Samples were stored at -20°C until analysis.

Similarly, for determination of insulin content, animals were anesthetized, the pancreas exposed via a ventral mid-line incision. Following excision, pancreata were blotted dry, weighed and then homogenized in 5 ml of ice cold 2 N acetic acid. Homogenates were then placed in a boiling water bath (10 minutes), and centrifuged (10 minutes; 15,000 rpm; 4 °C). The supernatants were collected, and stored at -20 °C for assaying. For determination of insulin, supernatants were thawed, vortexed and assayed for insulin content using an in-house insulin RIA (Control animals 1:10⁴ dilution; STZ rat 1:10³ dilution) and normalized for protein concentration (BCA; Pierce, Rockford, IL).

**MEASUREMENT OF INSULIN SENSITIVITY**

*Composite Insulin Sensitivity Index.* Estimation of insulin sensitivity from OGTT blood glucose and plasma insulin profiles was performed using the composite insulin sensitivity index (CISI) proposed by Matsuda and DeFronzo (Matsuda and DeFronzo 1999). Calculation of the index was made according to the equation,

\[
\text{CISI} = \frac{10,000}{\sqrt{(\text{FBG} \cdot \text{FPI}) \cdot (\text{MG} \cdot \text{MI})}} \quad \text{...Eq. 1}
\]

where the terms FBG and FPI correspond to fasting blood glucose (mM) and fasting plasma insulin (μU/ml) concentrations respectively, and MG and MI correspond to the mean glucose (mM) and insulin (μU/ml) concentrations (AUC/120 minutes) over the course of the OGTT.
Insulin Sensitivity in Soleus Muscle Strips. Uptake of $^{14}$C-labeled glucose in soleus muscle strips was measured as an indicator of skeletal muscle insulin sensitivity. In brief, after an overnight fast and 18 hours after the last dose of P32/98, animals were anesthetized and the soleus muscles of both hind-limbs were exposed and isolated. Strips of approximately 25-35 mg were pulled from the muscle (the two outer-thirds of each muscle were used) and after weighing, were fixed onto stainless steel clips at their resting length. Once mounted, the soleus strips were allowed to stabilize for thirty minutes in a Krebs-Ringer bicarbonate buffer supplemented with 3mM pyruvate, continuously gassed with 95% O$_2$:5% CO$_2$ and held at 37°C in a shaking water bath. These conditions were maintained for the duration of the experiment unless otherwise stated.

In order to assess glucose uptake in response to insulin, muscle strips underwent two pre-incubations (30 and 60 minutes respectively) followed by one half-hour test incubation. Both the second pre-incubation and the test incubation contained 0.0, 0.7, 1.3, or 4.8 nM insulin. The test incubation was performed in media supplemented with [${}^{3}H$]-inulin (0.1μCi/ml) as a measure of extracellular space, and the non-metabolizable glucose analogue [$^{14}$C]-3-O-methylglucose (0.05 μCi/ml) for measurement of glucose uptake. After incubation, each strip was blotted dry, digested with proteinase K (0.25 μg/ml) and the radioactivity of the muscle digests measured with a liquid-scintillation-counting dual-isotopic program.

Insulin Sensitivity in Isolated Adipocytes. Insulin effects on glycogen synthase (GS) activity, acetyl-CoA carboxylase (ACC) levels, and glycerol release were measured in isolated adipocytes as indicators of adipose tissue insulin sensitivity (Thomas et al. 1968, Brownsey and Denton 1982). In brief, 3 cm$^3$ samples of epididymal adipose tissue were obtained from anaesthetized animals and subjected to a 16-minute collagenase digestion (0.5 mg/ml). Recovered adipocytes were washed three times, and allowed to stabilize for one hour in 37°C Krebs buffer repetitively gassed with 95% O$_2$: 5% CO$_2$. Two milliliter aliquots of the adipocyte suspension containing 0, 0.7, 1.3, 4.8 or 9.0 nM insulin were incubated for 30 minutes and immediately flash frozen on liquid nitrogen and stored at -70 °C. Prior to ACC and GS assessment, stored samples were thawed, homogenized in buffer pH 7.2 containing 20 mM MOPS, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 2.5 mM Benzamidine, pH 7.2), and centrifuged (15 min @ 15,000g).

For measurement of ACC activity, 50 μl aliquots of supernatant, pre-incubated in the presence or absence of 20 mM citrate, were added to 450 μl of [$^{14}$C]-HCO$_3$ containing assay buffer pH 7.4 (50 mM HEPES, 10 mM MgSO$_4$, 5 mM EDTA, 5.9 mM ATP, 7.8 mM glutathione, 2 mg/ml BSA, 15 mM KHCO$_3$ and 150 μM Acetyl CoA). After three minutes, the reaction was arrested by the addition of 200 μl of 5 M HCl. Samples were dried for 6 hours, resuspended in 400μl of distilled water, combined with 3 mls of scintillation cocktail (Econo II SC; Fisher, USA) and counted on a Beckman LS 6001C β-counter. GS activity was measured as follows: 25 μl of the cell extracts prepared as indicated above.
were added to assay buffer pH 7.0 (75 mM MOPS, 75 mM NaF, 10 mg/ml glycogen, 2 mM UDP-[\textsuperscript{14}C]-
glucose) held at 30 °C in the presence or absence of 15 mM glucose-6-phosphate. Each reaction was
stopped by spotting 50 µl of the reaction mixture onto Whatmann 3MM filter paper and immersing the
paper in 66% ethanol. After three ethanol washes, the samples were air-dried and the [\textsuperscript{14}C] activity
(UDP-[\textsuperscript{14}C]-glucose incorporation into glycogen) determined. Finally, for measurement of insulin-
mediated inhibition of lipolysis, two milliliter aliquots of adipocyte suspension were pre-stimulated for
10 minutes with 0, 0.3, 0.6, 1.5, or 4.75 nM insulin after which lipolysis was stimulated with a
maximally stimulating dose of isoproterenol (10\textsuperscript{-7} M final concentration). The reaction was allowed to
proceed for 30 minutes at 37°C after which the samples were boiled (10 minutes) and centrifuged at 4°C
for 15 minutes (12,000 x g). The aqueous phase of the supernatant was recovered and stored at -70°C.
Glycerol determinations were made using a colorimetric glycerol kit (Boeh.-Mannheim; Germany).

**Euglycemic-Hyperinsulinemic Clamp.** After twelve weeks of P32/98 treatment, animals were
anaesthetized with sodium pentobarbital (Somnotol\textsuperscript{TM}; 36 mg/kg) and a midline incision made on the
ventral aspect of the neck. Chronic cannulae were then inserted into the left carotid artery (PE-50; Clay
Adams, USA) and the right jugular vein (dual PE-10 cannula encased in Silastic tubing; Dow Corning,
USA), brought around the neck subcutaneously and passed through a small skin incision at the base of
the neck. After four (or more) days of recovery with at least two days of consecutive weight gain, the
animals underwent a euglycemic-hyperinsulinemic clamp. Treatment was discontinued the day of
catheter implantation and re-initiated two days later; the final bolus of inhibitor was given at 1700h the
day prior to the clamp. The protocol consisted of three sequential 90 minute periods: priming, which
involved a bolus (2 μCi) followed by continuous infusion of D-[3-\textsuperscript{3}H]-glucose (0.03 μCi/min;
Amersham, QU), and two insulin infusion steps (HumulinR; Eli Lilly Canada Inc.; Montreal, Canada)
of 5 and 15 mU/kg/min respectively (30 and 90 pmol/kg/min). Blood samples (30 μl) taken every 5
minutes, were centrifuged briefly and the plasma analyzed for glucose using the glucose oxidase
method as above. During the last 30 minutes of each period, three 150 μl blood samples were taken at
fifteen minute intervals for determination of plasma tracer and insulin levels. Samples were
deproteinated (BaOH\textsubscript{2}/ZnSO\textsubscript{4}), evaporated to dryness (to remove [\textsuperscript{3}H]-H\textsubscript{2}O), and tracer levels measured
using a liquid scintillation counter after resuspension in dH\textsubscript{2}O.

Hepatic glucose output (HGO) and glucose disposal rate (GDR) were calculated according to a
modification of the method of Steele (Steele 1959) as follows:

\[
GDR = \frac{R_{g^*}}{SA_p} \quad \text{...Eq. 2}
\]

\[
\text{and,} \quad HGO = \left( R_{g^*} \cdot \frac{G_p}{G_{p^*}} \right) + R_{g^*} - R_g \quad \text{...Eq. 3}
\]

where, \( R_g \) and \( R_{g^*} \) are the steady state rates of D-glucose and D-[3-\textsuperscript{3}H]-glucose infusion respectively,
and \( G_p \) and \( G_{p^*} \) are the steady state plasma concentrations of the same. In summary, HGO was
calculated by subtracting the glucose infusion rate (GIR) from the tracer-determined rate of glucose
appearance into the plasma compartment. Similarly, at steady state the GDR is equal to the sum of the rates of endogenous (HGO) and exogenous (GIR) glucose entry into the plasma compartment.

Of 22 attempts, 14 animals (n=7) were successfully clamped with the remainder failing due to loss of patency of aortic cannulae over the recovery period. The data presented below are those compiled from the 14 successfully clamped animals.

ENZYME ACTIVITY DETERMINATION

DP IV Activity Assay. DP IV activity was measured spectrophotometrically (λ = 390 nm) using Gly-Pro-4-nitroanilide as a chromogenic substrate. One unit of DP IV activity is defined as the rate of appearance (μM/min) of yellow product, 4-nitroaniline, from 0.4 mM substrate in HEPES buffer (40 mM, pH 7.6, 30°C). The substrate cleavage reaction was initiated by substrate addition and progression of the reaction monitored over 20 minutes using a Dynatech MRX Microplate Reader. It is important to note that the assay involves a 10-fold sample dilution and therefore underestimates the actual degree of inhibition occurring in the undiluted sample when using rapidly reversible inhibitors such as P32/98.

PEPCK Activity Assay. Phosphoenolpyruvate carboxykinase activity was measured as follows. Liver samples (0.5 g) were homogenized in 1 ml of homogenization buffer (10 mM Tris-HCl, 1 mM EDTA, 0.25 M sucrose, 50 mM KCl, pH 7.2), and centrifuged for 30 minutes at 10,000g and 4°C. Following protein determination (BCAprot, Pierce; Rockville, USA), samples containing 100 μg of protein were combined with reaction buffer (50 mM Tris-HCl, 2 mM MnCl₂, 2.5 mM phosphoenolpyruvate, 10 mM NaHCO₃, 5 U/ml malate dehydrogenase, 0.15 mM β-NADH) in a 96-well plate. The reaction was initiated with the addition of 0.4 mM dGDP (final concentration) and followed at 340 nm on a microtiter plate-reader. One unit of PEPCK activity corresponds to the conversion of 1 μmole of β-NADH to NAD in one minute.

DETERMINATION OF PLASMA LIPIDS AND LONG-TERM GLYCEMIA

Measurement of FFA, cholesterol, triglycerides, fructosamine and HbA₁c levels in plasma or blood samples from the VDF and/or STZ studies were all performed using commercially available kits according to the supplied instructions. Triglycerides were measured using the TG (glycerol phosphate oxidase-Trinder 10) kit from Sigma Diagnostics (St. Louis, USA; ), cholesterol with the Cholesterol CII kit from Wako Chemicals Inc. (USA; cholesterol/cholesterol oxidase based), free fatty acids with the NEFA C kit from Wako Chemicals GmbH (Germany; based on the acylation of coenzyme A free fatty acids in the presence of acyl-coA-oxidase), and fructosamine with the Raichem Fructosamine Assay (Raichem, CA; based on the ability of ketoamine groups of glycated proteins to reduce terazolium salts at high pH). All assays were performed in 96-well plates and read spectrophotometrically using a microtiter plate reader (Dynatech MRX). Blood HbA₁c was measured using the Bio-Rad HbA₁c Micro Column Test kit (Bio-Rad Inc, CA).
IMMUNOHISTOCHEMISTRY AND β-CELL MASS DETERMINATION

For studies on the VDF rat, pancreata were removed from anesthetized animals and placed directly into fixative (44% formaldehyde, 47% distilled H₂O, 9% glacial acetic acid) for 48 hours after which they were washed and stored in 70% ethanol. After paraffin embedding, 5 μm tissue sections were cut, mounted onto slides, and dried ready for staining. In order to assess β-cell area, sections were stained with a guinea pig anti-insulin primary antibody followed by peroxidase conjugated goat anti-guinea pig secondary. Slides were developed using diaminobenzidine and counterstained with hematoxylin. Analyses were performed using Northern Eclipse Software (Empix Imaging, Mississauga, ON, Canada) as previously described (Finegood et al. 2001). Briefly, the relative cross-sectional area of β-cells to non-β-cell tissue was determined from captured digital images by adjusting the detection threshold for brown staining (β-cells). Adipose tissue area was also determined by tracing regions occupied by fat cells and subtracted from the total pancreatic tissue value. Relative cross-sectional areas calculated from analysis of ~70% of each slide were then related back to the original pancreatic masses obtained prior to fixation.

For studies on the STZ rat, pancreata were removed, fixed and sectioned as above. To account for variation between pancreatic regions, the pancreata were cut into ~4 mm blocks which were then randomly inserted into a cassette and paraffin embedded. β-cell counts were made from all blocks (~10-15) in a cassette thereby ensuring assessment of a randomized set of cross-sections in all three planes throughout each pancreas. Once embedded, pancreata were sectioned (5 μm), and the sections mounted onto slides and dried ready for staining. Sections were stained for insulin (Rbt α-insulin H-86; Santa Cruz Biotechnology Inc. Santa Cruz, CA) as per the manufacturer's instructions and the nuclei counterstained using DAPI (0.3 μM; Molecular Probes, Eugene, OR) to facilitate quantification of β-cell and islet parameters. Quantification was performed manually as a high degree of variability was observed in insulin staining within islet sections from animals exposed to streptozotocin. Determinations of insulin immunoreactive cell number (insulin-IR; β-cell number), and total cross-sectional islet cell number (nuclear stain) were made in islets from 250 mm² of pancreatic tissue section per animal (~100 islets); 3-4 animals were counted in each experimental group.

