INVESTIGATION OF THE EFFECT OF $IN\ VIVO$ INHIBITION OF DIPEPTIDYL PEPTIDASE IV (DP IV) ON GLUCOSE TOLERANCE IN ZUCKER RATS AND OF DP IV EXPRESSION IN PANCREATIC BETA CELLS

by

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Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are two hormones released from the gut post-prandially which act at the pancreatic \( \beta \) cell to potentiate glucose-induced insulin secretion. DP IV is the primary enzyme responsible for the cleavage and inactivation of the incretins and it is known that inhibition of the enzyme by the specific inhibitor Isoleucine-thiazolidide (Ile-thiazolidide), prevents degradation of GIP and GLP-1. It was hypothesised that inhibition of the enzyme would prolong the biological half-lives of the incretins, thereby enhancing insulin release post-prandially and improving glucose tolerance. In addition to the circulation, a site where a portion of this proteolysis occurs may be the \( \beta \)-cell, the incretin target tissue. DP IV has been previously localised to the pancreatic islets and specifically to the \( \alpha \)-cells, and its presence has yet to be demonstrated in the \( \beta \)-cell. Using conscious, unrestrained Zucker rats (n=6) Ile-thiazolidide was administered (20 \( \mu \)mol) simultaneously with an oral glucose challenge (OGTT) and resulted in rapid and sustained inhibition of DP IV activity in obese and lean animals. The integrated insulin response in both phenotypes was significantly different between the inhibitor-treated and control groups (obese, 1696.6 \( \pm \) 80.1 vs. 725.5 \( \pm \) 88.3 \( \mu \)U, respectively; lean, 2324.1 \( \pm \) 177.4 vs. 1890.8 \( \pm \) 67.9 \( \mu \)U, respectively). The integrated glucose response to an OGTT was reduced by 39% in inhibitor-treated obese animals versus 22% in treated lean rats. Pre-treatment of animals with Ile-thiazolidide, Pro-Ile-thiazolidide or Gly-Pro-Ile-thiazolidide prior to OGTT significantly inhibited DP IV activity as before and resulted in a more rapid insulin response compared to the previous protocol. Gly-Pro-Ile-
thiazolidide demonstrated the greatest effect on insulin secretion with a peak of 218.0 ± 22.5 μU/ml. Pro-Ile-thiazolidide and Gly-Pro-Ile-thiazolidide improved glucose tolerance in obese rats while Ile-thiazolidide did not. Due to a possible "priming" effect on the enteroinsular axis by pre-treatment with inhibitor, this protocol was of greater value in improving glucose handling in the obese Zucker rat, a model of NIDDM. In an attempt to localise DP IV to the β cell, pancreata from lean and obese Zucker rats, and DP IV-negative rats were fixed in paraformaldehyde and cryosectioned (20 μm). Sections were double stained for insulin and DP IV and visualised using fluorescence microscopy. Double labelling was observed in both phenotypes with no difference between the two. These results indicate that DP IV is expressed in the pancreatic β-cell and may be co-secreted with insulin where it could cleave and inactivate the incretin hormones at their target cell.
Table of Contents

Abstract...........................................................................................................................................ii

Table of Contents .................................................................................................................................iv

List of Figures ........................................................................................................................................vi

List of Tables .........................................................................................................................................vi

Acknowledgements ...............................................................................................................................vii

Preface .....................................................................................................................................................viii

INTRODUCTION .......................................................................................................................................1

THE INCRETIN CONCEPT ......................................................................................................................1

♦ Historical Aspects...............................................................................................................................1
♦ Discovery and Distribution ..................................................................................................................2
♦ Secretion ..............................................................................................................................................5
♦ Actions at Pancreatic Islets ..................................................................................................................8
♦ Extrapancreatic Actions .......................................................................................................................10
♦ Role in Non-Insulin Dependent Diabetes Mellitus ...........................................................................14

DIPEPTIDYL PEPTIDASE IV ..................................................................................................................19

♦ Characterisation ................................................................................................................................19
♦ Enzymatic Considerations ..................................................................................................................21
♦ Mechanism of Action ..........................................................................................................................23
♦ Inhibition of DP IV .............................................................................................................................23
♦ Biological Role .....................................................................................................................................26

THE OBESE (fa/fä) ZUCKER RAT ........................................................................................................30

THESIS INVESTIGATION .......................................................................................................................31

CHAPTER 1: EFFECT OF ORALLY ADMINISTERED DP IV
INHIBITORS ON GLUCOSE TOLERANCE IN ZUCKER RATS..........................................................33

PROJECT RATIONALE ..........................................................................................................................33

MATERIALS AND METHODS ...............................................................................................................33

♦ Animals..............................................................................................................................................33
CHAPTER 2: DP IV EXPRESSION ON PANCREATIC β CELLS

PROJECT RATIONALE

MATERIALS AND METHODS

RESULTS

DISCUSSION

Future Directions

Summary

REFERENCES
List of Figures

Figure 1. Proposed steps in the development of NIDDM ........................................... 15
Figure 2. Effect of oral Ile-thiazolidide on DP IV activity, and plasma insulin and glucose in response to OGTT in Wistar rats ................................................................. 37
Figure 3. Plasma DP IV activity in response to oral Ile-thiazolidide in Zucker rats .......................................................................................................................... 38
Figure 4. Effect of oral Ile-thiazolidide on plasma insulin and glucose in response to OGTT in obese and lean Zucker rats ......................................................... 39
Figure 5. Effect of oral Ile-thiazolidide alone on plasma insulin and glucose in obese and lean Zucker rats ................................................................. 41
Figure 6. Time course of DP IV inhibition by Ile-thiazolidide ......................................... 42
Figure 7. Effect of oral Ile-thiazolidide administered prior to OGTT on plasma insulin and glucose in obese and lean Zucker rats ........................................ 44
Figure 8. Effect of oral Pro-Ile-thiazolidide administered prior to OGTT on plasma insulin and glucose in obese and lean Zucker rats ............................... 46
Figure 9. Effect of oral Gly-Pro-Ile-thiazolidide administered prior to OGTT on plasma insulin and glucose in obese and lean Zucker rats ............................... 48
Figure 10. Comparison of the effects of Ile-thiazolidide, Pro-Ile-thiazolidide and Gly-Pro-Ile-thiazolidide on DP IV activity, and plasma insulin and glucose in obese Zucker rats ................................................................. 50
Figure 11. Co-localisation of DP IV and insulin within the same islet of a Zucker rat .... 59
Figure 12. Co-localisation of DP IV and insulin within the same islet of a Zucker rat .... 61
Figure 13. Co-localisation of DP IV and insulin within the same islet of a DP IV-negative rat ............................................................................................................. 62

List of Tables

Table 1. Details of primary and secondary antibodies ......................................................... 58
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Preface

The generous gift of three specific DP IV inhibitors by Dr. Hans-Ulrich Demuth enabled the study presented in Chapter 1 of this thesis to be performed. Results from specific experiments using Isoleucine-thiazolidide in Zucker rats have been published as:

INTRODUCTION

THE INCRETIN CONCEPT

Historical Aspects

Unger and Eisentraut (1969) originally introduced the term enteroinsular axis "...to designate a proposed regulatory system in which the secretion of pancreatic islet hormones is under the partial influence of hormones of the gastrointestinal tract." Later, this definition was amended to include neural factors and direct substrate stimulation (Creutzfeldt 1979). For decades prior however, it was believed that absorbed nutrients could account for the insulin secretory response to a meal. Two separate studies showed that glucose acted directly to affect secretion of insulin (Metz 1960) and glucagon (Unger et al. 1961). The hypothesis that an unidentified intestinal factor could affect glucose levels was extensively investigated in the 1920's and 30's (Creutzfeldt 1979) but the possibility that the observed hypoglycaemia was a result of insulin stimulation could not be studied until the development of the insulin radioimmunoassay (RIA) (Yalow and Berson 1960). Investigations using bioassay (Dupré and Beck 1966) and immunoassay techniques (Elrick et al. 1964) demonstrated that the pancreatic response to glucose could not completely account for the resulting blood insulin levels following glucose ingestion. A study by McIntyre and colleagues (1964) reported a greater plasma insulin response, lower blood glucose levels and increased rate of glucose clearance from the blood following intrajejunal administration of glucose to healthy subjects as compared to the intravenous (i.v.) route. The relative contribution of these unidentified gastrointestinal hormones was quantified by Perley and Kipnis (1967) in an experiment
comparing the insulin response to oral and i.v. glucose loads in normal and obese, diabetic and non-diabetic subjects. They estimated that at least 50 % of the insulin response was stimulated by gut hormones, a finding which lent significant support to establishing the enteroinsular axis as a physiological link between the gut and endocrine pancreas.

In 1929 Zunz and LaBarre introduced the term "incretin" to describe the hormonal activity derived from the gut which may potentiate insulin secretion from the pancreas. This definition was later refined to include only those factors which are released by nutrients, particularly glucose, and demonstrate glucose-dependent insulin secretion (Creutzfeldt 1979). The latter criterion is of physiological importance in preventing inappropriate hypoglycaemia, that is, under normal circumstances incretins potentiate insulin secretion only when glucose levels are elevated above a given threshold level. Though a multitude of gut hormones have been investigated for incretin activity, to date only two hormones have been classified as true incretins, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (reviewed by Fehmann et al. 1995a).

**Discovery and Distribution**

GIP was initially identified by Brown and Pederson (1970a) based on its enterogastrone effects in a study wherein partially purified preparations of cholecystokinin (CCK), 10 % and 40 % pure, were compared for their effect on gastric acid secretion. Using a Bickel pouch of the parietal region of the canine stomach it was shown that i.v. infusion of the 40 % pure preparation yielded greater stimulation of acid secretion than did the 10 % pure preparation. The investigators concluded that an acid inhibitory factor may have been removed during CCK purification or a stimulating factor concentrated. The former
hypothesis was further investigated with results demonstrating that during pentagastrin-stimulated acid secretion from the dog stomach the 10 % pure preparation was again a more potent inhibitor of acid release than the more pure preparation (Pederson 1971). Isolation and purification of this acid inhibitor yielded a hormone which was termed gastric inhibitory polypeptide (Brown et al. 1969; Brown et al. 1970b). Subsequent investigations revealed the insulinotropic activity of the hormone and established it as its main physiological action leading to the alternate name, glucose-dependent insulinotropic polypeptide.

Using immunohistochemical techniques (Polak et al. 1973) and RIA (O’Dorisio et al. 1976) studies have shown GIP to be localised to the upper small intestine, primarily in the duodenum and to a lesser extent the jejunum, in dog, pig and man. In these regions the GIP-secreting cell was reported to be interspersed with several other types of endocrine cells including S (secretin) and L (enteroglucagon) cells (Polak et al. 1973). Buchan and colleagues (1978) later identified the K cell as that responsible for release of GIP though it has been demonstrated that the cellular ultrastructure differs slightly between species (Usellini et al. 1984).

While the discovery of GIP employed classical physiological techniques, GLP-1 was identified by molecular analysis of the biosynthesis of glucagon. Patzelt and Schug (1981) reported that a molecule which they termed the major proglucagon fragment (MPGF), was synthesised in parallel with glucagon in the pancreatic islet. Two subsequent studies elucidated the structure of glucagon mRNA as well as the glucagon gene and demonstrated that posttranslational processing of proglucagon produced MPGF which was shown to contain two glucagon-like substances, GLP-1 and GLP-2 (Bell et al. 1983a; Bell et al. 1983b). However, based on tissue expression, MPGF and more generally proglucagon,
undergo differential cleavage. In the pancreas, MPGF along with glucagon and glicentin-related pancreatic peptide are the predominant products though small but significant levels of GLP-1\textsubscript{1-37}, GLP-1\textsubscript{7-37} and GLP-1\textsubscript{7-36} amide have been reported (Ørskov \textit{et al.} 1987; Suda \textit{et al.} 1989; Mojsov \textit{et al.} 1990; Holst \textit{et al.} 1994). Conversely, enteric processing of proglucagon primarily generates glicentin and three peptides derived from MPGF: GLP-1, GLP-2 and intervening peptide with the naturally occurring form of GLP-1 being 7-36 amide (hereafter, GLP-1\textsubscript{7-36} amide will be referred to as GLP-1) (Kreymann \textit{et al.} 1988; Ørskov \textit{et al.} 1989; Suda \textit{et al.} 1989; Mojsov \textit{et al.} 1990). Detectable only in intestinal L cells and not in pancreatic α cells (Scopsi \textit{et al.} 1995) prohormone convertase PC3 has been reported to be the enzyme responsible for the conversion of MPGF to GLP-1\textsubscript{1-37} and cleavage to GLP-1\textsubscript{7-36} amide (Rouille \textit{et al.} 1997).

Immunoreactive GLP-1 has been localised to the pancreas and intestinal tract in all species studied, though in the pancreas co-localisation of this hormone with glucagon in the electron-dense core of the α cell is presumably due to cross-reaction with MPGF (Varndell \textit{et al.} 1985; Vaillant and Lund 1986; Eissele \textit{et al.} 1992). In the intestine from which active GLP-1\textsubscript{7-36} is secreted, immunohistochemical staining was observed in the L cell which show homogenous granule morphology. In pig and human, cell density was reported to increase from the proximal to distal small intestine and colon reaching a maximum in the rectum (Varndell \textit{et al.} 1985; Eissele \textit{et al.} 1992; Deacon \textit{et al.} 1995a) whereas rat demonstrated peak cell density in the ileum (Eissele \textit{et al.} 1992).
**Secretion**

Inherent in the classification as an incretin is the ability of a peptide to potentiate glucose-induced insulin secretion. It follows that subsequent to ingestion of a glucose load or meal, the incretin is released and its insulinotropic action is expressed. In the case of GIP, the reported basal and post-prandial levels in human vary considerably from 60-460 pM and 170-1470 pM, respectively, though the increase in GIP relative to basal was comparable (reviewed by Pederson 1994). Differences in the recognition ability of the antisera used in the GIP RIA is thought to account for some of the observed variability (Jorde et al. 1983a). Despite these inconsistent results it is widely accepted that carbohydrates and fat act as major secretagogues of this incretin.