Insulitis in the BB rat was determined after fixation (as above) of pancreata from 9-week old non-diabetic diabetes prone animals, both treated and control. Hematoxilin and eosin stained sections were analyzed under 200x magnification and rated using a subjective 5 point scale, as follows: 0, normal islet appearance and numbers with no infiltration of the islet; 1, infiltration in islet periphery, but core and mantle identifiable; 2, mixed islet appearances varying from unaffected to end stage, with overall mild inflammation; 3, infiltration into islet core with some distinguishable β-cells; 4, end stage islets with core fully infiltrated and no recognizable β-cells. Determinations were made in a randomized blinded fashion by the same individual in two separate sittings.
GIP/GLP-1 PROTECTION OF β-(INS-1)-CELLS IN VITRO

The rat insulinoma cell line INS-1 (832/13) was obtained as a generous gift from Dr. C.B. Newgard (Duke University, Durham, NC). Cells (passages 50-65) were cultured in RPMI 1640 supplemented with 10% FBS (Cansera, Rexdale, ON), 11 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM pyruvate, and 50 µM β-mercaptoethanol. Cells were plated into either 6-well (2 x 10^6 cells/well; caspase studies) or 96-well (7.5 x 10^4 cells/well; protection assays) coated tissue-culture plates (Becton Dickinson, Lincoln Park, NJ) 24 hours prior to experimentation. Unless otherwise stated the glucose concentration during all cell survival assays was 3 mM for the duration of the experiment in order to minimize complications associated with stimulation of proliferation, and glucose-dependent secretory products. Similarly in all experiments cells were serum starved overnight (3 mM glucose RPMI + 0.1% BSA) and subjected to a 30-minute STZ exposure the following day. After STZ exposure media was replaced and cells were allowed to recover for 24 hours. GIP and/or GLP-1 (10^{-7} M) were added 10 minutes prior to STZ (2 mM) during the dose-response and caspase-3/8 experiments; and 10 minutes prior to (Pre), immediately after (Post), or both 10 minutes prior to and immediately after (Pre + Post) the STZ exposure for the cell protection assays. “Post” and “Pre + Post” groups were therefore exposed to incretins for the duration of the 24 hour recovery period. Quantification of live vs. dead cell number was made manually using a conventional trypan blue exclusion method. Caspase-3 and -8 determinations were made with the EnzChek Caspase Assay Kits (Molecular Probes, OR) according to the manufacturer’s recommendations. Following treatment and cell lysis by freezing, caspase-3 and -8 activities were measured by exploiting the liberation of AMC (7-amino-4-methylcoumarin) from the caspase-3 and -8 specific substrates Z-DEVD-AMC and Z-IETD-AMC respectively. Liberation of AMC results in fluorescence excitation/emission profile at 360/460 nM. Assays were performed in a 96-well plate and read using a Bio-tek FL600 fluorescence plate reader the results of which were normalized for protein content of the samples (BCAprot).

BLOOD PRESSURE MEASUREMENT

Indirect measurement of systolic blood pressure was performed on conscious restrained animals in a 28-30°C chamber using a tail cuff method (Model 179 semi-automatic blood pressure analyzer, IITC Woodland Hills, CA). Animals were conditioned to restraint for two weeks prior to experimentation. The method involves measurement of the pressure of pulsation reappearance (upon gradual deflation of the tail cuff) detected by a photoelectric sensor. Five sequential noise free readings were averaged and taken as an individual systolic blood pressure measurement. Each set of readings was performed in a randomized singly blinded manner. The apparatus and method used was previously validated against direct intra-arterial cannulation.
DATA ANALYSIS

Data are presented as mean ± standard error of the mean (SEM), with the number of experiments shown in the figure legends. Statistical significance was set at the 5% level, and assessed using a student's t-test or analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison Test or the Newmann-Keuls Test as post hoc tests of significance where appropriate. Data analysis was done using the Prism software package (GraphPad, San Diego, CA).
RESULTS

STUDY I: DEGRADATION AND INACTIVATION OF GLUCAGON BY DP IV

Considering the importance of glucagon towards proper glucose homeostasis and the fact that the N-terminal sequence of glucagon falls into the theoretical substrate constraints for DP IV, an investigation was initiated into DP IV mediated glucagon metabolism (Hinke et al. 2000, Pospisilik et al. 2001).

Degradation of glucagon by purified DP IV and plasma was initially investigated using a bioassay measuring the hyperglycemic action of glucagon. In order to ensure maximal sensitivity of the bioassay, a dose-response investigation of glucagon degradation by DP IV was performed. A minimum effective dose of 0.31 U/ml was determined in a 3-hour incubation and was used for all subsequent experiments (Fig. 1). Incubation of full length glucagon in purified porcine DP IV or rat plasma resulted in a complete loss of hyperglycemic activity when injected into either conscious or anesthetized Wistar rats (Fig. 2&3). Whereas incubation in control (Fischer 344) rat plasma ablated the hyperglycemic effect of glucagon, the same protocol performed using DP IV-deficient Fischer 344 rat plasma (DP IV -/-) showed near-total retention of biological activity (Fig. 2). Similarly, addition of the DP IV-inhibitor P32/98 (50 μM) to the incubation medium was shown to abrogate any loss of bioactivity when administered via jugular infusion (Fig. 3). The latter protocol was developed after subcutaneous (s.c.) administration proved ineffective i.e. no protection of glucagon bioactivity was evidenced in the presence of P32/98 (data not shown). The latter finding was believed to be an artifact of the relative rapidity of diffusion of the inhibitor away from the injection site rather than an inability of the inhibitor to protect glucagon.

Figure 1. Dose dependency of glucagon degradation by dipeptidyl peptidase IV. (A) The ability of glucagon to elicit hyperglycemia following subcutaneous injection was shown to be progressively compromised following a 3 hour incubation (37°C) with increasing concentrations of DP IV. (B) Area under the curve (AUC) analysis reveals the same trend. Blood glucose measures were made using a SureStep glucose analyzer after subcutaneous injection of 7 nmol/kg of glucagon incubate into male Wistar rats (N=4; ~250g).
Figure 2. Bioactivity of DP IV- and plasma- mediated glucagon degradation products. (A) Glucagon (7 nmol/kg) was resuspended in 1ml of either saline, normal rat plasma, DP IV(-/-) rat plasma, or 0.31 U/ml purified porcine DP IV in saline and incubated for 3 hours (37°C) prior to subcutaneous injection into conscious, unrestrained male Wistar rats (N=6; ~250g). (B) Corresponding area under the curve (AUC) analyses.

Figure 3. Inhibition of DP IV and plasma degradation of glucagon by P32/98 as monitored by bioassay. Glucagon (7 nmol/kg) was incubated for 3 hours at 37°C in (A) saline and purified DP IV or (B) rat plasma, either in the presence or absence of P32/98, and administration via jugular cannula to anaesthetized male Wistar rats (N=4; ~250g).

In order to assess the physiological relevance of DP IV-mediated glucagon degradation in vivo, two experimental scenarios were employed, first, the counterregulatory response to a twenty-four hour fast (Fig. 4), and second, to insulin induced hypoglycemia (Fig. 5&6). In a 24-hour food deprivation study in Wistar rats, all plasma glucagon measurements made after P32/98 administration were shown to be reduced (~30%). Corresponding blood glucose levels, however, were shown to be unchanged suggesting enhanced activity of the reduced circulating glucagon pool. Examination of the counterregulatory response to insulin-induced hypoglycemia showed similar results. Wistar rats administered P32/98 concomitant with a 1.5 U/kg i.p. insulin injection, exhibited a reduced
hypoglycemic excursion in the face of a reduced glucagon response to hypoglycemia (Fig. 5). It is important to note that the antibody used to measure circulating glucagon during these studies does not discriminate between N-terminally truncated and intact forms of the glucagon molecule, therefore an elevation in circulating active glucagon may be masked under a reduced total glucagon concentration e.g. in the absence of N-terminal degradation. When the same study was repeated in DP IV -/- rats, a similarly blunted hypoglycemic response was observed. Interestingly though, this was found in the face of an exaggerated glucagon response, a stark contrast to the inhibitor treated animals (Fig. 6).

Figure 4. DP IV-inhibition reduces the glucagon requirement for euaglycemia during fasting. Male Wistar rats (n=5; ~250g) were fasted for 24 hours during which time they received 3 oral doses of either water (control) or 10 mg/kg P32/98. Blood samples were taken via the tail vein as indicated above. Blood glucose determinations were made using a hand-held glucose meter while plasma glucagon was measured using a radioimmunoassay directed against the C-terminal end of glucagon (thus measuring both full-length and truncated metabolites).

Figure 5. DP IV-inhibition enhances the counterregulatory response towards insulin-induced hypoglycemia. Male Wistar rats (n=8; ~250 g) were administered a single 1.5 U/kg insulin dose (T = 0 min) concomitant with either saline (control) or a 10 mg/kg of the DP IV-inhibitor P32/98 in saline (P32/98). Blood samples were taken via the tail vein as indicated above. Blood glucose determinations were made using a hand-held glucose meter while plasma glucagon was measured using a radioimmunoassay directed against the C-terminal end of glucagon (thus measuring both full-length and truncated metabolites).
Figure 6. DP IV-deficient rats have an enhanced counterregulatory response towards insulin-induced hypoglycemia. Male Wistar (n=8; ~250 g; Control) and DP IV-deficient Fischer rats were administered a single 1.5 U/kg insulin dose. Blood samples were taken via the tail vein at the times indicated above. Blood glucose determinations were made using a hand-held glucose meter while plasma glucagon was measured using a radioimmunoassay directed against the C-terminal end of glucagon (thus measuring both full-length and truncated metabolites).

Further evidence supporting the relevance of DP IV-mediated glucagon degradation in vivo was obtained through a refinement of the initial glucagon bioassay. The caveats of the first methodology included relatively inaccurate glucose measurement, use of pharmacological glucagon doses and interference of compensatory mechanisms on observed changes in blood glucose, primarily that caused by insulin-mediated recovery from hyperglycemia. Using the more sensitive Beckman Glucose Analyzer II, and a somatostatin infusion to suppress endogenous glucagon and insulin secretion, we were able to examine the effect of DP IV-inhibition on a physiological rise in circulating glucagon (7 pmol/kg; ~ 100 pM rise) given as an i.v. bolus. Inclusion of P32/98 into the injection medium increased the efficacy of the glucagon bolus by approximately 80% over control animals. Surprisingly an equimolar injection into DP IV -/- animals elicited a minimal response, well below that of the controls.

Figure 7. Enhancement of glucagon bioactivity by P32/98. A 7 pmol/kg glucagon bolus was administered via jugular cannula to anaesthetized male Wistar rats (N=4) concomitant with 10 mg/kg of P32/98 or a saline control. Endogenous glucagon and insulin secretion were suppressed through a continuous infusion of somatostatin (0.4 μg/kg/min). * P < 0.05 P32/98 vs. Control; # P < 0.05 DP IV -/- vs. Control.
To characterize the metabolism of glucagon by DP IV further, an *in vitro* and *in vivo* examination was made of the proposed metabolites of the cleavage reaction. Bioassay of synthetic glucagon\textsubscript{3-29}, [pGlu\textsuperscript{3}]glucagon\textsubscript{3-29}, and glucagon\textsubscript{5-29} indicated that these peptides had negligible hyperglycemic activity at 10-fold higher doses than native glucagon (Fig. 8C). Binding and activation properties of these peptides at the human glucagon receptor (hGlucR; transfected into CHO-K1 cells) were shown to be severely compromised relative to the parent compound with a 10-20 fold right-shift in binding affinity and marked reductions in both potency and efficacy of receptor activation (Fig. 8A & B; Table II). Co-stimulation studies using both the truncated metabolites (variable dose) and native glucagon (fixed concentration) provided evidence that the two pure metabolites of DP IV-degradation serve as competitive antagonists at pharmacological doses (Fig. 8D; Table II).

**Figure 8.** Receptor binding, activation and *in vivo* bioactivity of glucagon and synthetic DP IV and plasma degradation products. (A) Competitive binding displacement curves, and (B) concentration-dependent cAMP generation in hGlucR-CHO-K1 cells. (C) *In vivo* bioactivity of synthetic glucagon fragments measured in response to subcutaneous injection into conscious unrestrained male Wistar rats. (D) Determination of potential antagonism of glucagon-mediated cAMP generation by synthetic glucagon fragments in hGlucR-CHO-K1 cells. Refer to Table II for potency, efficacy and antagonistism properties. Experimental work for A, B, D was performed by Simon A. Hinke (Ph.D.) and published in (Hinke *et al.*, 2000); used with permission.
In a parallel investigation, the potential of N-terminally modified, DP IV-resistant glucagon analogues to effect hyperglycemia and to bind and activate the hGlucR was examined. Of four analogues previously shown to be DP IV-resistant, only D-Ser₂-glucagon₁₋₂₉, showed enhanced bioactivity in vivo. The remaining three peptides showed a reduction in ability to induce hyperglycemia (Fig. 9C). In vitro analysis revealed that D-Ser₂-glucagon₁₋₂₉ alone shared similar binding and activation properties with native glucagon. The remaining peptides exhibited reduced receptor-activation properties concomitant, in two cases (D-Gln₃- and [P]-Ser₂-glucagon₁₋₂₉), with reduced binding affinity (Fig 9A & B; Table II). Thus, a mechanism was provided that could rationalize the in vivo data. Further examination of the biological potency of these peptides was performed after incubation with purified DP IV, plasma, or DP IV -/- plasma. The results are summarized in Figure 10.

Figure 9. Receptor binding, activation and in vivo bioactivity of glucagon and synthetic DP IV-resistant glucagon analogues. (A) Competitive binding displacement curves, and (B) concentration-dependent cAMP generation in hGlucR-CHO-K1 cells. (C and D) In vivo bioactivity of synthetic glucagon analogues measured in response to subcutaneous injection into conscious unrestrained male Wistar rats. Refer to Table II for potency and efficacy properties. Experimental work for A and B performed by Simon A. Hinke (Ph.D.) and published in (Hinke et al., 2000); used with permission.
Figure 10. Bioactivity of synthetic DP IV-resistant glucagon analogues after incubation with purified DP IV, plasma, and DP IV(-/-) plasma. In vivo bioactivity was measured in response to subcutaneous injection into conscious unrestrained male Wistar rats, after a 3 hr incubation in either saline (A), saline and DP IV (B), plasma (C), or DP IV(-/-) plasma. (E) A summary of the integrated blood glucose responses from all incubation conditions.
Table II. Summary of binding and activation properties of DP IV-degradation products and DP IV-resistant analogues of glucagon at the human glucagon receptor.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Binding (nM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; cAMP Release (nM)</th>
<th>Max. cAMP Release (fmol/1000 cells)</th>
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<tbody>
<tr>
<td>Glucagon&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>8.83 ± 0.60</td>
<td>0.39 ± 0.06</td>
<td>32.8 ± 1.4</td>
</tr>
<tr>
<td>Glucagon&lt;sub&gt;3-29&lt;/sub&gt;</td>
<td>155 ± 29*</td>
<td>239 ± 20*</td>
<td>17.0 ± 1.9*</td>
</tr>
<tr>
<td>pGlu&lt;sub&gt;3&lt;/sub&gt;-Glucagon&lt;sub&gt;2-29&lt;/sub&gt;</td>
<td>48.7 ± 4.1*</td>
<td>131 ± 34*</td>
<td>22.3 ± 1.1*</td>
</tr>
<tr>
<td>Glucagon&lt;sub&gt;3-29&lt;/sub&gt;</td>
<td>45.3 ± 9.4*</td>
<td>182 ± 33*</td>
<td>10.5 ± 1.0*</td>
</tr>
<tr>
<td>D-Ser&lt;sub&gt;2&lt;/sub&gt;-Glucagon&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>23.6 ± 6.4*</td>
<td>1.41 ± 0.41*</td>
<td>38.1 ± 3.8*</td>
</tr>
<tr>
<td>(P)-Ser&lt;sub&gt;2&lt;/sub&gt;-Glucagon&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>69.1 ± 5.7*</td>
<td>30.3 ± 7.2*</td>
<td>31.8 ± 2.0</td>
</tr>
<tr>
<td>Gly&lt;sub&gt;2&lt;/sub&gt;-Glucagon&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>16.6 ± 5.5</td>
<td>1.70 ± 0.67*</td>
<td>31.9 ± 1.3</td>
</tr>
<tr>
<td>D-Gln&lt;sub&gt;3&lt;/sub&gt;-Glucagon&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>164 ± 79*</td>
<td>15.9 ± 10.1*</td>
<td>45.5 ± 3.2*</td>
</tr>
</tbody>
</table>

* P<0.05 relative to Glucagon<sub>1-29</sub>
STUDY II: LONG-TERM EFFECTS OF DP IV-INHIBITION IN THE VDF RAT

What follows is a summary of the results of two sequential studies examining the long-term effects of DP IV-inhibition on glucose homeostasis in the Vancouver Diabetic Fatty (VDF) rat model of obesity related type-2 diabetes. The data were obtained from 26 male VDF rats all of which underwent a three month oral treatment regimen with the DP IV-inhibitor P32/98 (10 mg/kg twice daily).

**Body Weight, Blood Glucose, and Food and Water Intake.** VDF rats treated with P32/98 displayed a gradual 12.5% (25 g) reduction in weight gain over the three month treatment period (control: 211 ± 8 g; treated: 176 ± 6 g) (Fig. 11A). Measurements of food and water intake, which decreased over the course of the experiment, showed no significant differences between experimental groups (Fig. 11B). Bi-daily monitoring of blood glucose revealed no difference in morning or evening blood glucose values between the experimental groups, though neither group displayed notably hyperglycemic values (Fig. 11C). Morning blood glucose levels over the course of the experiment averaged 5.0 ± 0.1 mM in the treated and 5.3 ± 0.1 mM in the control animals. Evening blood glucose values averaged 6.7 ± 0.1 mM and 7.0 ± 0.2 mM respectively. Hematocrit, measured at four week intervals, indicated no adverse effects of the blood sampling protocol employed, averaging between 43.4 % and 45.3 % in both groups (Fig. 11D).

![Figure 11](image-url)

Figure 11. Metabolic parameters measured in VDF rats treated with either the DP IV inhibitor P32/98 (20 mg/kg/day; open circles) or with control injection vehicle (1% cellulose; closed squares)(n=6). Over the three month treatment regimen, body weight (A), food and water intake (B), morning and evening blood glucose (C) were measured every two days. To ensure that blood sampling was not excessive, hematocrit was measured at 4 week intervals (D). Statistical significance (p<0.05) is indicated by an asterisk.
**Blood Glucose, Insulin, and DP IV Profile.** After six weeks of treatment, a 24-hour profile of blood glucose, insulin and DP IV activity levels was generated by regular blood sampling (3 hr intervals), interrupting neither treatment administration nor the light/dark cycle. The profile confirmed that administration of P32/98 caused significant inhibition of DP IV activity over the majority of the 24-hour cycle, with at least 65% inhibition during the feeding cycle (Fig. 12A). The integrated blood glucose excursion in the treated animals was 75% that of the controls, peaking at 7.7 ± 0.3 mM as compared to 9.8 ± 0.6 mM for the untreated animals (Fig. 12B). The corresponding plasma insulin profile exhibited not only a decrease in peak insulin values, but also of “basal”, non-feeding, values (~0800 to 1800 h) in the treated animals (Fig. 12C).

![Graph A](image)

![Graph B](image)

![Graph C](image)

**Figure 12.** A twenty-four hour profile of plasma DP IV activity (A), blood glucose (B), and plasma insulin (C) levels in VDF rats after six weeks of treatment either with (open circles) or without (solid squares) the DP IV inhibitor P32/98 (n=6). Treated animals were administered 10 mg/kg P32/98 twice daily as indicated by the arrows, while the control group received only the 1% cellulose injection vehicle. Statistical significance (p<0.05) is indicated by an asterisk.
**Oral Glucose Tolerance.** Three oral glucose tolerance tests (OGTTs), performed in the absence of circulating P32/98 and at one month intervals, were used to monitor the progression of the disease state in the control animals and to document any improvements displayed in the treated group. The initial OGTT, administered after four weeks of treatment, showed significant decreases (~2 mM) in basal, 45, 60, and 90 minute blood glucose values in the treated group despite overlapping plasma insulin excursions (Fig. 13A). Data from the second OGTT were very similar to the first; with the exception that the 120 min blood glucose value was also significantly lowered in the treated group (10.8 ± 0.8 vs. 12.3 ± 0.8 for the control animals); once again the insulin profiles were superimposable (data not shown). The final OGTT, performed after 12 weeks of treatment, showed a marked difference in glucose tolerance between the two groups with significantly decreased blood glucose values observed at all time points. Peak blood glucose values in the treated group averaged 12.0 ± 0.7 mM, 8.5 mM less than that of the control animals (Fig. 13B). Also, blood glucose in the treated group had returned to 9.2 ± 0.5 mM after 120 minutes, a 40% reduction compared to the controls. Active GLP-1 levels (GLP-1a), measured during the final OGTT using an N-terminally directed ELISA, were found to be unchanged (Fig. 13B). Despite this lack of altered GLP-1a levels, the early phase insulin response measured in the treated group exceeded that of the control animals by 43%. However, the integrated insulin responses between the two groups showed no significant difference. Analysis of the OGTT data using the composite insulin sensitivity index of Matsuda and DeFronzo (Matsuda and DeFronzo 1999), revealed a progressive increase in estimated insulin sensitivity of the treated animals relative to the controls (Fig. 13C).
Figure 13. Oral glucose tolerance tests (OGTT) administered to both DP IV inhibitor treated (open circles/bars) and control (solid squares/bars) VDF rats after four (A) and twelve (B) weeks of treatment (n=6). Blood glucose and plasma insulin measurements were performed in both series of tests, while the active fraction of plasma GLP-1 was also measured at twelve weeks. The inset in B shows the integrated plasma insulin responses for the twelve week OGTT. Statistical significance (p<0.05) is indicated by an asterisk. (C) Composite insulin sensitivity index calculated using the 4 and 12 week OGTTs shown in A and B, and normalized to control values.

Comparison of the oral glucose tolerance tests over the course of the experiment revealed a progressive decline in both fasting and peak blood glucose values in the animals treated with P32/98, improvements that were not observed in the control group (Fig. 14A&B). Peak insulin values did not differ significantly between the two experimental groups until the final, 12 week, OGTT, at which time the peak insulin levels in the treated animals exceeded those of the control animals by an average of 43% (Fig. 14C). Plasma DP IV activity, measured at the start of each OGTT, was significantly increased in the treated group by week 8 of the study and the elevation maintained at week 12 (Fig. 14D).
Figure 14. Comparison of fasting (A) and peak (B) blood glucose, peak plasma insulin (C), and fasting plasma DP IV activity (D) measured during OGTTs performed at four week intervals in control (solid squares) or DP IV inhibitor treated (open circles) VDF rats (n=6). Statistical significance (p<0.05) is indicated by an asterisk.

Pancreatic Glucose and Incretin Responsiveness, β-cell Mass, and Islet Morphology. A low-to-high step glucose perfusion protocol was performed on the pancreata of half of each group of animals. The shift from 4.4 to 8.8 mM glucose perfusate caused a 3.2-fold increase (3.42 ± 1.02 pmol/min to over 12.5 pmol/min) in insulin secretory rate in the pancreata from the treated animals while failing to elicit any significant response in the control pancreata until well over twenty minutes of high glucose perfusion (Fig. 15A). The three month oral DP IV inhibitor regimen yielded no significant differences in β-cell area, or islet morphology. Islets from control and treated animals comprised 1.51 ± 0.04 % and 1.50 ± 0.03 % of the total pancreatic area, respectively (Fig. 15B). Large, irregularly shaped islets with significant β-cell hyperplasia, characteristic of the fa fa Zucker rat, were observed in both groups.
Figure 15. Glucose-stimulated insulin release and β-cell area measured in VDF rats treated for three months with the DP IV-inhibitor P32/98. (A) Low-to-high glucose perfusion of pancreata from VDF rats after twelve weeks of treatment with (open circles) or without (solid squares) the DP IV inhibitor P32/98 (n=3). (B) β-cell area determinations made in the second half of each cohort.

In addition to perturbations in the glucose-dependence of β-cell function, a reduced incretin effect has also been observed in type-2 diabetic patients, an abnormality believed to be linked to defective GIP-receptor expression. An examination of the incretin responsiveness of VDF pancreata to a step-wise concentration-escalation protocol for both GIP and GLP-1 revealed an amelioration of this condition. Whereas no difference between experimental groups was found during graded infusion of GLP-1 (responsiveness was robust in both groups), the P32/98 treated animals displayed a marked improvement in responsiveness to GIP over controls, particularly at low concentrations (Fig. 16).

Figure 16. Sensitivity of the pancreatic insulin response to GIP and GLP-1 after long-term DP IV-inhibitor treatment. Glucose-stimulated insulin release was measured in VDF rats after three months of treatment with the DP IV-inhibitor P32/98 (open circles) or with a 1% cellulose control solution (solid squares). (A) A low-to-high glucose perfusion followed by three 15 minutes incremental GLP-1amide infusion steps with steady-state concentrations of 10, 30 and 50 pM. (B) A parallel protocol for GIP with steady-state concentrations of 50, 150 and 250 pM. These peptide concentrations were chosen to mimic expected basal, and low- and high- fed plasma concentrations for the full length, insulinotropic peptides in a conscious animal (N=5).
Plasma Lipids and Pancreatic Incretin Responsiveness. Recent studies on the phenomenon of reduced GIP-efficacy and GIP-receptor downregulation by Lynn and colleagues have suggested hyperglycemia induces the downregulation of the GIP receptor while increased FFA levels ameliorate the process (Lynn et al. 2003). Examination of plasma FFA, cholesterol, and triglycerides in the VDF rat over the 3-month course of DP IV-inhibitor treatment revealed no change in the progression of dyslipidemia in the treated animals (Fig. 17). Hemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}) levels, however, did show a significant reduction after 14 days of P32/98 treatment providing evidence of an overall reduction in hyperglycemia.

Figure 17. Plasma lipid and glyated hemoglobin measures taken over a 12 week treatment of VDF rats with the DP IV inhibitor P32/98. Plasma cholesterol (A), triglycerides (B), non-esterified (free) fatty acids (C), and hemoglobin Al\textsubscript{c} levels were all measured using commercially available assays in blood samples collected weekly at 1700 h.