Several early studies established that orally administered glucose caused a significant increase in circulating levels of GIP (Cataland et al. 1974; Pederson et al. 1975; Falko et al. 1980; Williams et al. 1981). Portal venous levels of GIP markedly increased 2 min after glucose ingestion while the insulin concentration rose after 5 min suggesting a causal link between oral glucose loading, GIP and insulin secretion (Cataland et al. 1974). Sykes et al. (1980) further characterised the ability of various monosaccharides and disaccharides to stimulate the release of GIP and reported glucose, sucrose and maltose as effective secretagogues with galactose less so. Fructose, mannose and lactose did not stimulate GIP compared to controls. This group concluded that transport of glucose from the gut was occurring via a sodium-dependent hexose transporter given that addition of phloridzin, an inhibitor of this process, was able to abolish both the absorption of glucose and the resultant GIP response.
The efficacy of fat, particularly long-chain triglycerides, in releasing GIP was also investigated and found to be greater and more prolonged than that of glucose (Falko et al. 1975; Pederson et al. 1975; Krarup et al. 1985) while medium-chain triglycerides were shown to be weak stimulants (O'Dorisio et al. 1976). Clinically, post-prandial GIP levels were recently reported to be elevated in patients with hypertriglyceridemia (Gama et al. 1997). The question whether triglycerides or their hydrolytic products caused secretion of this incretin was studied in patients who demonstrate defective fat lypolysis (Ross and Shaffer 1981) and in pancreatectomised dogs (Ohneda et al. 1983). Both groups reported the absence of a GIP response following fat loading compared to controls whereas administration of pancreatic enzymes elicited significant increases in plasma GIP levels. The former group carried out an additional experiment to show that long-chain fatty acids increased GIP secretion 4-fold over basal, supporting the hypothesis that the products of hydrolysis are primarily responsible for fat-induced GIP release. As an emulsifier of fat entering the duodenum, bile has also been demonstrated to have a role in potentiating GIP secretion in response to fat ingestion (Yoshidome et al. 1995). Additionally, inhibition of chylomicron formation in gut cells by administration of a surfactant was found to completely block GIP release by fat (Creutzfeldt and Ebert 1985).

Studies of the modulation of GIP release by amino acids have been contentious. Mixed amino acids including arginine, histidine, isoleucine, leucine, lysine and threonine have been reported to stimulate (O'Dorisio et al. 1976; Herrmann et al. 1995) or have no effect (Williams et al. 1981) on release of the peptide. Mixtures of methionine, phenylalanine, tryptophan and valine have generated no such response (Thomas et al. 1978).
As with GIP, reported levels of circulating GLP-1 have varied considerably though basal and postprandial values generally lie within the low picomolar range (reviewed by Fehmann et al. 1995a). The stimulants of GLP-1 secretion from the intestine are much the same as those for GIP though there have been conflicting results regarding the role of glucose. Studies in human and perfused rat colon have reported rapid release of the incretin following administration of a glucose solution (Kreymann et al. 1987; D'Alessio et al. 1993; Plaisancié et al. 1995). Others, using administration to the rat intestinal lumen (Roberge and Brubaker 1991) and cultured canine L cells (Damholt et al. 1998) have shown glucose not to directly increase levels of GLP-1. A study of the structure-activity of sugars infused into the terminal ileum of dogs demonstrated glucose, galactose, maltose and sucrose, among others, to secrete the incretin while fructose, fucose, mannose, xylose and lactose had no such effect (Shima et al. 1990).

Unlike glucose, oral fat administration has consistently been demonstrated to rapidly stimulate GLP-1 and prolong its release as compared to oral glucose loads (Roberge and Brubaker 1991; D'Alessio et al. 1993; Knapper et al. 1995a). Some investigators have reported a small but significant secretory effect of mixed amino acids (Göke et al. 1993; Hermann et al. 1995) while others have not observed this effect (D'Alessio et al. 1993; Plaisancié et al. 1995).

Release of GLP-1 has also been found to be partially controlled by intestinal regulatory peptides such as gastrin releasing peptide (GRP), calcitonin gene-related peptide (CGRP), and GIP, all of which had a stimulatory role in rat (Plaisancié et al. 1994; Brubaker 1991), while somatostatin (Plaisancié et al. 1994; Brubaker 1991) and galanin (Herrmann-Rinke et al. 1996) were reported to inhibit GLP-1 secretion. Furthermore, cholinergic
agonists have also been found to cause incretin release (Plaisancie et al. 1994; Brubaker 1991; Herrmann-Rinke et al. 1995).

The observation that GLP-1 is detectable in the circulation just minutes after ingestion of a meal and the fact that virtually all nutrients are absorbed by the time the bolus reaches the distal small intestine, has given rise to speculation of a feed-forward mechanism whereby GLP-1 is stimulated by some previously released factor. Roberge and Brubaker (1991) first proposed this hypothesis after observing that the GLP-1 responses to duodenal and ileal fat in rat were qualitatively and quantitatively identical despite the discrepancy of L cell distribution between these two tissues. It has since been suggested that GIP plays a role in this duodenal-ileal axis given that it is released postprandially and has been shown to significantly stimulate GLP-1 secretion in rat. However, infusion of exogenous GIP in human has not been shown to increase GLP-1 release (Nauck et al. 1993). It is apparent therefore that further study is required as conclusions about GLP-1 secretion in human can not be drawn based upon observations made from animal models.

**Actions on Pancreatic Islets**

Using the CCK preparations previously investigated by Brown and Pederson (1970a), Rabinovitch and Dupré (1974) reported that the 10% pure preparation was a more effective insulinotrope than the 40% pure preparation. In a subsequent study utilising slightly less GIP as in the 10% pure preparation, Dupré and co-workers (1973) showed in human that an i.v. solution of peptide and glucose stimulated insulin release to a greater extent than i.v. glucose alone, thus confirming GIP as an insulinotropic hormone. Further study by Andersen et al. (1978) using a glucose clamp technique conclusively demonstrated a role for endogenous
GIP in insulin release following oral glucose in human. These data were supported by Pederson et al. (1975) who demonstrated in dog a greater integrated insulin response to oral as compared to i.v. glucose, whereas when GIP was added to the latter, the insulin response was not different than that for the oral load.

It was apparent that GIP activity was glucose dependent, an observation quantified in man (Elahi et al. 1979) and the perfused rat pancreas (Pederson and Brown 1976). The former study reported that concomitant infusion of glucose 7.9 mmol/l above basal and GIP markedly elevated circulating insulin while GIP and glucose 3 mmol/l above basal, or GIP alone, showed little or no insulin effect, respectively. Experiments in the perfused pancreas have demonstrated glucose thresholds of 4.5 mmol/l (Jia et al. 1995a) and 5.5 mmol/l (Pederson and Brown 1976).

The action of GIP on the pancreatic α cell have proven less clear. The peptide has been reported as showing no glucagonotropic effects in human (Elahi et al. 1979), dog or the perfused porcine pancreas (Lauritsen 1983). Pederson and Brown (1978) however, demonstrated a stimulatory effect of GIP in the perfused rat pancreas at glucose concentrations less than 5.5 mM, and Opara and Go (1991) observed that in isolated perifused murine islets GIP was able to reverse the suppressive action of high glucose concentrations on glucagon secretion.

At the δ cell Schmid et al. (1990) demonstrated GIP to only weakly stimulate somatostatin release.

As with GIP, GLP-1 was found early on to have insulinotropic activity following a meal or glucose load. One of the first investigators reported a glucose-dependent increase in insulin release with addition of the peptide (Schmidt et al. 1985), a result later supported by
Holst and colleagues (1987) who showed GLP-1, in a dose-dependent manner, to stimulate insulin secretion from the perfused porcine pancreas. Similar results have been reported in the perfused rat pancreas (Ørskov et al. 1988; Komatsu et al. 1989), conscious dog (Kawai et al. 1990), sheep (Faulkner and Pollock 1991) and man (Kreymann et al. 1987). Nathan et al. (1992) reported in human an attenuation of GLP-1 action at a glucose level of 5.0 mM and complete suppression of insulintropic activity below 2.8 mM.