Euglycemic-Hyperinsulinemic Clamp. Baseline glucose, insulin and DP IV activity (Treated: 35.6 ± 1.7 mU/ml vs. Control: 24.4 ± 1.0 mU/ml) values measured at the outset of the clamp were comparable to those observed during the OGTT approximately 10 days earlier; the treated group exhibited reduced fasting plasma glucose concomitant with unchanged plasma insulin values (see Fig. 13). Fasting plasma glucose levels appeared slightly elevated under clamp conditions, a phenomenon attributable to the difference in measurement technique, and in keeping with our own observations and with the literature on measurement of whole blood vs. plasma glucose levels (Weitgasser et al. 1999).
Figure 18 shows clearly that steady-state insulin levels between groups were comparable, rising ~ 0.7 nM during the first insulin step and a further ~ 2 nM during the second. These values, though hyperinsulinemic, correspond closely to plasma values measured both during the oral glucose tolerance test (Fig. 13) and during normal feeding (Fig. 12). P32/98-treated animals displayed an immediate requirement for exogenous glucose infusion (GIR) when infused with 5 mU/kg/min insulin; under the same conditions, control animals showed a reduced and significantly delayed (~ 25 minutes) response to the same stimulus (Fig. 18). Elevation of the insulin infusion rate to 15 mU/kg/min elicited a further increase in GIR in both groups with plateau levels reaching 39.2 ± 5.3 and 26.8 ± 4.5 μmol/kg/min in the treated and control groups respectively.

Figure 18. Plasma glucose (Gp), plasma insulin, and glucose infusion rate (GIR) during a euglycemic-hyperinsulinemic clamp in conscious VDF rats after 12 weeks of treatment with (open circles) or without (solid squares) the DP IV-inhibitor P32/98 (n=7). Insulin infusion rate (0, 5, or 15 mU/kg/min; shown above) was held constant during each of three 90 minute periods. Blood sampling, and glucose and insulin infusions were made through chronic carotid and jugular catheters, respectively, inserted ~5 days earlier under general anesthesia (sodium pentobarbital). Statistical significance (p<0.05) is indicated by an asterisk.
**Hepatic Glucose Output.** Hepatic glucose output (HGO), calculated from plasma tracer levels, was significantly reduced in the treated compared to control animals, during each stage of the clamp (Fig. 19 A,B). Basal HGO averaged $6.5 \pm 0.9$ μmol/kg/min less in the treated than in the control group ($12.7 \pm 0.9$ μmol/kg/min). A similar magnitude difference was found at each insulin infusion level (Fig. 19 A,B). To further investigate the changes in hepatic insulin sensitivity, liver PEPCK levels and fasting plasma glucagon levels were measured. Measurement of PEPCK activity in terminal liver biopsies showed no significant difference between the treated and untreated animals, suggesting a non-PEPCK dependent pathway for the left shift in insulin sensitivity. PEPCK activity averaged $25.9 \pm 2.9$ and $31.5 \pm 4.3$ mU/mg tissue in the treated and control groups respectively (Fig. 19C). Values for the treated animals were comparable to those obtained in control Wistar rats ($24.7 \pm 0.2$ mU/mg). No difference was detected in fasting plasma glucagon levels either with time or as a result of the treatment (Fig. 19D).

Figure 19. Steady-state hepatic glucose output (A,B) calculated during a euglycemic-hyperinsulinemic clamp in conscious VDF rats (n=7) treated twelve weeks with either the DP IV-inhibitor P32/98 (open bars/circles) or with a 1% cellulose control solution (solid bars/squares). Upon termination, liver biopsies were obtained from the same animals for measurement of phosphoenol pyruvate carboxykinase (PEPCK) activity (C). Also, fasting plasma glucagon levels (D) were measured at the outset of the experiment as well as one week prior to the clamp. Statistical significance relative to controls is indicated by one (p<0.05) or two asterisks (p<0.01).
Peripheral Glucose Disposal. Calculation of glucose disposal rate (GDR) revealed little or no response to insulin in the control animals (Fig. 20), consistent with previous clamp studies of obese Zucker rats (Terrettaz et al. 1986). GDR in the treated group, however, showed a return of insulin responsiveness (steady state levels 105 % and 216 % above basal during each of the two insulin steps respectively), concurrent with a reduction in basal values (Fig. 20). These findings were confirmed in vitro using isolated soleus muscle strips incubated with $^{14}$C-labeled glucose and exposed to increasing concentrations of insulin. Soleus muscle strips taken from the treated animals exhibited significantly higher rates of glucose uptake both in the basal and in the insulin stimulated state. Glucose uptake in the non-stimulated state was 22% higher in the treated rats (Fig. 20). The insulin-stimulated rise in glucose uptake was enhanced in the treated group compared to the controls (control: 58.5 ± 3.5; treated: 87.5 ± 10.4 cpm/mg tissue at 800 μU/ml insulin).
Insulin Sensitivity in Isolated Adipocytes. Using glycerol release as an indicator, inhibition of isoproterenol-stimulated lipolysis by insulin was examined in isolated epididymal adipocytes (Fig. 20). Concentration-response of inhibition by insulin showed a left-shift in adipocytes isolated from treated animals when compared to controls; estimated half-maximal concentrations (EC\textsubscript{50}) were 0.29 ± 0.01 nM and 1.11 ± 0.01 nM respectively (P<0.01). Further, glycogen synthase (GS) and acetyl coA
carboxylase (ACC) activity were measured in isolated adipocytes. ACC levels in adipose tissue from both experimental groups were minimal (approaching limits of detection), lacked insulin responsiveness, and showed no difference between the two groups. GS activity also appeared insensitive to insulin, though the enzyme activity at all measured insulin concentrations was higher in the treated animals than in their control littermates (Fig. 20).

**Blood Pressure.** Cardiovascular dysfunction (CAD and hypertension) is a common secondary complication of human type-2 diabetes, a condition believed to be linked to insulin resistance and shown in some instances to be ameliorated by glucose lowering and insulin sensitizing therapy. Considering the marked improvements in glucose tolerance and insulin sensitivity evidenced in the first set of DP IV-inhibitor treated animals, it was hypothesized that P32/98 treatment would also reduce the mild hypertension displayed by the VDF rat. Using an indirect tail cuff method, systolic blood pressure (SBP) was measured in 12 VDF rats undergoing the same bidaily P32/98 dosing regimen previously described. Control animals were shown to have developed a mild hypertension prior to the experiment outset (12 weeks of age; ~150 mm Hg SBP), a condition that exacerbated slightly plateauing at ~160 mm Hg. The P32/98 treated group, in contrast, displayed an immediate reduction in SBP relative to the controls that was sustained over the 12-week treatment course. The average reduction in SBP relative to controls was 11 ± 1 mm Hg. In a follow-up study, no effect of P32/98 treatment was observed on SBP, oral or intraperitoneal glucose tolerance, or body weight in spontaneously hypertensive (SH) rats treated over the eight week course of hypertension development (ages 6-14 weeks).

![Figure 21](image-url)

**Figure 21.** Effects of long-term DP IV-inhibitor treatment on systolic blood pressure in the VDF rat. Using an indirect tail cuff method systolic blood pressure was measured in VDF rats treated either with (open circles) or without (closed squares) the DP IV-inhibitor P32/98. Blood pressure readings were made in the morning, >16 hours after the inhibitor/control dose in acclimatized animals after a 15-minute equilibration period.
STUDY III: LONG-TERM EFFECTS OF DP IV-INHIBITION IN THE STZ RAT

The following section outlines the results obtained during a study of the long-term effects of DP IV-inhibitor treatment in the STZ rat, a model of insulin-insufficiency, and islet plasticity following gross β-cell destruction. Studies were carried out in Wistar rats administered a single high dose of streptozotocin (50 mg/kg i.v.; citrate buffer control), and treated either with or without the DP IV inhibitor P32/98 (Pospisilik et al. 2003).

Acute Inhibition of DP IV and Intraduodenal Glucose Tolerance. Initially, an IDGTT was performed in a small group of STZ animals to confirm the ability of DP IV-inhibitors to increase circulating active GLP-1 levels in this model. A 10 mg/kg dose of P32/98 was sufficient to protect an additional 40% of the total GLP-1 secretion, allowing peak glucose-stimulated active GLP-1 levels to reach 45 ± 7 pM (vs. 14 ± 1 pM for control; Fig 22). Further, an earlier and more robust plasma insulin peak was evidenced after P32/98 treatment as was a concomitant reduction in blood glucose (Fig.22).

Figure 22. DP IV inhibition enhances active GLP-1 levels, early phase insulin release and improves intraduodenal glucose tolerance. Blood glucose (A), plasma insulin (B), plasma active (C) and total (D) GLP-1 levels measured during an IDGTT (1 g/kg) performed in anesthetized STZ-Wistar rats either in the presence (open circles) or absence (closed squares) of the DP IV-inhibitor P32/98 (10 mg/kg). Statistical significance (P<0.05) vs. control is indicated with an asterisk.
**Body Weight Gain and Nutrient Intake.** STZ-induced diabetes in rats is accompanied by a marked reduction in weight gain despite increased caloric intake, a direct result of insulin insufficiency (Fig. 23). Over the course of the present study, the early and late treatment groups (established to differentiate between potential cytoprotective and regenerative effects of DP IV inhibition) gained 129 ± 7 g and 92 ± 4 g respectively: 2.3-fold and 1.7-fold the STZ-control value (Fig. 23). Further, the STZ-induced doubling in food intake exhibited by the diabetic controls was decreased 48% and 23% in the early and late groups respectively. Changes in food and water intake paralleled one another, with improvements evident in both treatment groups within one week of treatment initiation (Fig. 23).

**Figure 23.** Body weight (A), food (B), and water (C) intake measurements from Wistar rats (n=20) exposed to a single high-dose of streptozotocin (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Control (solid squares) and STZ-control (open squares) animals were administered a 1% cellulose solution while the early treatment group (open triangles; treatment initiated 1 week before STZ admin.) and the late treatment group (open circles; treatment initiated 1 week after STZ admin.) were administered 10 mg/kg P32/98 twice daily by oral gavage. Food and water intake were measured by subtraction. Statistical significance (P<0.05) for the early group vs. STZ-control is indicated with an asterisk, while that for the late group vs. STZ-control is indicated with a number sign.
Circulating Glucose, Insulin, and DP IV. The initial STZ-induced increase in morning blood glucose values averaged ~20 mM above normal (Fig. 24A). This rapid rise in glycemia, which stabilizes within 4 days, was markedly attenuated in the early treatment group plateauing ~5 mM below the untreated controls. In contrast to a gradual deterioration in morning blood glucose in the untreated STZ-controls, both treatment groups displayed significant reductions in morning glycemia within one week of treatment initiation. The reduction in morning glycemia evident in the late treatment group, unlike the abrupt, immediate reduction seen in the early groups, was progressive over the six weeks of treatment. Measurement of plasma fructosamine levels (an indicator of long-term glycemic control) revealed significant reductions in both treatment groups corroborating the morning blood glucose data (Fig. 24B). Once again, the early treatment group exhibited an immediate blunting of the STZ-induced rise in plasma fructosamine, while the late group showed a more progressive reduction, achieving significance during the final two weeks of the study.

Figure 24. Weekly monitoring of blood glucose (A) and plasma insulin (B), fructosamine (C), and DP IV activity levels (D) in Wistar rats (n=20) exposed to a single high-dose of streptozotocin (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Control (solid squares) and STZ-control (open squares) animals were administered a 1% cellulose solution while the early treatment group (open triangles; treatment initiated 1 week before STZ admin.) and the late treatment group (open circles; treatment initiated 1 week after STZ admin.) were administered 10 mg/kg P32/98 twice daily by oral gavage. Blood glucose was measured on a hand-held blood glucose meter, insulin and fructosamine using commercially available kits, and DP IV activity using an in-house assay. Statistical significance (P<0.05) for the early group vs. STZ-control is indicated with an asterisk, while that for the late group vs. STZ-control is indicated with a number sign.
Whereas untreated animals displayed a 0.08 nM drop in plasma insulin within the first two weeks following STZ-exposure, the pre-treated animals presented only a 0.04 nM drop, a 43% improvement over the untreated controls. As before, the late treatment group displayed a more gradual reversal, with a 40% elevation in morning plasma insulin levels detected by week 6 of the experiment (Fig. 24C).

Morning plasma DP IV activity measured immediately prior to P32/98 administration showed an STZ-induced elevation to a plateau level approximately 50% above normal within four days in all three STZ groups (Fig. 24D).

**Oral Glucose Tolerance.** Seven weeks after STZ injection all groups underwent an oral glucose tolerance test (OGTT). Fasting blood glucose values in the treated animals averaged 5.4 mM less and 2.0 mM less than their untreated diabetic littermates (early and late respectively), though neither group achieved normalcy (Fig. 25A). Integrated blood glucose responses over the 120 minute course of the OGTT were decreased by 33% and 20% in the early and late treatment groups respectively, with concomitant 240% and 45% increases in integrated insulin responses (Fig 25 insets). Glucose-responsiveness of insulin secretion, which was largely ablated in the diabetic controls, was partially restored in the early treatment group (Fig. 25B).

**Figure 25.** Oral glucose tolerance tests performed on Wistar rats (n=20) exposed to a single high-dose of streptozotocin (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98 for seven weeks. Blood glucose (A) and plasma insulin (B) were measured during a 1 g/kg OGTT in control (solid squares) and STZ-control (open squares) animals, an early treatment group (open triangles; treatment initiated 1 week before STZ admin.) and late treatment group (open circles; treatment initiated 1 week after STZ admin.) which received 10 mg/kg P32/98 twice daily by oral gavage for seven weeks following STZ administration. Insets show area under the curve calculations. Statistical significance (P<0.05) for the early group vs. STZ-control is indicated with an asterisk, while that for the late group vs. STZ-control is indicated with a number sign.
**Pancreatic Insulin Secretion and Content.** The partial restoration of glucose responsiveness of insulin release observed during the OGTT was accompanied by increased glucose-stimulated insulin secretion during pancreas perfusion. Under basal (4.4 mM glucose) conditions, insulin secretion from pancreata of both treated groups was elevated (190% and 77% for the early and late treatment groups respectively) compared to the untreated control levels (Fig. 26). Basal secretion from the early treatment group equaled normal Wistar controls (0.21 nM). Furthermore, first phase insulin release in response to a 4.4 mM to 8.8 mM glucose perfusion step, was partially restored in both treatment groups with peak insulin levels reaching 0.87 nM and 0.59 nM in the early and late groups respectively (~70% and ~50% of control, 400% and 250% of STZ-control; Fig. 26A).

**Figure 26.** Insulin response to glucose (A) and insulin content (B) in pancreata isolated from Wistar rats (n=20) exposed to a single high-dose of streptozotocin (50 mg/kg) and treated either with or without the DP IV inhibitor P32/98. Glucose stimulated insulin secretion was assessed during an *ex vivo* pancreas perfusion and pancreatic insulin content determinations were made for entire pancreata excised under anesthesia. Inset shows area under the curve for the 5th to 10th minutes. Statistical significance (*P*<0.05) for the early group vs. STZ-control is indicated with an asterisk, while that for the late group vs. STZ-control is indicated with a number sign.
In keeping with the elevation in basal insulin secretion evident during perfusion, parallel increases were observed in whole pancreas insulin content after long-term DP IV-inhibitor treatment (Fig. 26B). The late and early treatment groups exhibited greater than 2- and 8-fold higher total pancreatic insulin levels than the untreated STZ injected group (corresponding to 5% and 17% of control). Data presented in Figure 26, normalized against protein concentration, were representative of total pancreatic insulin as no difference was evidenced in pancreatic mass between groups (Control 1.91 ± 0.10g; STZ-Control 1.93 ± 0.11g; Early Treatment 1.84 ± 0.07g; and Late Treatment 1.86 ± 0.06g).

**β-cell Mass and Islet Morphology.** Pursuant to evidence of increased pancreatic insulin content, morphometric analysis of islet β-cell distribution was performed. Examination of islet number per unit area in all three STZ-exposed groups showed an increase in the number of small islets (1-20 cells in cross-section) (Fig. 27A). The effect was most profound in the treated groups, with marked increases in islets with a cross-sectional area ≤10 cells. Determination of β-cell area as a fraction of cross-sectional islet cell number revealed that the majority of islets ≤20 cells in cross-sectional area contained a near-normal fraction of β-cells (60-80 %) while those of larger size showed a marked reduction in β-cell fraction (Fig. 27B). Significant increases in β-cell fraction were evidenced in the early treatment group in all subsets of islets >10 cells in cross-section, while a significant increase in the late group was only seen in the group 11-20 cells in cross-section (Figs. 27B&D). The logical interpretation of these two results, in functional terms, is that β-cells in small islets make up a larger fraction of the functional β-cell mass (insulin-IR mass) in the three STZ groups. Expressed as the fraction of the total pancreatic β-cell number, β-cells of smaller islets were shown to comprise a significantly larger fraction of the total β-cell mass in all three STZ-exposed groups (Fig. 27C). Interestingly, islets with a cross sectional area of 11-20 cells appeared particularly sensitive both to the toxic effects of streptozotocin and to the protective effects of the treatment displaying marked increases in β-cell fraction, and fraction of total pancreatic β-cell number in response to the DP IV-inhibitor regimen (Fig. 27B,C,I,J).

Qualitative analysis of insulin immunoreactivity revealed a number of interesting features. Whereas insulin-IR cells in the islets of STZ-control animals displayed relatively diffuse and weak staining (Fig. 27F), islets in both treatment groups contained a marked number of cells displaying more intense insulin staining (Fig. 27G&H). These insulin-"bright" (IN<sup>bright</sup>) cells appeared relatively rotund and polarized while those of STZ-control islets appeared diffuse and elongated, and lacked distinct polarity (Fig. 27E-J).
Figure 27. Immunohistochemical analyses of islet sections stained for insulin. Islets were binned according to cross-sectional endocrine cell number (0-5, 6-10, 11-20, 21-50, 51-100, 100-350), and reported as the largest islet size of any given bin. (A) Number of islets per unit area; (B) fraction of islet endocrine cells immunoreactive (IR) for insulin per size bin; (C) insulin-IR cells as a fraction of the total number of insulin-IR cells per subject; (D) β-cell fraction of total islet endocrine cells combined for all sizes and number of insulin-IR cells per unit pancreatic area. Insulin staining from (E) normal Wistar rat, and (F) STZ-control, (G) early P32/98 treated, (H) and late P32/98 treated Wistar rats exposed to 50 mg/kg STZ 7 weeks earlier. Islets that contained ~20 cells in cross section appeared particularly sensitive to both STZ (I) and to the protective effects of the DP IV-inhibitor treatment (J; early treatment group).
**Incretin-mediated cell survival of β-cells in vitro.** In an attempt to provide a mechanism of action for the immunohistochemical and the *in vivo* data, an examination was made of the ability of GIP and GLP-1 to promote cell-survival in INS-1 (832/13) cells transiently exposed to STZ. After a 30-minute STZ-exposure followed by a twenty-four hour recovery period, STZ was shown to elicit cytotoxic effects in the low mM range, with an EC₅₀ of 2.1 mM (Fig. 28A). GLP-1 and GIP were shown independently to partially reverse STZ-induced cytotoxicity with EC₅₀ values of 0.04 nM and 0.75 nM respectively (Fig. 28B). STZ-induced cytotoxicity under control conditions involved 3- and 7-fold increases in caspase-3 and -8 activity respectively, increases that were largely ablated by the addition of the incretins (Fig. 28 C&D). Further, only minimal differences in cytoprotection were observed when the interval of GIP or GLP-1 administration was varied between pre-treatment and recovery treatment (Fig. 28 E&F). Addition of GIP and GLP-1 in combination showed no significant additive effect (data not shown).
Figure 28. GIP and GLP-1 stimulation of β-(INS-1 832/13)-cells protects against STZ-induced apoptotic cell death. (A) STZ dose-response curve in INS-1 832/13 cells; (B) dose-dependency of GIP/GLP-1 mediated inhibition of STZ-induced cell death; (C, D) GIP and GLP-1 mediated suppression of STZ-induced caspase-3 and -8 activation. Further examination of prevention of STZ-induced cell death by GIP (E) and GLP-1 (F), in the presence (open bars) or absence (closed bars) of the DP IV inhibitor P32/98 (10 μM) showed no strong dependency on whether peptide was added prior to (Pre), immediately after (Post), or before and after (Pre + Post) a 30 minute STZ (2 mM) exposure.
Plasma Lipids and Hepatic PEPCK. Table III summarizes the results of plasma triglyceride, free fatty acid, and cholesterol measurements made on fasting samples obtained seven weeks into the experiment. An indication of insulin insufficiency and a characteristic of type-1 animal models, the STZ-controls displayed significant increases in circulating levels of all three lipids. Treatment with the DP IV-inhibitor P32/98 resulted in a lasting reduction in plasma lipids with the early treatment group returning to control TG and cholesterol levels (Table III). Another metabolic abnormality characteristic of the STZ-rat is an increase in hepatic PEPCK activity. Early treatment with the DP IV-inhibitor P32/98 reversed this defect, while delayed treatment elicited a non-significant, 46% reduction in the STZ-induced rise in PEPCK activity (Table III).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ-Control</th>
<th>Early-Treatment</th>
<th>Late-Treatment</th>
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</thead>
<tbody>
<tr>
<td>PEPCK (mU/kg)</td>
<td>22.7 ± 1.7</td>
<td>46.3 ± 4.3††</td>
<td>24.2 ± 2.4 *</td>
<td>35.9 ± 4.8 †</td>
</tr>
<tr>
<td>TGs (mM)</td>
<td>0.50 ± 0.14</td>
<td>2.37 ± 0.28††</td>
<td>0.94 ± 0.11 **, †</td>
<td>1.61 ± 0.22 * ††</td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.28 ± 0.04</td>
<td>0.52 ± 0.05††</td>
<td>0.24 ± 0.03 *</td>
<td>0.41 ± 0.02 ††</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>1.59 ± 0.16</td>
<td>2.53 ± 0.31† †</td>
<td>1.54 ± 0.10 *</td>
<td>1.75 ± 0.12 *</td>
</tr>
</tbody>
</table>

TGs = triglycerides, NEFA = non-esterified fatty acids. Statistical significance vs. STZ controls are indicated by a single (P<0.05) or double (P<0.01) asterisk; and vs. controls by a single (P<0.05) or double (P<0.01) cross.
STUDY IV: LONG-TERM EFFECTS OF DP IV-INHIBITION IN THE BB RAT

Having demonstrated relatively profound improvements in β-cell mass and function in the STZ model, the potential applicability of DP IV-inhibition in the treatment of type-1 diabetes was established (Pospisilik et al. 2003). In order to shed further light on the topic, and more specifically to address the potential immunosuppressive aspects of the intervention, a preliminary study was initiated in the BB rat, a well-characterized model of spontaneous autoimmune diabetes. Upon weaning, diabetes prone BB rats were initiated on a treatment course of twice daily P32/98 (50 mg/kg) by oral gavage. DP IV-inhibitor treatment was shown to delay onset of diabetes by 10 ± 2 days in addition to partially preventing diabetes in a subset of the animals (21% reduction in incidence; Fig 29). Treated animals that did develop diabetes were maintained on DP IV-inhibitor therapy post-onset. Relative to control diabetics, they displayed small reduction in daily blood glucose prior to administration of a subcutaneous bovine insulin implant. Post-implantation, animals continued to display an improved morning blood glucose profile (Fig. 30A). However, removal of insulin therapy (implant excision) after four weeks rendered both groups of animals completely glucose intolerant (Fig. 30B).

Figure 29. Long-term DP IV-inhibitor treatment delays the onset of diabetes in the BB rat. Diabetes incidence curves from three BB rat cohorts (n=20) treated either with (from 3 or 6 weeks of age) or without the DP IV inhibitor P32/98 (50 mg/kg twice daily by oral gavage).
Figure 30. Long-term DP IV-inhibitor treatment shows disparate effects on glycemia in the diabetic BB rat. While under the influence of 2 U/day bovine insulin (subcutaneous implant, Linplant, ON), BB rats treated with the DP IV-inhibitor P32/98 twice daily by oral gavage (50 mg/kg) showed a reduction in morning blood glucose levels (A). After removal of daily insulin therapy no difference in oral glucose tolerance was observed between groups.

As is evident from the incidence curves presented in Figure 29, not all diabetes prone BB rats develop diabetes. Both pre-diabetic and ultimately non-diabetic animals however have been reported to display very mild glucose intolerance. In order to examine the effect of DP IV-inhibitor therapy on these subsets of animals, morning blood glucose was monitored over the course of the experiment in addition to administration of an oral glucose tolerance test two weeks after initiation of the treatment. While no difference was observed in daily values, a fasted glucose tolerance test revealed a small but significant improvement in glucose tolerance in the treated animals, characterized by a reduced glucose excursion (associated with a reduction in fasting blood glucose), concomitant with a more pronounced early phase insulin release (Fig. 31).

Figure 31. Long-term DP IV-inhibitor treatment shows disparate effects on glycemia in the pre-diabetic BB rat. Non-diabetic diabetes prone BB rats treated with the DP IV-inhibitor P32/98 twice daily by oral gavage (50 mg/kg) showed no divergence in morning blood glucose levels (A). When challenged with an oral glucose tolerance test (1g/kg) after 2 weeks of treatment the treated group displayed a reduction in fasting and challenged glucose levels concomitant with a more pronounced early insulin release profile (B).
In an attempt to provide further insight into the *in vivo* effects mentioned above, islets from pre-diabetic animals were examined both functionally (perfused pancreas) and morphologically (histochemistry). Pancreas perfusion responses to a low-to-high glucose perfusion in the control animals were relatively blunt with a poorly defined first-phase and only a small second phase of insulin secretion. Parallel perfusions performed on pancreata from treated animals however showed marked first- and second-phase responses, suggesting an improved glucose-responsiveness of insulin secretion (Fig. 32 A). Neither total pancreatic insulin measurements (data not shown) nor insulitis analysis (Fig. 32 B) made on pancreata from separate animals showed any difference between treated and control animals supporting the notion that the differences observed during pancreas perfusion were inherent to the β-cells themselves.

**Figure 32.** Chronic inhibition of DP IV improves glucose stimulated insulin release in non-diabetic BB rats in the absence of an alteration in insulitis. Glucose stimulated insulin secretion was assessed during an *ex vivo* pancreas perfusion (A). Insulitis was scored in H&E stained sections of pancreata from both groups (*n*=4; 40 islets scored per rat).
ON THE METABOLISM OF GLUCAGON BY DIPEPTIDYL PEPTIDASE IV

There has been considerable controversy in the literature regarding the specific tissues and enzymes responsible for the degradation of glucagon. The current study specifically examined the possible involvement of the ubiquitous enzyme, dipeptidyl peptidase IV in the metabolism of glucagon. Parallel assessment of glucagon degradation products by the group of Dr. H-U Demuth using capillary zone electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identified the specific molecular species produced from glucagon metabolism both in human serum and by purified porcine DP IV (Pospisilik et al. 2001). Together these studies provide strong evidence that DP IV is a primary candidate for enzymatic degradation of glucagon.