All in vivo models studied to date have demonstrated GLP-1 to inhibit glucagon release (Ørskov et al. 1988; Komatsu et al. 1989; Fehmann et al. 1995b; Freyse et al. 1997) while other investigators using in vitro techniques have observed no effect (D’Alessio et al. 1989) or glucagonotropic action (Ding et al. 1997).

These conflicting reports may be partially accounted for by the stimulatory action of GLP-1 on the δ cell, a result consistently demonstrated in both in vitro (Fehmann et al. 1995b; Heller and Aponte 1995) and in vivo preparations (Schmidt et al. 1985; Ørskov et al. 1988). The inhibitory paracrine action of somatostatin in the intact islet has been well established and is thought to have a significant role in indirectly decreasing glucagon secretion in the presence of GLP-1.

**Extrapancreatic Actions**

Though much investigation has focused on the insulintropic action of GIP and GLP-1 these two hormones are known to have significant activity at other sites primarily adipose tissue, the liver and stomach. There is an extensive and sometimes conflicting literature regarding the lipolytic and lipogenic properties of GIP. Dupré and co-workers (1976) showed GIP to reduce glucagon-induced lipolysis in rat adipocytes by acting at the glucagon receptor
and inhibiting cAMP production. This antilipolytic effect was supported by subsequent studies which demonstrated a significant increase in lipoprotein lipase activity with the addition of GIP, and an even greater response when GIP and insulin were added together (Eckel et al. 1979; Knapper et al. 1995b). Furthermore, incubation of rat adipocytes with GIP has been demonstrated to enhance cellular sensitivity to insulin by increasing both receptor affinity and glucose uptake (Starich et al. 1985). In contrast GIP has also been reported to have some lipolytic action which is readily reversible in the presence of insulin as was shown by Beck and Max (1983). It was observed that 2 ng/ml of GIP significantly decreased fatty acid incorporation into adipose tissue by 9%, while the addition of 100 μU/ml of insulin with GIP increased uptake to 113.2% above basal. An inhibitory effect of insulin on GIP-induced glycerol release from adipocytes has similarly been shown (McIntosh et al. unpublished observations). Other studies have reported that while cAMP concentration and glycerol release are increased above basal in the presence of GIP alone, the peptide in combination with glucagon, isoproteronol or to a lesser extent secretin, markedly reduces glycerol liberation and concomitantly, cAMP levels (Ebert and Creutzfeldt 1987; Hauner et al. 1988). It seems probable that the physiological role of GIP in adipose tissue is a lipogenic one postprandially. However, given the potency of fat as a secretagogue, in the unstimulated preprandial state, this hormone may contribute to lipolysis to maximally stimulate its own release and further potentiate insulin secretion after a meal, following which the anabolic actions of GIP predominate (McIntosh et al. unpublished observations).

In the perfused rat liver GIP only slightly decreased glucagon stimulated glycogenolysis whereas with the additional presence of insulin at a concentration insufficient to inhibit glucagon on its own, inhibition of glucose output was significantly reduced
(Hartmann et al. 1986). This is in agreement with findings in dog by Andersen and co-workers (1984).

Though first identified on the basis of its inhibitory effect on the acid secretion from the denervated canine stomach (Brown and Pederson 1970a), at physiological doses in normal man GIP has been reported to have very little enterogastrone effect on pentagstrin-stimulated acid secretion (Maxwell et al. 1980; Nauck et al. 1992). It has since been suggested that GIP acts by stimulating release of a mediator, likely somatostatin, and thereby indirectly inhibits acid release (reviewed by McIntosh 1995). This would explain the enhanced enterogastrone activity of GIP on the denervated stomach given that vagal input decreases somatostatin release.

In a recent study by O'Harte and colleagues (1998) it was demonstrated that GIP increased glucose uptake and oxidation as well as glycogen and lactate production in mouse abdominal muscle; all results were significantly reduced by glycation of GIP.

As there have been conflicting reports regarding GIP and fat metabolism, so too is the case with GLP-1. A study by Oben et al. (1991) observed the stimulatory effect of this hormone on acetate incorporation into fatty acids at physiological concentrations using explants of rat adipose tissue. A supporting study by Perea and group (1997) noted an increase in glucose uptake in isolated rat adipocytes, and increased glycogen synthesis, glucose oxidation and lipogenesis in fat explants in the presence of 0.1-10 nM GLP-1, though this was significantly less potent than 1 nM insulin. Conversely, at a dose of 1 μM GLP-1 significantly increased cAMP production and glycerol release from isolated adipocytes however, at the same concentration glucagon was more than twice as effective (Ruiz-Grande

An investigation studying the effect of GLP-1 on isolated rat hepatocytes showed increases in glycogenesis and glycogen synthase \( a \) activity both of which were abolished with the addition of glucagon (Valverde et al. 1994; Villanueva-Penacarrillo et al. 1994). It has been postulated that GLP-1 affects hepatic glucose metabolism indirectly through its action on pancreatic hormones (Larsson et al. 1997). This was supported by a rat liver perfusion study wherein 10 nM GLP-1 had no effect on glucose, cAMP or ketone body output whereas the same dose of glucagon demonstrated significant stimulation (Murayama et al. 1990).

In the perfused rat stomach GLP-1 has been found to stimulate somatostatin and inhibit gastrin release (Eissle et al. 1990) whereas no such effects were observed in the pig (Ørskov et al. 1988). Conflicting results for gastric acid secretion have also been reported in human (Nauck et al. 1992; Wettergren et al. 1997) as well as for the role of neural mediation on GLP-1 inhibited acid release (Eissle et al. 1990; Wettergren et al. 1997). With regard to gastric motility, GLP-1 has been reported to cause a significant decrease in human (Willms et al. 1996; Nauck et al. 1997), an observation shown to be antagonised by GLP-1_{9-36}, the primary metabolite of GLP-1 degradation (Wettergren et al. 1998).

As with GIP, GLP-1 has recently been implicated in glucose uptake and oxidation, glycogenesis and lactate production in isolated mouse abdominal muscle, again a result inhibited with glycation of the peptide (O'Harte et al. 1997). GLP-1 may also have a role in central satiety given the observations of Turton and colleagues (1996) where intracerebroventricular injection of GLP-1 significantly inhibited feeding in fasted rats and injection of the antagonist GLP-1_{9-36} inhibited this response.
Role in Non-Insulin Dependent Diabetes Mellitus

Despite an extensive literature and intensive investigation non-insulin dependent diabetes mellitus (NIDDM, Type 2 diabetes mellitus) continues to be poorly understood particularly with regard to its etiology. Genetic susceptibility is thought to have an important role in disease development given the increased concordance rates for NIDDM in identical twins compared to fraternal twins or other siblings. Additionally, selected ethnic groups such as the Pima Indians, Nauruans and Asian Indians show an increased incidence of NIDDM compared to other populations, and though NIDDM has been found to be associated with different genetic markers depending on the patients' ethnicity, no actual genetic linkage has been reported (Bennett 1996). Several other factors contribute to the etiology of NIDDM including increasing age, lack of physical activity, a diet high in fat, and obesity.