When examining degradation of glucagon by purified DP IV, the sequential loss of dipeptides resulted in the expected molecular weights for the truncated N-terminal fragments glucagon$_{3-29}$ and glucagon$_{5-29}$. However, incubation of glucagon$_{1-29}$ in serum consistently gave rise to a peptide product 17 Daltons less than the expected weight for glucagon$_{3-29}$: glucagon$_{3-29}$-17Da (Pospisilik et al. 2001). Hence it appeared that in serum, DP IV first cleaved the N-terminal dipeptide from glucagon, and a serum enzyme subsequently catalyzed the cyclization of the amino terminal glutamine residue (Gln)$^3$, resulting in the loss of an NH$_3$ group (17 Da). Likely candidates for this cyclization reaction include transglutaminases, well documented serum enzymes which cause the formation of new γ-amide bonds between γ-carboxy-amine groups of glutamine and primary amines (Aeschlimann and Paulsson 1994). No intermediate glucagon$_{3-29}$ was observed in the study, therefore the DP IV-catalyzed N-terminal cleavage reaction appeared to be rate determining. Furthermore, as glucagon$_{5-29}$ was not observed, it appeared that the cyclization step occurred much more rapidly than the second DP IV-mediated truncation step (cleavage of glucagon$_{3-29}$ to glucagon$_{5-29}$). In order to render the peptide resistant to further degradation by DP IV, the cyclization event must involve either the α-amine or γ-carboxy-amine of Gln$^3$. Products with the correct molecular mass could include pyroglutamyl-glucagon$_{3-29}$ ([pGlu]$^3$glucagon$_{3-29}$), cyclo$^{1-12}$-glucagon$_{3-29}$, cyclo$^{1-20}$-glucagon$_{3-29}$ or cyclo$^{1-24}$-glucagon$_{3-29}$ (H-U Demuth personal communication; (Pospisilik et al. 2001). When used as a standard for HPLC, MALDI-TOF MS, and CE, synthetic [pGlu]$^3$glucagon$_{3-29}$ could not be distinguished from the serum-catalyzed glucagon$_{3-29}$-17 Da. Hence, the authors concluded that it was likely that [pGlu]$^3$glucagon$_{3-29}$ was the product formed in serum though the possibility of other cyclic derivatives warrant further investigation. The characteristics of this cyclization are consistent with the activity of the serum enzyme, glutaminyl cyclase (Fischer and Spiess 1987).

Thus, in vitro enzymology studies identified DP IV as the primary enzyme involved in the metabolism of glucagon in the circulation. In order to substantiate the in vitro results and determine the
in vivo consequences of N-terminal truncation and cyclization, further studies were carried out using a number of bioassays in rats. Incubation of glucagon in normal rat plasma or purified porcine DP IV resulted in a complete loss of the hyperglycemic response to glucagon in vivo (Fig. 2). Consistent with the in vitro assays, use of P32/98 prevented the loss of bioactivity of glucagon incubated in serum or DP IV (Fig. 3). Further evidence supporting DP IV-mediated metabolism of glucagon was provided by incubation of glucagon in DP IV-negative rat plasma. As expected, glucagon incubated in DP IV-/- plasma retained bioactivity, in contrast to control Fischer rat plasma (Fig. 2). Concurrent evaluation of the binding and activation characteristics of synthetic truncated glucagon fragments using CHO-K1 cells transfected with the human glucagon receptor revealed that all three potential degradation products (glucagon_{3-29}, [pGlu^3]glucagon_{3-29}, and glucagon_{5-29}) showed only weak agonist activity (20-60 % efficacy at pharmacological doses) with 5- to 18-fold lower binding affinities than the native glucagon molecule (Fig. 8; Table II). Over the physiological range these peptides can be considered non-stimulatory, a conclusion consistent with previous studies using truncated forms of the glucagon molecule (Frandsen et al. 1981).

With regard to the physiological relevance of DP IV-mediated glucagon degradation in vivo, it is important to consider several points: First, the majority of the DP IV activity in the circulation is membrane bound, endothelial DP IV (at least one order of magnitude higher than plasma DP IV) (Mentlein 1999), and therefore that the majority of the conversion of glucagon to its truncated form, in the circulation, likely occurs at the blood vessel wall rather than in the plasma matrix. Among peptide substrates, glucagon exhibits the highest affinity for DP IV tested so far (Nausch et al. 1990, Demuth and Heins 1995), in addition to a second order rate constant (194 mM^{-1}sec^{-1}) on par with those of the incretins GIP, and GLP-1 (220 and 430 mM^{-1}sec^{-1} respectively; (Mentlein et al. 1993b)). Taken together with the fact that the circulating concentration ranges of the three hormones are similar and the physiological impact of DP IV activity on incretin action, it is likely simply from a theoretical standpoint that DP IV would play a significant role in the clearance of active glucagon from the circulation. Examination of the acute effect of P32/98 treatment on Wistar rats during two glucagon-demanding physiological scenarios, fasting and insulin-induced hypoglycemia, provided in vivo proof of the concept (Figs. 4-6). Further, generation of a sensitive glucagon biosassay exploiting the insulinostatic and glucagonostatic actions of somatostatin, corroborated the point, revealing an 80% increase in glucagon-stimulated glucose release in the presence of P32/98 (Fig. 7). Interestingly, concurrent examination of the responsiveness of DP IV -/- animals to those same challenges (fasting, hypoglycemia, and low dose glucagon) revealed an apparent insensitivity of the DP IV -/- model to glucagon (Figs. 4-7). Differing degrees of compensation in this regard may in part explain the controversy in the literature regarding glucose tolerance in DP IV -/- rodent models (CD26 null mouse and the DP IV -/- rat used in this study; (Pederson et al. 1996, Marguet et al. 2000). In addition, these
data serve as an important reminder of the limitations of knockout or monogenic experimental models and reinforce the need for multiple experimental strategies in the definition of physiological action (Leiter 2002).

Comparison of the second order rate constants of the three glucoregulatory peptides discussed so far, with those of previously characterized bioactive peptide DP IV substrates, now become very fitting. That is to say that glucagon, GIP, and GLP-1 ($k_{cat}/K_m$ ~200-400 mM$^{-1}$sec$^{-1}$), which must move through the circulation (crossing two endothelial barriers) to reach their target tissues are afforded a relative DP IV-resistance over, for example, the locally acting neuropeptide DP IV-substrates NPY, GRH, and PYY which are degraded much more rapidly ($k_{cat}/K_m$ =2000 and 12000 mM$^{-1}$sec$^{-1}$, respectively; (Mentlein et al. 1993a)). Potential overstimulation of local paracrine pathways (e.g. glucagon stimulation of insulin secretion) by the relatively stable peptide hormones is likely avoided by co-release of DP IV and localization of DP IV to the target tissues. A recent finding of Grondin and colleagues, the localization of DP IV to the pancreatic $\alpha$-cell secretory granule (Grondin et al. 1999) gives support to this idea, as well as giving rise to the possibility of local effects of the truncated peptide fragments (dipeptides and N-terminally truncated).

Further support for the concept of DP IV-mediated glucagon degradation as a relevant physiological regulatory mechanism was provided by our parallel characterization of synthetic DP IV-resistant glucagon analogues. Peptides were generated with modifications of the $P_1$ or $P_{1}'$ residue on the basis of similar successful attempts to confer DP IV-resistance to the GIP and GLP-1 backbone (Hinke et al. 2003, Meier et al. 2003). Whereas three of four analogues tested failed to demonstrate enhanced bioactivity (later shown to be due to incomplete DP IV-resistance, reduced binding and activation characteristics, or susceptibility to non-DP IV mediated hydrolysis), D-Ser$^2$-glucagon was shown to elicit a prolonged and increased hyperglycemic response when compared to native glucagon (Fig. 9 & 10; (Hinke et al. 2000). D-Ser$^2$-glucagon was the sole analogue that displayed comparable binding and activation properties to native glucagon when tested in CHO-K1 cells transfected with the human glucagon receptor (Fig. 9).

As the glucagon degradation products (glucagon$\text{3-29}$ and [pGlu$\text{3}$]glucagon$\text{3-29}$, or other possible cyclized derivatives) are found in human serum, measurements of glucagon immunoreactivity by ‘side-viewing’ or C-terminally directed antibodies likely cross-react with these peptides. As of yet there has been no reported immunoassay specific for N-terminally intact or N-terminally truncated, cyclized glucagon. The majority of studies using antibodies for the measurement of glucagon are therefore likely misleading in this respect. Though unlikely to significantly alter our general understanding of glucagon physiology, these findings do provide a potential underpinning for previous reports of glucagon stability in plasma (Alford et al. 1976, Marki 1983), and warrant a re-evaluation of the pathophysiological role of glucagon in diabetes. Frandsen et al found that glucagon$\text{3-29}$ cross-reacted with at least two antisera
tested (K5563 and K4023) (Frandsen et al. 1981). Many such antibodies probably also cross-react with glucagon
3-29 and [pGlu3]glucagon3-29 (or other cyclized derivatives). Assays now exist specifically directed towards N-terminally intact GIP and GLP-1, assays that have contributed tremendously towards our understanding of incretin physiology. Development of comparable glucagon assays is warranted and will surely be repeated for glucagon. Such assays, in addition to more discriminate methods for identification of molecular species in biological samples, will be necessary for definitive characterization of the conversion of glucagon3-29 to [pGlu3]glucagon3-29 in vivo.

Considerable attention has been given to the development of DP IV-resistant GLP-1 (Deacon et al. 1998b, Ritzel et al. 1998) and GIP (O'Harte et al. 1999, Hinke et al. 2002) analogues, and to inhibition of DP IV (Pauly et al. 1996b, Deacon et al. 1998a, Pederson et al. 1998b) as therapeutic strategies in the treatment of type-2 diabetic patients. Generation of synthetic incretin analogues with increased resistance to DP IV has led to peptides with increased circulating half-lives and enhanced antidiabetic effects owing to prolonged insulinotropic activity. Similarly, inhibition of DP IV with the specific inhibitors, (P32/98) ile-thia and val-pyr, have been shown to improve glucose tolerance in animal models, through enhancing the activity of endogenous incretins (Pauly et al. 1996b, Deacon et al. 1998a, Pederson et al. 1998b). Given that DP IV is also responsible for degrading glucagon, the improved glucose tolerance seems counter-intuitive, as inhibition of DP IV would also enhance the activity of endogenous glucagon. However, postprandially, both the stimulation of, and thus the rate of endogenous glucagon secretion, are at a minimum, while those of the incretins are at a maximum. Thus any enhancement of glucagon activity is likely masked by a much greater relative enhancement of incretin activity. Additionally, remaining intact negative feedback loops for glucagon continue to be active (Figs. 4, 5) while the secretion stimulus for the incretins continues. It follows then, that when considering the design of DP IV inhibitors as antidiabetic agents, rapidly reversible inhibitors that are quickly cleared from the circulation are most desirable. Considering the data presented so far, it appears that the physiological consequences of DP IV inhibition would shift in tandem with this transition in regulatory control, from insulin enhancing (incretin stabilization) to glucagon enhancing (glucagon stabilization), due to the relative changes in concentration of these circulating hormones in the postprandial state. When taken together with the fact that approximately two thirds of the 24-hour cycle is spent in the fasting state, primarily under the control of glucagon, it may be that slow-binding, irreversible or stable inhibitors which sustain DP IV inhibition into the fasting state, would likely become counterproductive as antidiabetic drugs.
DP IV-INHIBITION IN THE VDF RAT: IMPLICATIONS FOR THE TREATMENT OF TYPE-2 DIABETES

The protection of full-length GIP$_{1-42}$ and GLP-1$_{7-36}$ amide (incretins) in the circulation using dipeptidyl peptidase IV (DP IV) inhibitors represents a significant advancement in the search for new, effective alternative treatments for diabetes. Since the early 1990's, numerous studies have revealed a pleiotropy of anti-diabetic effects triggered by interaction of the incretins with their respective cell-surface receptors. Among them, are stimulation of β-cell insulin biosynthesis, glucose-competence, proliferation, differentiation, growth and cell-survival, inhibition of glucagon secretion, and several reports indicating stimulation of glucose uptake in muscle cells (Mizuno et al. 1997, Yang et al. 1998, Buteau et al. 1999, Xu et al. 1999, Stoffers et al. 2000, Hui et al. 2001, Trumper et al. 2001, Ehses et al. 2002). Early studies on DP IV-inhibitor efficacy established the proof-of-concept for these compounds on an acute scale (Pauly et al. 1996a, Pederson et al. 1998b, Balkan et al. 1999, Ahren et al. 2000). However, investigations performed on an acute scale are unlikely to exploit the longer term incretin actions mentioned above, effects that require alteration in intracellular protein function and gene expression. The focus of the current study, therefore was to characterize the effects of long-term DP IV-inhibitor therapy on glucose tolerance, β-cell function, and insulin sensitivity in a model of type 2 diabetes, the Vancouver Diabetic Fatty rat (VDF; fa/+) Zucker diabetic syndrome, and caused a progressive improvement in glucose tolerance, insulin sensitivity and β-cell glucose responsiveness.

Daily monitoring revealed a 12.5% decrease in body weight gain (4% reduction in final body weight) in the treated animals compared to untreated controls (Fig. 11 A). Though not statistically significant, mean food intake in the treated animals averaged 0.4 g/day/rat (41 g/rat over the course of the study) less than those in the control group. It is possible that the cumulative 41 g/rat non-significant difference in food intake over the course of the experiment might partially account for the decreased weight gain in the treated animals. These findings rule out neither the possibility that the gastric inhibitory actions of the incretins nor the reported satiety effects of GLP-1, played a role in the decrease in weight gain.

Monitored on a bi-daily basis, morning and evening blood glucose values showed no significant response to the inhibitor treatment, a likely reflection of two points. First, the blood sampling times (0800 h and 1700 h) corresponded to post-absorptive and early feeding states respectively, with blood glucose values in the ranges 4.5-5.5 mM and 6.0-8.0 mM. In light of the hypothesized, glucose-dependent mechanism of action of the treatment, large decreases in glucose values would not be anticipated at these glycemic levels. Secondly, both morning and evening blood samples were collected immediately prior to drug dosing, at times of minimum DP IV inhibition where the potential for any acute therapeutic effects of the treatment were at a minimum. Both points are supported by the 24 hour profile shown in Figure 12.
The unaltered post-absorptive blood glucose values notwithstanding, DP IV inhibitor treatment effectively reduced both prandial blood glucose and blood glucose responses to an OGTT (Figs. 12-14). During the 24 hour profile the control animals exhibited a 105 % rise in plasma insulin in response to a 5.2 mM increase in blood glucose, while the treated animals displayed a larger, 160 % insulin response to a much smaller glucose excursion (3.0 mM). While these differences were likely due, at least in part, to an acute increase in circulating incretin levels induced by P32/98, the pronounced early phase insulin peak exhibited during the OGTT was not (the OGTT took place after complete drug washout). The latter data were suggestive not only of increased insulin sensitivity but also of enhanced β-cell glucose responsiveness. Ultimately, an increase in β-cell glucose responsiveness was clearly demonstrated through pancreas perfusion. Upon exposure to an elevated (8.8 mM) glucose perfusate, pancreata from the control animals showed an absence of first phase insulin release while those from the treated group exhibited an immediate, 3.2-fold insulin response (Fig. 15). The absence of early phase insulin release seen in the control group is characteristic of the VDF rat and is a hallmark of type 2 diabetes (Lynn et al. 2001). Considering the lack of altered β-cell area or islet morphology, these data suggest that long-term treatment with P32/98 causes an improvement in the ability of the existing β-cell population to sense and respond to increases in glucose concentration. These findings are consistent with the reported effects of GLP-1 on β-cell differentiation as well as numerous reports showing glucose-sensitizing effects of GIP and GLP-1 both in islets and in immortalized β-cell models (Wang et al. 1995, Wang et al. 1996). Further, demonstration of DP IV-inhibitor induced prevention of, or recovery from a GIP insensitive state (Fig. 16 B) is novel and of great import to the field of diabetes research. The data are in agreement with the recent link made, once again by Lynn et al., between hyperglycemia and GIP-receptor downregulation in vivo (and associated islet insensitivity to GIP; (Lynn et al. 2003) and imply that proper glycemic control of diabetic patients may be sufficient to restore GIP sensitivity.

Elevated fasting blood glucose in the face of hyperinsulinemia, and poor clearance of an oral glucose load, respectively, are consistent with the hepatic and muscle insulin resistance described in the fa/fa Zucker rat. Findings of the present study show that DP IV-inhibitor treatment at least partially corrected both of these metabolic deviations, providing evidence for improvements in both sites of insulin resistance. Indirect evidence included an increased glucose-to-insulin ratio evident during the post absorptive state of the 24-hour profile (Fig. 12) as well as reduced fasting values of the 12-week OGTT (Figs. 13 & 14). These data are consistent with a decrease in insulin resistance in the treated animals. This was shown to be significant at both 4 and 12 weeks using the composite insulin sensitivity index of Matsuda and DeFronzo (Matsuda and DeFronzo 1999). The results of the 24-hour glucose/insulin/DP IV profile and the OGTT were corroborated in vitro by glucose uptake measurements made in soleus muscle strips and, in vivo, by euglycemic-hyperinsulinemic clamp, data which clearly demonstrated enhanced insulin-stimulated glucose uptake (Fig 20 A).
Extensive literature exists on the obese Zucker rat, including a number of studies examining insulin resistance by means of euglycemic-hyperinsulinemic clamp (McIntosh and Pederson 1999). In one such study, Terrettaz and associates tested HGO and GDR responses in obese Zucker rats over a wide range of insulin concentrations allowing a comprehensive evaluation of both responsiveness (the efficacy or magnitude of the response to insulin) and sensitivity (the potency or half-maximal concentration of the response to insulin) to the hormone (Terrettaz et al. 1986). The authors concluded that the obese animals demonstrate marked hepatic insulin resistance (characterized by a fully responsive yet right-shifted hepatic glucose output) and severely impaired glucose disposal (characterized by a total lack of responsiveness over a wide range of insulin concentrations) when compared to their lean littermates (Terrettaz et al. 1986). Similar to these early reports, the control VDF rats in the current study showed a responsive yet right-shifted hepatic glucose response to insulin, and an extremely blunted peripheral response (Figs. 19 & 20). It is apparent that long-term DP IV-inhibitor treatment partially reversed both of these functional pathologies, causing a left-shift in the HGO response to insulin (Fig. 19) and partial restoration of peripheral insulin-stimulated glucose uptake (Fig. 20). The primary physiological consequence of these improvements appears to be a reduction in fasting plasma glucose and a marked enhancement of glucose tolerance. Similar changes in HGO and GDR have been shown for several classes of oral anti-diabetic agents in obese Zucker rats (Bowen et al. 1991, Shibata et al. 1998, Hevener et al. 2000).

The finding that P32/98 treatment improves hepatic insulin sensitivity supports the demonstration of reduced hyperinsulinemia in the basal fed state and reduced fasting plasma glucose in the same model (Fig. 12; Pospisilik et al. 2002). Similar reductions in insulinemia and fasting plasma glucose have been shown in a number of studies using long-term GLP-1 (or GLP-1 mimetic) treatment (Larsen et al. 1996, Rachman et al. 1997, Young et al. 1999). In keeping with the previous discussion of HGO sensitivity to insulin, the defining feature of P32/98-treatment on hepatic insulin sensitivity appeared to be a left-shift in insulin-responsiveness. Since fasting insulin levels did not differ between groups, a reduction in basal HGO in the treated animals is implicit. And since basal (fasting) glucose output from the liver is primarily determined by the rates of gluconeogenesis and glycogenolysis (both of which are potently stimulated by glucagon), a potential underpinning for these results might have been a reduction in glucagon levels in the treated group. Examination of fasting (complete drug washout) glucagon levels, however, though measured using an antibody blind to N-terminal truncation, did not support such a hypothesis (Fig. 19D). Also, PEPCK activity was measured as an index of gluconeogenic enzyme expression, a group of enzymes that exhibit coordinate changes in expression in response to insulin and glucagon stimulation. Since PEPCK does not undergo short-term regulation via phosphorylation or allosteric effectors, and since, physiologically, it is rate limiting for gluconeogenesis, PEPCK activity measurement provides an indicator of gluconeogenic potential. Though a non-
significant (~20%) decrease in PEPCK activity was demonstrated in the treated animals, the exact mechanisms responsible for the HGO sensitivity-shift remain unclear and warrant further study (Fig. 19C).

Whereas treatment-induced alterations in HGO preferentially affected sensitivity to insulin rather than responsiveness, alterations in GDR appeared to comprise shifts in both responsiveness and sensitivity to insulin. Control animals displayed a basal GDR of 12.6 ± 1.1 μmol/kg/min, nearly two-fold that of the treated group (7.1 ± 1.2 μmol/kg/min). Further, the P32/98-treated group displayed 2- and 3-fold responses to 5 and 15 mU/kg/min insulin, respectively (Fig. 20), whereas the control animals showed their first sign of peripheral insulin responsiveness (46%) only during the latter infusion step. These data suggest a marked left-shift in insulin-stimulated peripheral glucose uptake and are consistent with the demonstration of increased glucose uptake in soleus muscle (Fig. 20A; (Pospisilik et al. 2002), and with several reports of incretin-stimulated increases in glucose uptake (Wang et al. 1996, Yang et al. 1998).

Though somewhat controversial, both GIP and GLP-1 (and exendin-4) have been reported to increase muscle insulin sensitivity through the stimulation of glycogen synthesis and glucose uptake (Alcantara et al. 1997, Mizuno et al. 1997, O'Harte et al. 1998, Yang et al. 1998). Additionally, a number of whole animal studies using GLP-1 or related GLP-1 receptor agonists have observed similar improvements in glucose tolerance and insulin sensitivity. Young and associates showed that long-term administration of the GLP-1 agonist Exendin-4 causes glucose lowering, and insulin sensitizing effects in a number of diabetic animal models including the fa/fa Zucker rat (Young et al. 1999). Also, sub-chronic infusion of GLP-1 has been shown to elicit improvements in glycemic control, glucose tolerance and insulin sensitivity (Freyse et al. 1997, Rachman et al. 1997, Zander et al. 2002). Our findings are consistent with these previous investigations.

On that note, although the literature on DP IV-inhibition focuses primarily on a mechanism of action involving the enhancement of circulating active GIP and GLP-1, the role of other peptide substrates of DP IV in the improvements evidenced here, and previously, should not be discounted. Natural substrates of DP IV include all tested members of the glucagon/VIP superfamily of polypeptides (Lambeir et al. 2001), including glucagon (Hinke et al. 2000, Pospisilik et al. 2001), as well as a number of neuroendocrine and immune factors (Mentlein 1999). Many of these peptides play significant roles in the regulation of energy metabolism and are likely to have contributed towards the improvements associated with DP IV-inhibitor treatment. For instance, recent work by the group of Schuit and associates, has highlighted the importance of the counter-regulatory hormone glucagon in the maintenance of glucose competence of the β-cell and in proper insulin secretion (Huypens et al. 2000). Considering that blood glucose is maintained by circulating glucagon levels for approximately
two thirds of the 24-hour cycle, DP IV-inhibitor induced enhancement of N-terminally intact glucagon (active; glucagon\textsubscript{1-29}) is likely to contribute towards the reported improvements in β-cell function.

In addition to the examination of HGO and GDR \textit{in vivo}, an \textit{in vitro} examination of adipocyte insulin sensitivity was performed three days after the clamp. The VDF Zucker rat displays excessive fat accumulation and pronounced hyperlipidemia (including elevated free fatty acids; FFA), pathologies intimately associated with human type-2 diabetes. It has been suggested that over 50% of the insulin resistance in diabetic patients is FFA-induced, with elevated plasma FFA stimulating insulin secretion, peripheral glucose underutilization, and hepatic glucose overproduction (Boden 1997, McGarry 2002). Physiologically, these FFA effects likely serve to preserve glucose stores when supply is limited. However, in times of plenty (e.g. a typical western diet) these effects become counterproductive, inhibiting the utilization of glucose (Boden 1997, McGarry 2002). The demonstration that insulin-induced inhibition of lipolysis is sensitized in adipocytes from DP IV-inhibitor treated animals, suggests a potential mechanism of action for the improvements in hepatic and peripheral insulin sensitivity discussed above. A reduction in FFA release from adipocytes, secondary to a sensitized inhibition of lipolysis by insulin, might attenuate the glucose sparing effects of plasma FFA, thereby reducing the severity of insulin resistance. These findings warrant further investigation into the effects of DP IV-inhibition on lipid metabolism.

Reductions in insulin resistance, whether diet, exercise or drug induced, have been associated with a reduced risk for the development of both CAD and hypertension. Having convincingly demonstrated a DP IV-inhibitor mediated amelioration of the diabetic condition in the VDF rat, including an increase in insulin sensitivity, we examined the effect of the same treatment regimen on blood pressure. Using an indirect tail cuff method for measurement, systolic blood pressure (SBP) in the treated animals was shown to decrease slightly from \(~160\) mm Hg to \(~150\) mm Hg, a small but significant reduction that was maintained over the entire treatment course. In a follow-up investigation in SH rats, an identical P32/98-treatment regimen showed no effect on the progressive development of hypertension in these non-diabetic animals. Parallel assessments of glucose tolerance, both oral and intraperitoneal, indicated a lack of drug effect, suggesting that the observations made in the VDF rat were linked to the treatment-induced improvements in glucose homeostasis (glycemia and insulin sensitivity). Based on the preliminary nature of the data, however, no mechanistic conclusions can be made regarding these changes. Interestingly, a number of known DP IV substrates, including VIP, SP, GIP and GLP-1, have been implicated in the regulation of vasoactive tone in a number of vascular beds.

An important facet shared by the OGTT, the muscle glucose uptake and the pancreas perfusion protocols was that cessation of drug treatment occurred 18 hours prior to these experimental procedures. Any divergence between groups, therefore, reflected long-term, lasting changes in metabolic state, rather than an acute effect of the drug. Drug washout was confirmed by DP IV activity
measurements. Interestingly an increase in DP IV activity was observed in the treated animals over the course of the study, likely a compensatory response to chronic inhibition of DP IV activity in the treated group (>90% of the 24 hour cycle). The significance of this finding is not fully understood since the circulating, soluble form of DP IV measured in the present study, represents only 5-10% of the entire DP IV pool (Mentlein 1999). Therefore, further investigation is warranted into the effects of DP IV inhibitor therapy on other sources of DP IV activity in the circulation (particularly lymphocytic and endothelial DP IV). It is likely that the compensatory change in circulating DP IV levels could be avoided by once daily treatment and/or a lower inhibitor dose. Therapeutic doses required to improve glucose tolerance on an acute scale in humans (~0.2 mg/kg) are a hundred-fold lower than those used in the present study (Demuth et al. 2000, Glund et al. 2000).

Several therapeutic strategies exploiting the anti-diabetic effects of GIP and GLP-1 are currently being pursued. DP IV inhibition slows the rate of incretin inactivation but leaves the nutrient-dependent mechanism of incretin release intact, and the glucose dependence of their anti-diabetic actions unaltered. The present study shows for the first time that long-term DP IV-inhibitor therapy leads to enhanced peripheral insulin sensitivity and β-cell glucose and GIP responsiveness, improvements that culminate in markedly improved glucose tolerance well beyond the clearance time of the drug. Recently, Ahren and colleagues, in a study in early-stage type-2 diabetic humans, provided corroborative evidence for these findings, demonstrating improved glucose tolerance and reduced fasting plasma glucose in two groups of patients treated daily with the DP IV-inhibitor NVP DPP728 (Ahren et al. 2002). In conclusion, the findings of this study exemplify the importance of the non-insulinotropic effects of GIP and GLP-1 in the regulation of β-cell function. In conclusion, the data presented here establishes the potential utility of DP IV-inhibitors in the treatment of type-2 diabetes mellitus.