NIDDM is generally predicted by the presence of fasting and stimulated hyperinsulinemia which in the initial stages of the disease, generally results in normal glucose tolerance. Another characteristic of NIDDM is resistance of the peripheral tissues to the actions of insulin though it remains unclear whether this resistance is a cause or effect of the hyperinsulinemic state. In rare cases a mutation in the insulin receptor or mutations of the glucokinase gene may underlie the diabetic state, however in the vast majority of patients the genetic basis for this disease remains elusive (Bennett 1996). Increased insulin resistance eventually leads to impaired glucose tolerance and chronic hyperglycemia which in turn perpetuates the hyperinsulinemia. Following development of dysfunctional glucose handling the β cell demonstrates decreased responsiveness to various secretagogues including glucose, such that the insulin response to nutrients or a glucose challenge is markedly attenuated and hyperglycemia worsens. Lack of insulin-dependent inhibition of hepatic glucose output
further contributes to grossly elevated glucose levels. Decompensation of the β cell may be caused in part by the direct action of the glycemia itself resulting in glucotoxicity, and by lipotoxicity caused by build-up of intracellular triglycerides (Unger 1997). Figure 1 proposes a two-step model for the development of NIDDM.

![Figure 1. Proposed steps in the development of NIDDM. Genetic and metabolic factors are hypothesised to contribute to disease onset. IGT, impaired glucose tolerance (reproduced from Bennett 1996).](image)

The hypothesis that both primary genetic defects and secondary metabolic abnormalities contribute to development of NIDDM has generated much investigation into the role of incretins in the evolution of the disease and as potential therapies. Comparing normal and diabetic patients following an oral glucose load or isoglycemic glucose infusion Nauck and co-workers (1986) showed the incretin effect in the latter group to be reduced by 50 % compared to normals. In apparent contradiction to this report, numerous experiments have been carried out which show elevated levels of both GIP and GLP-1.
In studies on obese patients with impaired and normal glucose tolerance, normal weight patients with impaired glucose tolerance and those with NIDDM, the majority of reports have shown an increased concentration of GIP both in basal and stimulated states compared to normal weight patients with normal glucose tolerance (Crockett et al. 1976; Creutzfeldt et al. 1978; Coxe et al. 1981; Salera et al. 1982; Mazzaferri et al. 1985). Ross et al. (1977) observed no significant difference in fasting levels between NIDDM and healthy patients though hypersecretion of GIP following glucose ingestion was reported. Others have demonstrated no difference in circulating GIP either pre- or postprandially between obese and lean nondiabetics (Jorde et al. 1983b; Service et al. 1984), NIDDM patients and normal controls (Nauck et al. 1986), lean and obese NIDDM patients, and lean NIDDM patients and lean controls (Service et al. 1984). However, the postprandial response in obese NIDDM patients was actually blunted by 42 % compared to obese nondiabetics (Service et al. 1984). Willms et al. (1978) and Ebert et al. (1979) found that the exaggerated release of GIP in obese subjects following a test meal or glucose load could be reversed by modest weight reduction. The range of GIP responses to nutrient ingestion is substantially larger in diabetic patients than in healthy controls and could not be correlated to insulin secretion (Creutzfeldt et al. 1983; Nauck et al. 1986).

The insulinotropic activity of exogenous GIP in patients with NIDDM has been extensively studied and has generally been shown to have a significant though greatly reduced action compared to controls, with little or no attendant decrease in plasma glucose levels (Krarrup et al. 1987; Jones et al. 1987; Jones et al. 1989). Similar results were found in the perfused diabetic rat pancreas (Suzuki et al. 1990). Other investigators have reported the
virtual absence of any insulin releasing action during GIP infusion in diabetics (Nauck et al. 1993; Elahi et al. 1994).

Given that the majority of experimenters have reported hyperGIPemia following nutrient intake, the role of GIP in the reduced incretin effect observed in the state of NIDDM has been postulated to involve a receptor defect (Holst et al. 1997). As discussed below, the diabetic β cell retains its responsiveness to GLP-1 thereby discounting any dysfunction of the intracellular machinery. Two missense mutations have recently been identified in the coding region of the GIP receptor gene, one of which displayed an attenuated cAMP response to GIP stimulation however, neither of the mutations were associated with NIDDM in patients of Japanese origin (Kubota et al. 1996). Yet, because this disease is polygenic in nature it must be considered that such a mutation may act as a risk factor in its development. Holst and group (1997) also suggested that the different ethnic origins of the patients (Japanese versus primarily Caucasian in previous studies) may correlate with differences in the genetic component of NIDDM, i.e. in Caucasian diabetics the mutation may lie outside of the coding region of the receptor gene.

As an incretin GLP-1 has likewise been studied for its role if any, in the etiology of NIDDM, and where incretins lead controversial reports follow. Elevated fasting levels have been reported by Hirota and colleagues (1990) and Nauck and group (1993) compared to normal subjects. Ørskov et al. (1991) described similar findings but attributed the increase to the presence of a cross-reactive proglucagon fragment not GLP-1. Vaag et al. (1996) observed no difference in basal GLP-1 concentration between identical twins discordant for NIDDM and normals. Postprandial levels have been reported to be increased over controls (Hirota et al. 1990; Ørskov et al. 1991) thought to be due in part to an impaired suppression
of pancreatic GLP-1 and/or enhanced release of intestinal GLP-1 (Hirota et al. 1990). Elevated colonic levels of the incretin have been found in streptozotocin-induced diabetic rats (Kreymann et al. 1988). Unchanged (Nauck et al. 1993) and attenuated (Vaag et al. 1996) responses compared to controls following oral glucose challenge have also been reported.

A potential therapeutic role for exogenous GLP-1 in the management or treatment of NIDDM has stimulated considerable interest and has revealed a markedly greater antidiabetogenic action as compared to exogenous GIP (Nauck et al. 1993; Elahi et al. 1994). Infusion (Nathan et al. 1992; Nauck et al. 1993; Elahi et al. 1994) or subcutaneous administration (Schirra et al. 1998) of GLP-1 to NIDDM patients has reportedly increased the insulin response to nutrients resulting in reduced postprandial hyperglycemia (Nathan et al. 1992; Willms et al. 1996; Schirra et al. 1998). The glucagonostatic action and ability to inhibit gastric emptying (Gutniak et al. 1992; Willms et al. 1996; Schirra et al. 1988) as well as stimulation of glycogenesis, glucose uptake and metabolism (Morales et al. 1997) were preserved in the NIDDM. These actions combined can result in fully normalised fasting glucose levels and improved glucose tolerance. Although the majority of studies have employed exogenous concentrations of GLP-1 which were slightly above physiological values, the potential as an effective therapy in NIDDM cannot be dismissed.

In diabetic patients GLP-1 retains its physiological capability to stimulate cAMP production and ultimately induce insulin release. Dachicourt and co-workers (1997) showed that infusion of GLP-1 to diabetic rats prior to an i.v. glucose challenge significantly improved glucose tolerance by enhancing the insulin response, and increasing the insulinogenic index as well as the rate of glucose disappearance. They postulated that artificially raising the intracellular content of cAMP in β cells of diabetic rats restores their
responsiveness to glucose. GLP-1 is also known to restore the first-phase insulin response which is typically absent in NIDDM, thereby attenuating the hyperglycemia observed following nutrient intake (Rachman et al. 1996; Willms et al. 1996; Schirra et al. 1998). The importance of GLP-1 in maintaining normal glucose tolerance and insulin levels becomes apparent in considering the GLP-1 receptor knock-out mouse which demonstrates both fasting and stimulated hyperglycemia, and a markedly decreased insulin response 30 min following oral glucose loading (Scrocchi et al. 1996). Interestingly though there has been no evidence reported of obesity or abnormal feeding behaviour in these mice (Scrocchi et al. 1996; Scrocchi et al. 1998) suggesting that the regulation of food intake and weight is multifaceted and GLP-1 plays only a part.

**DIPEPTIDYL PEPTIDASE IV**

*Characterisation*

Dipeptidyl peptidase IV (DP IV, CD 26, EC 3.4.14.5) is a proline specific peptidase originally discovered by Hopsu-Havu and Glenner (1966) in the rat liver and later purified from a variety of bacterial, insect and mammalian sources. Of the latter group, DP IV activity has been localised to tissues including the stomach, spleen, colon, testes, lung, vascular endothelium, the pancreatic Islets of Langerhans (Mentzel et al. 1996) and specifically the α-cell (Poulsen et al. 1993). However, it is in placental tissue and on the brush borders of the intestinal enterocytes and proximal tubules of the kidney where DP IV activity is most highly concentrated (Yaron and Naider 1993).

A gene located on the long arm of chromosome 2 has been shown to encode human DP IV (Bernard et al. 1994; Abbott et al. 1994; Darmoul et al. 1994). The promoter
responsible for its transcription has the sequence characteristics of a housekeeping gene promoter yet is able to initiate transcription in a tissue specific manner (Böhm et al. 1995). The product of gene expression is a homodimer with each identical subunit having a molecular mass of 100-130 kDa depending on the species and cell type as well as the degree of glycosylation of which there exist nine sites on DP IV (Reutter et al. 1995; Cunningham and O’Connor 1997). cDNA analysis showed rat DP IV to be comprised of 767 amino acids with approximately 85% sequence homology between rat and human DP IV, and 92% between rat and mouse (Reutter et al. 1995). The primary configuration of the protein was likewise deduced and shown to have five distinct structural domains (Reutter et al. 1995). A short N-terminal cytoplasmic region and 22 amino acid transmembrane domain representing the signal peptide remain intact during biosynthesis and together act to anchor the protein to the membrane (Reutter et al. 1995). The first extracellular region contains a high density of potential glycosylation sites, followed by a cysteine-rich region probably involved in disulphide-bridge formation, and finally the C-terminus possessing the active site of the serine protease (Reutter et al. 1995).

As mentioned previously, DP IV has ubiquitous distribution as a cell-associated glycoprotein, however enzyme activity is also present in a soluble form in urine, plasma and serum. It was originally assumed that these two forms were equivalent. However, Duke-Cohan et al. (1995) reported on a novel form of DP IV in human serum, distinct from that bound to the membranes of T lymphocytes. The soluble form was shown to exist as a trimer composed of subunits having a mass of 175 kDa. In a subsequent study, this same group (1996) demonstrated that serum DP IV is antigenetically, biochemically and enzymatically identical to an antigen released from activated T lymphocytes. The appearance of the T cell
derived form in their tissue culture medium correlated exactly with decreased expression of
the antigen on the cell surface. This group concluded that the 175 kDa serum form of DP IV
is released from activated T lymphocytes perhaps as a result of shedding small membrane
vesicles, and that it is distinct from the smaller form which is continuously membrane-
associated. Though these two forms were shown to be very closely related enzymatically, this
group suggested that they may be products of two different genes which would explain the
size discrepancy.

**Enzymatic Considerations**

DP IV is a proline-specific serine peptidase which differs from other serine proteases
such as the trypsin and subtilisin families in several areas. This enzyme is larger in size than
the trypsin-like serine proteases and its active site is located in the C-terminal region of the
primary sequence (Demuth and Heins 1995). Additionally, the topology of the catalytic
residues is Ser-Asp-His versus His-Asp-Ser as in trypsin-like serine peptidases and these
differences have lead to the inclusion of DP IV in a novel subclass of serine proteases, the
prolyl oligopeptidases (Cunningham and O’Connor 1997).

The enzymatic region of DP IV as mentioned previously, is located in the C-terminus
and is composed of the catalytic triad Ser$^{624}$Asp$^{702}$His$^{734}$ as identified in mouse (David *et al.*
1993). In humans Gly-Trp-Ser$^{631}$-Tyr-Gly has been found to form the active site, which is in
accordance with the consensus sequence proposed for serine proteases (Gly-X-Ser-X-Gly)
(Ogata *et al.* 1992). Additionally it was reported that substitution of the serine residue
resulted in a complete loss of enzymatic activity, and that replacement of Tyr$^{632}$ with either
Gly or Leu but not Phe, or replacement of either of the two Gly residues caused a loss of
surface DP IV expression suggesting that the sequence Gly-X-Ser\textsuperscript{631}-(Tyr)-Gly is critical for normal enzyme activity (Ogata et al. 1992; Fujiwara et al. 1992).

It has been well established that DP IV is highly specific with regard to substrate type and configuration. This enzyme is known to cleave dipeptides from the N-terminus and although proline in the penultimate position is optimally accommodated into the binding pocket, alanine, hydroxyproline, serine, glycine, valine and leucine are also accepted though with decreasing specificity constants (Demuth and Heins 1995). There is also a strict requirement that the amino acid residue in the P\textsubscript{1}-position be in the L-configuration and if proline occupies this site, the same requirement exists for the P\textsubscript{2}-position. However, either D- or L- residues are accepted in the N-terminal site if alanine occupies the penultimate position, although peptides possessing the D-configuration are hydrolysed more slowly (Demuth and Heins 1995). Affinity of DP IV for its substrate is known to increase with the presence of an aromatic or aliphatic amino acid in position P\textsubscript{2}. Additionally, the scissile P\textsubscript{1}-P\textsubscript{2} bond must be in the \textit{trans} formation and the N-terminus must be unprotected and protonated in order to optimise enzyme activity. Interestingly, it has been shown that the efficiency of enzymatic cleavage of a substrate can be affected by peptide chain length. For example increasing the N-terminal fragment of substance P from 4 to 11 amino acids caused a decrease in catalytic efficiency (Kikuchi et al. 1988) whereas removing a dipeptide from the C-terminus of the 7-residue chain of \(\beta\)-casomorphin increased efficiency (reviewed by Yaron and Naider 1993). Furthermore, lower rates of degradation of growth hormone releasing hormone (GHRH) were observed for GHRH\textsubscript{1-11} and GHRH\textsubscript{1-3} as compared to GHRH\textsubscript{1-44}, GHRH\textsubscript{1-29} and GHRH\textsubscript{1-20} (Bongers et al. 1992).
Mechanism of Action

The catalytic cycle of DP IV is very similar to that of other serine proteases with regard to the principal reactions - substrate-enzyme binding to form a intermediate compound, acylation, release of the dipeptide, and reformation of the enzyme by deacinylation (Demuth and Heins 1995). Interaction of DP IV with its substrate initiates activation of the serine residue by proton transfer to histidine allowing for a nucleophilic attack on the carbonyl carbon of the amino acid residue in P₁ forming a tetrahedral intermediate. At this step Brandt’s group (1996) proposed a novel mechanism of stabilisation of the intermediate during DP IV catalysis which could perhaps be extended to serine proteases in general. The oxyanion produced as a result of the nucleophilic attack would subsequently attack the carbonyl carbon of the P₂ residue generating a stable oxazolidine ring. A neutral compound was suggested to form by the transfer of an hydrogen atom from the protonated N-terminus to the negatively-charged oxygen in P₂. Breakdown of this intermediate into an acyl enzyme and dipeptide is followed by a reaction of the former with water producing a second tetrahedral compound, the deacylation of which re-establishes the serine and histidine residues and releases the remaining peptide (Demuth and Heins 1995). Kinetic investigations have established acylation as the rate-limiting step for peptides possessing alanine in the penultimate position from the N-terminus, and deacylation as that when proline is in this position (Rahfeld et al. 1991a).

Inhibition of DP IV

While the products of DP IV cleavage have been shown to be among the best competitive inhibitors of this enzyme with Kᵢ values in the range of 10-100 μM, synthetic
inhibitors have been generated as a means of studying the mechanism and biological roles of this enzyme (Demuth and Heins 1995). These manufactured compounds fall within two general classes—binding-mediated and mechanism-based inhibitors. More specifically, the former class includes affinity labels and transition-state analogues (Demuth 1990).

The structure of the affinity label is such that it resembles a natural substrate and can therefore interact with the catalytic site of the enzyme inhibiting its activity, generally in a covalent, irreversible fashion (Demuth 1990). Of the affinity labels, the most investigated inhibitors are the peptide halomethyl ketones which act by alkylating the active site histidine of serine proteases such as DP IV (Demuth 1990). However, two major limitations of this type of compound are their high electrophilic reactivity which makes them very susceptible to degradation, as well the significant levels of non-specific alkylation observed prior to reaching the target enzyme (Demuth 1990). More recent studies have investigated halomethyl ketone derivatives with an additional methyl side chain near the reactive site as a means of decreasing such secondary reactions (Wikström et al. 1989).

The second of the binding-mediated inhibitors are the transition-state analogues which are compounds vulnerable to nucleophilic attack by the active site of the protease, but which lack a scissile carbonamido linkage present in a natural substrate. These analogues can bind reversibly to the target molecule three- to six-fold stronger than the corresponding carbonyl substrates (Demuth 1990). There are several examples of transition-state analogues, perhaps the most potent of which are dipeptides containing boroPro, the boronic analogue of proline, displaying Kᵢ values as low as 2-3 nM (Flentke et al. 1991). The compounds are very unstable however, with half lives ranging from 2-30 min for Ala-boroPro, to 1.5 h for Pro-boroPro (Flentke et al. 1991). A second example are nitrile inhibitors which presumably act
by forming an imidate adduct with the active site serine with $K_i$ values in the low to submicromolar range (Li et al. 1995). An advantage to the nitriles is their stability, potency and facility of synthesis (Li et al. 1995). A third class of transition-state analogues, the thiazolidides, has been shown to be very effective in inhibiting DP IV activity, for example Isoleucine-thiazolidide demonstrates a $K_i=130$ nM and Valine-thiazolidide a $K_i=270$ nM (Demuth and Heins 1995). The structure of this type of inhibitor contains a ring which mimics a natural proline-containing substrate however, its non-scissile carbonamide bond allows for the formation of a tetrahedral intermediate. Introduction of a sulphur atom into this pyrrolidide ring thus generating the thiazolidide, seems to increase the affinity of the enzyme-inhibitor interaction (Tsuru et al. 1988).

The second of the synthetic DP IV inhibitors are the mechanism-based variety which take advantage of the catalytic machinery of the enzyme in order to become fully functional inhibitors (Demuth 1990). Specifically, the acyl enzyme inhibitors are a group of compounds which undergo nucleophilic attack by the target enzyme forming an acyl enzyme, a product normally generated by the interaction of enzyme and substrate (Demuth 1990). In the former case however, the deacylation step becomes rate-determining for the entire process. Recently developed are the phosphonates and phosphonate esters which are thought to have their action via this mechanism (Boduszek et al. 1994; Lambeir et al. 1996). While the phosphonates have been demonstrated to be specific, they are not very potent, and the phosphonate esters have been shown to be degraded in plasma (Boduszek et al. 1994; Lambeir et al. 1996).

Finally, the enzyme-activated inhibitors require the catalytic activity of the enzyme to release their latent inhibitory properties thereby allowing the formation of stable bonds with
all intermediates and transition states of the mechanistic process (Demuth 1990). A well studied example are the diacyl hydroxylamines which are activated by enzymatic cleavage of the -NH-O- bond, releasing an inactivating nitrogen intermediate (Silberring et al. 1993). Though they are of only modest potency (Li et al. 1995), the covalent, irreversible nature of this diacyl hydroxylamines-enzyme interaction has lead to their designation as “suicide” inhibitors (Yaron and Naider 1993).

Long used as inhibitors of DP IV, diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu) have recently been reconsidered as potential substrates where their inhibitory role is an artifact originating from their substrate-like structure (Rahfeld et al. 1991b). Rahfeld’s group demonstrated that when DP IV catalysis of a substrate with an optimum configuration takes place in the presence of either diprotin A or B or a related tripeptide, the latter acts as a competitive inhibitor where the $K_i$ value of the tripeptide is virtually equivalent to its $K_m$ value.

**Biological Role**

The diversity of DP IV’s actions reflects its ubiquitous distribution. It has been implicated in such processes as renal transport and intestinal digestion of proline-containing peptides. In a study investigating the latter, it was reported that a strain of rats lacking DP IV fed a proline rich diet experienced significant weight loss while normal controls maintained their weight and demonstrated a marked increase in the expression of intestinal brush border DP IV (Tirupathi et al. 1993). This enzyme is also involved in fibronectin-mediated cellular adhesion, blood clotting and immunological activation of immunocompetent cells (Yaron and Naider 1993). The latter is a role which has been heavily investigated recently due to the
potential link between DP IV and cellular infection by the human immunodeficiency virus-1 (HIV-1).

The role of DP IV in the immune system is an interesting and somewhat controversial one. The enzyme was originally localised to the surface of activated T lymphocytes as CD 26, a cell surface molecular marker (Fox et al. 1984; Dang et al. 1991). The exact mechanism by which DP IV acts to stimulate T lymphocytes, particularly CD4+ helper T cells has yet to be elucidated, though it has been reported that an association occurs between DP IV and CD 45 (Torimoto et al. 1991) as well as adenosine deaminase (ADA) in humans (Kameoka et al. 1993; Morrison et al. 1993).

Of considerable debate is whether DP IV activity is required for T-cell activation or if the presence of the molecule is sufficient. Tanaka and co-workers (1993) reported decreased activation of transfected Jurkat cells lacking functional DP IV activity, others have demonstrated no such reduction in activation (Steeg et al. 1995). The use of DP IV inhibitors and their effect on T-cell activation also has been investigated with similarly conflicting results. Schön et al. (1987) reported a greatly decreased cell response while others attributed this effect to be due to non-specific cytotoxic effects. In fact DP IV Jurkat cells were shown to be equally susceptible to specific DP IV inhibitors as DP IV cells (Hegen et al. 1993).

DP IV may also have a role in HIV-1 infection of healthy T-lymphocytes though this too remains highly controversial. Callebaut and co-workers (1993) reported that expression of DP IV on murine T-lymphocytes served as a necessary co-factor for the entry of HIV-1, and that inhibitors and monoclonal antibodies directed against DP IV could inhibit viral expression. Other investigators have been unable to confirm these results (Morimoto et al. 1994; Morimoto and Schlossman 1995). More recently it has been reported HIV-1 Tat, a
regulatory protein required for transactivation of viral genes and replication, is able to bind to and inhibit DP IV, thereby suppressing lymphocyte proliferation (Wrenger et al. 1997). The underlying mechanism may be the interference of Tat with the DP IV/CD 45/ADA interaction which provides an intriguing possibility given that a defect in ADA expression or function is known to result in severe combined immunodeficiency disease in humans (Subramanyam et al. 1995). In light of these controversial data it is apparent that more investigation is required in order to elucidate the role, if any, of DP IV in HIV-1 infection and disease progression.

As a protease, one of the primary roles of DP IV is to cleave peptides rendering them inactive or active. In the case of promelittin, the precursor of the main constituent in honey bee venom, cleavage by the enzyme which has been localised to the extracts of venom glands results in the active melittin (Kreil et al. 1980). A similar process has been proposed in the generation of mature antifreeze protein in winter flounder and in the production of many other hormones from their polypeptide precursors (Yaron and Naider 1993). DP IV may also be involved in release of β-casomorphine from the parent molecule casein which is normally resistant to the proteolytic action of most other proteinases; additionally, β-casomorphine itself further hydrolysed by DP IV to a biologically inactive form (Kreil et al. 1983).

Equally important is the role of DP IV in the cleavage and inactivation of various other physiologically relevant hormones including substance P (Nausch and Heymann 1984; Ahmad et al. 1992), neuropeptide Y (Mentlein et al. 1993a; Medeiros dos Santos and Turner 1996), peptide YY (Mentlein et al. 1993a) and growth hormone releasing hormone (GHRH) (Frohman et al. 1986; Frohman et al. 1989; Kubiak et al. 1989).

Frohman et al. (1989) reported that biologically active GHRH\textsubscript{1-44} was cleaved by DP IV to the inactive form, GHRH\textsubscript{3-44}, a result supported by their concurrent finding that DP IV-
resistant GHRH analogues displayed prolonged biological activity. GIP and GLP-1 belong to the same peptide family as GHRH which shares the N-terminal X-Ala motif, the cleavage site of DP IV. Biologically active GIP<sub>1-42</sub> and GLP-1<sub>7-36</sub> both possess an alanine moiety in this position, therefore, studies were undertaken to investigate incretins as possible DP IV substrates. Mentlein and co-workers (1993b) reported that the two hormones were degraded to the inactive forms, GIP<sub>3-42</sub> and GLP-1<sub>9-36</sub>, *in vitro* by human placenta purified DP IV with K<sub>m</sub> values of 34±3 and 4.5±0.6 μM, respectively and V<sub>max</sub> values of 3.8±0.2 and 0.97±0.05 μmol·min<sup>-1</sup>·mg<sup>-1</sup>, respectively. These results suggested that at physiological (nanomolar) concentrations, the enzyme could play an important role in the *in vivo* inactivation of GIP and GLP-1. Incubation of the incretins with human serum produced the same degradation products, and addition of specific DP IV inhibitors abolished this observation (Mentlein *et al.* 1993b). These results were supported by a similar *in vitro* study by Deacon and co-workers (1995b) as well as an *in vivo* investigation. The latter reported that subcutaneous or intravenous GLP-1<sub>7-36</sub> administration to both healthy and NIDDM patients resulted in rapid cleavage of the peptide to the inactive metabolite GLP-1<sub>9-36</sub> (Deacon *et al.* 1995c).

The circulating half-lives of biologically active GIP<sub>1-42</sub> and GLP-1<sub>7-36</sub> in humans have been determined by immunoreactive studies to be approximately 20 min (Sarson *et al.* 1982) and 4-11 min (Kreymann *et al.* 1987), respectively, with the kidney as the main site of GIP clearance (O’Doriso *et al.* 1977) and GLP-1 catabolism (Ruiz-Grande *et al.* 1993). Kieffer *et al.* (1995) further investigated the biological half-lives of the incretins. Using high performance liquid chromatography (HPLC) it was shown that within two minutes of [¹²⁵I]-GIP or [¹²⁵I]-GLP-1 administration into rats, over 50% of the peptide was cleaved to its inactive form. In addition, this study reported the presence of intact, iodinated-peptide even
after a twenty minute incubation with serum from a DP IV-deficient strain of rat. They concluded therefore that DP IV was an important mediator of GIP$_{1-42}$ and GLP-1$_{7-36}$ degradation.

**THE OBSESE (fa/ fa) ZUCKER RAT**

The obese Zucker rat has long been utilised as a model of hyperinsulinemia and impaired glucose tolerance, characteristics of the early stages of NIDDM. The phenotype occurs as a result of a mutation in the fa gene which encodes the leptin receptor. A single point mutation within this gene which is located on chromosome 5, results in a glutamine to proline substitution at position 269 (Chua et al. 1996; Iida et al. 1996;) greatly reduced leptin binding (Yamashita et al. 1997) and therefore hyperphagia.

The underlying mechanisms leading to elevated fasting and stimulated insulin levels are thought to be manifold. Increased parasympathetic output (Jeanrenaud 1985), overactivity of β cell glucokinase leading to increased glucose cycling (reviewed by Chan 1995), and loss of the glucose threshold for insulinotropic action of GIP (Chan et al. 1984; Chan et al. 1985) and GLP-1 (Jia et al. 1995b) resulting in increased sensitivity to basal glucose levels, are all suggested to have a role in the hyperinsulinemic state. Additionally, the liver in the obese Zucker rat, when presented with chronically high levels of insulin, shows a reduced ability to remove insulin from the circulation; thus hyperinsulinemia acts to perpetuate itself (Jeanrenaud 1985).

Though the insulin output is generally sufficient to maintain fasting and stimulated normoglycemia, diabetic variants of the obese Zucker have been identified, displaying reduced insulin responses and chronic hyperglycemia. As a result of increased peripheral
resistance to the action of insulin, and decreased inhibition of hepatic gluconeogenesis and
glycogenolysis by insulin, chronic hyperglycemia develops. Furthermore, lack of functional
leptin binding and activity at the β cell prevents oxidation of free fatty acid, resulting in
esterification and intracellular accumulation of triglycerides (Unger 1997). Gluco- and
lipotoxic β cell damage ensue leading to a markedly reduced insulin capacity. Defects in
mitochondrial glucose oxidation are also observed in the obese Zucker rat which reduce ATP
output and affect insulin release (reviewed by Chan 1995).

**THESIS INVESTIGATION**

Recent studies by ourselves and others have demonstrated conclusively that DP IV is
a major enzyme responsible for the cleavage and biological inactivation of the incretins GIP
and GLP-1 (Pauly *et al.* 1996; Kieffer *et al.*, 1995; Deacon *et al.*, 1995a). These results have
enabled the initiation of various experiments in which the circulating half life of incretins is
increased by inhibiting the activity of the enzyme DP IV. Studies by Pauly *et al.* (1998) as
well as Deacon and group (1998a) have observed that infusion of specific DP IV inhibitors,
Isoleucine-thiazolidide (Ile-thiazolidide) and Valine-pyrrolidide, respectively, potentiated the
insulinotropic action of the incretins.

While both of these studies reported improved glucose tolerance through inhibition of
DP IV activity, investigations were carried out on animals which demonstrate normal glucose
handling. Additionally, the inhibitors were administered under anaesthesia by intravenous
infusion. The first part of this project was therefore to study the effect of DP IV inhibition on
glucose tolerance in the conscious obese Zucker rat using a protocol of orally administered
inhibitors which represents a more accurate physiological system. It is of interest then to
determine if there are long term effects on glucose handling in these rats following simultaneous oral administration of DP IV inhibitor with a glucose challenge as well as to establish the time course of enzyme inhibition over several hours. Furthermore, the optimal administration protocol has yet to be established, that is, whether improvement in glucose tolerance is greater when the inhibitor is given simultaneously or prior to the glucose challenge. Ile-thiazolidide has previously been shown to effectively inhibit plasma DP IV activity (Pauly et al. 1996; Pauly et al. 1998). Additionally, two other inhibitors which have been shown effective in vitro are