DP IV-INHIBITION IN THE STZ AND BB RAT: IMPLICATIONS FOR THE TREATMENT OF TYPE-1 DIABETES

Traditionally, the impetus for research into incretin physiology, and into DP IV-inhibitor-mediated enhancement of these endocrine axes, has been the potential of GIP and GLP-1 not only to stimulate insulin secretion, but also to enhance β-cell secretory function (glucose competency and insulin production), parameters altered in type-2 diabetes. And while the potential of exploiting the glucagonostatic actions of GLP-1 in the treatment of type-1 diabetes has also been investigated, research into the therapeutic potential of the incretins towards the autoimmune disorder has been minimal (Gutniak et al. 1992, Dupre et al. 1995, Creutzfeldt et al. 1996). Recent studies have added stimulation of β-cell growth and differentiation, and inhibition of β-cell apoptosis to a growing list of “anti-diabetic” incretin effects (Holz et al. 1993, Dachicourt et al. 1996, Buteau et al. 1999, Xu et al. 2002).
1999, Stoffers et al. 2000, Hui et al. 2001, Trumper et al. 2001). It was these latter findings that provided the initial rationale for an investigation into the effects of long-term DP IV-inhibition on glucose homeostasis and islet integrity in the STZ model of type-1 diabetes. Through the demonstration of β-cell protection, stimulation of islet neogenesis, and enhancement of overall glucose homeostasis, we have shown for the first time the potential for DP IV-inhibitors as a therapeutic strategy for the treatment of type-1 diabetes.

One hallmark of type-1 diabetes, in both humans and animal models is limited weight gain and low body mass index, direct consequences of insulin insufficiency. Lack of insulin-stimulated nutrient uptake stimulates hyperphagia, while hyperglycemia promotes polyuria and, consequently, polydipsia. Thus body weight, and food and water intake serve as indicators of proper glucose handling. In the current study, DP IV-inhibitor treatment resulted in partial reversal of STZ-induced changes in all three of these metabolic indicators, suggesting improvements in glucose homeostasis and nutrient uptake (Fig. 22). Increased weight gain, concomitant with reduced food intake in the P32/98-treated animals indicates improved anabolic (likely insulin-dependent) function, a notion supported by weekly morning glucose, insulin and fructosamine measurements (Fig. 23), OGTT data (Fig. 24), and pancreas perfusions (Fig. 25).

That said, it is important to note that all of these measures (weekly monitoring, OGTT, and pancreas perfusion) were all performed well after drug washout (~16 hours after the last dose of P32/98) and therefore represent cumulative effects of long-term DP IV-inhibition rather than the effects of acute incretin enhancement. The blunting and partial reversal of STZ-induced deviations in weekly glucose and fructosamine, and experimentally challenged blood glucose values indicate a marked improvement in oral glucose tolerance in the treated animals (Fig 23&24). Parallel blood glucose profiles during the OGTT, coupled with only minimal enhancement of glucose-responsiveness of insulin secretion (Figs. 24B inset & 25A inset), suggested that the improvements in glucose tolerance were secondary to enhanced total insulin-secretory capacity rather than enhanced insulin sensitivity (Fig. 24). The relatively limited enhancement of β-cell function (responsiveness) evidenced in both the OGTT (Fig. 24B inset) and the pancreas perfusion (Fig. 25A inset) was in contrast to a relatively profound improvement in glucose responsiveness found in the previous studies of long-term DP IV-inhibition in obese Zucker rats (Pospisilik et al. 2002). To further define the pancreatic effects of DP IV-inhibition in the STZ model, and to help provide a potential underpinning for the in vivo data, immunohistochemical analyses of pancreatic sections and in vitro cytoprotection studies were performed.

Long-term DP IV-inhibitor treatment was shown to both preserve (early treatment) and increase (early and late) β-cell number through an apparent stimulation of islet neogenesis, and β-cell regeneration (differentiation from precursor cells) and/or enhanced insulin biosynthesis (Fig. 26). Evidence for preservation of a population of β-cells, aside from the enhancement of β-cell precursor
differentiation, comes largely from the dichotomy of the effects of early vs. late treatment with P32/98. All indices of glucose handling examined showed a profound and immediate improvement in the early treatment group relative to the late treatment group (Figs. 22&23). Also, the substantial increases in β-cell number, pancreatic insulin content, and islet β-cell fraction in the early treatment group relative to late, provide strong evidence for a cytoprotective effect of DP IV-inhibitor treatment on β-cells (Figs. 25&26). The potential of the incretins as mediators of these improvements was highlighted by dose-dependent reversal of STZ-induced cell death in the INS-1 (832/13) β-cell line (Fig. 27). These data, which included suppression of aberrant caspase-3 and -8 activity, support an anti-apoptotic mechanism of action, however they do not exclude the potential contribution of incretin effects on cell-proliferation. Growth limiting conditions (serum free, low glucose media) were used, however, to minimize proliferation, a strategy that proved effective as evidenced by the lack of change in total cell number between STZ-exposed experimental groups (data not shown).

Protective effects aside, the immunohistochemical analysis showed evidence for what was likely either enhanced insulin biosynthesis or β-cell regeneration, as well as for islet neogenesis. Evidence for the latter included a significant increase in islets of the smallest size subsets in both DP IV-inhibitor treated groups (Fig. 26A). These islets were found to contain a near-normal β-cell fraction (60 – 85 %) and to be comprised almost exclusively of intensely stained, morphologically normal β-cells (IN

In contrast, larger islets contained a broad range of insulin-immunoreactive cells suggesting their presence during the STZ insult. The increase in number of very small, intensely stained islets in the treated groups is consistent with previous reports of GLP-1 stimulated β-cell differentiation, islet budding, and islet neogenesis (Perfetti et al. 2000, Stoffers et al. 2000, Tourrel et al. 2001).

What was the nature of the IN

bnght cells contained within the larger, more mature islets? Teitelman and colleagues showed, in two separate studies, the presence of a wave of intra-islet β-cell regeneration / differentiation that peaked within the first few days following high-dose STZ administration in CD-1 mice, and disappeared 7 to 30 days after exposure (Femandes et al. 1997, Guz et al. 2001). These intra-islet β-cell precursor cells were shown to be of PP-cell or δ-cell origin, staining positive for the pancreatic-duodenum homeobox-1 (PDX-1) and either pancreatic polypeptide or somatostatin (PDX-1+/PP+ and PDX-1+/SOM+ respectively). These PDX-1+/PP+/IN+ and PDX-1+/SOM+/IN+, β-cell precursors were found within 24 hours of exposure to high-dose STZ and were shown to be preferentially induced by normalization of hyperglycemia (Femandes et al. 1997, Guz et al. 2001). If the IN

bnght cells observed in the present study are the mature product of these adult β-cell precursors then it would seem as though DP IV-inhibitor treatment, likely through the enhancement of GIP and GLP-1 levels, enhanced the differentiation of intra-islet precursors and/or prolonged their survival in a severely hyperglycemic environment. GLP-1 has been shown to enhance PDX-1 expression in vitro,
and in vivo to stimulate differentiation of β-cell precursors within the islets of old glucose-intolerant rats (Perfetti et al. 2000). Additional data from the current study that support such a conclusion include: 1. a doubling of pancreatic insulin-content despite a minimal increase in insulin-IR cell number in the late treatment (versus STZ-control); 2. the finding that IN^bright cells were detected in the STZ-control animals, and that there was a 1.3-fold increase in islet β-cell fraction in larger islets (>50 cells in cross section) in the late treatment group versus the STZ controls (Fig. 26). Despite the strength of these findings, a confident description of the mechanisms underlying the increases in β-cell and islet number, and in pancreatic insulin content will require a detailed temporal examination of this model at various stages of treatment.

Notwithstanding the fact that GIP and GLP-1 are discussed as the primary mediators of protective and reparative processes reported above, there remain a number of endogenous substrates of DP IV whose enhancement might also play a role (Mentlein 1999). As mentioned before, glucagon (Hinke et al. 2000, Pospisilik et al. 2001), VIP (Lambeir et al. 2001) and PACAP (Lambeir et al. 2001) have all been shown to be relatively good substrates for DP IV and to play significant roles in the regulation of β-cell function and development (Inagaki et al. 1996, Ahren 2000, Jamen et al. 2000, Zhu et al. 2003). The contribution of these and other peptides towards the present findings is very likely to be significant. In addition to non-incretin substrate involvement, effects of DP IV-inhibitors on immune function (including clearance of apoptotic β-cells) cannot be ruled out as contributing factors towards our findings. DP IV-inhibitors have been shown to suppress a number of T-cell, B-cell and NK cell specific immune functions (De Meester et al. 1999).

Though the functional role of DP IV in the immune system has yet to be clearly elucidated, its definitive involvement makes the investigation of DP IV-inhibition in type-1 diabetes intriguing. The potential for combined immunosuppressive and incretin-enhancing effects is a unique therapeutic paradigm. Having described the stimulatory effects of DP IV inhibitors on β-cell survival and regeneration, and on overall glucose tolerance in a type-1 model that highlights insulin deficiency, β-cell apoptosis and β-cell regeneration (the STZ rat), we sought to investigate the same in an autoimmune model of the disease. Long-term treatment of diabetes-prone BB rats from the age of 3 weeks (the postulated outset of the autoimmune progression), led not only to a delay in diabetes incidence (~10 days) but also to a reduction in incidence i.e. the treatment prevented the disease in a subset of the diabetes prone animals. The amelioration of metabolic dysfunction evidenced in both the diabetic and non-diabetic cohorts are consistent with prolonged incretin enhancement through DP IV-inhibition in type-2 diabetes (Pospisilik et al. 2002), though they do not preclude the involvement of the hypothesized immunosuppressive effects of DP IV-inhibitors.

Attempts at identifying the mechanisms underlying the DP IV-inhibitor mediated changes in susceptibility towards, and severity of the diabetic syndrome of the BB rat are certainly beyond the
scope of this preliminary study. Though the involvement of both incretin and immunosuppression mechanisms is likely, the data would suggest that any immunosuppressive effect of DP IV-inhibition (by P32/98 at the given dose) was likely minimal. Considering the findings of previous studies using DP IV-inhibitors in experimental autoimmune disorders a greater delay and reduction in incidence would have been expected. Additionally, the lack of effect of the treatment on pre-diabetic insulitis, though crude in measure, does not provide support for an immunosuppressive mechanism. Through a mechanism associated with transforming growth factor (TGF)-β1 upregulation, Steinbrecher and colleagues showed a marked reduction in clinical severity and a greater than 30 day delay in average onset in experimental autoimmune encephalitis in mice treated only five times with the DP IV inhibitor Lys[Z(NO$_2$)]-pyrrolidide (Steinbrecher et al. 2001). Similarly impressive findings have been achieved in a chemically-induced model of arthritis, in stress-induced abortion (a T-cell mediated process), and in allograft rejection of cardiac transplants (Korom et al. 1997, Tanaka et al. 1997, Ruter et al. 2002). Indeed, the juxtaposition of these findings indicates that the subtleties both of the autoimmune etiologies of these disorders and of the relationship between DP IV-inhibition and CD26-signal stimulation require further elucidation. The idea that further developments in this arena may allow specific targeting of these related actions remains attractive and certainly warrants further study.

**SUMMARY AND CONCLUSIONS**

Since the initiation of this thesis investigation, all members of the glucagon superfamily of polypeptides have been shown to be substrates of DP IV in vitro, and further examination in vivo has established the physiologically relevance of this enzymatic regulatory module for many. The current study shows clearly that DP IV is a primary enzyme involved in the degradation and inactivation of glucagon. While counterregulatory studies revealed an important role for DP IV in the inactivation of glucagon under physiological conditions, bioassays of the synthetic degradation products, shown to lack any hyperglycemic activity both in vitro and in vivo, have left the physiological role of these truncated peptides unknown. The compiled results comprise a major step in the characterization of glucagon metabolism and thus contribute towards our understanding of diseases involving abnormal glucose counterregulation. The fairly promiscuous substrate specificity of DP IV supports a housekeeping role for the enzyme. Shared substrate properties such as those for the glucagon superfamily allow inactivation of overlapping endocrine signals by a single enzyme, an efficient means to effect rapid secretory control of endocrine signaling, and represent a unique pharmacological target for the treatment of several metabolic disorders. Indeed, it is this idea of rapid, broad-scope endocrine inactivation effecting rapid secretory regulation of hormone activity that reconciles the apparently counterintuitive physiological circumstance where DP IV serves as a metabolic mechanism for both glucagon and the potent insulin secretagogues GIP and GLP-1.
The success of DP IV-inhibitors as a therapeutic strategy in the treatment of diabetes is owed in great part to the pleiotropic nature of its primary effectors, the incretins GIP and GLP-1. Previously believed to be mere enhancers of insulin secretion, the incretins have been shown to possess numerous non-insulin dependent functions including the stimulation of β-cell growth, survival and function as well as modulation of peripheral energy disposal (liver, muscle and fat) (Alcantara et al. 1997). The findings reported in this thesis extend these concepts and further exemplify the importance of the non-insulinotropic effects of GIP and GLP-1 in the regulation of glucose homeostasis. The addition of improved β-cell function (glucose-competence and incretin effect), and hepatic and peripheral insulin sensitivity to the list of beneficial metabolic effects of long-term DP IV-inhibitor therapy provides strong support for the use of these compounds in the treatment of type-2 diabetes.

Further, the demonstration that DP IV-inhibition stimulates islet neogenesis and β-cell regeneration, increases pancreatic insulin content, and significantly improves overall glucose tolerance in a model of type-1 diabetes sets the foundation for the examination of the applicability of DP IV-inhibitors in the treatment of insulin-dependent diabetes. The added immunomodulatory potential of DP IV-inhibitors purports a unique therapeutic paradigm for the treatment of type-1 diabetes combining immunosuppressive effects with β-cell protection (anti-apoptosis) and functional enhancement. Is there a possibility that DP IV-inhibition could fill the void for an inexpensive, benign, preventative therapy for type-1 diabetes? Data from the BB rat suggests that the possibility is real.
REFERENCES


Larsen, J. J. Jallad, and P. Dansbo. 1996. One week continuous infusion of GLP-1 (7-37) improves glycemic control in NIDDM. *Diabetes* 45: 233A (abstr.).


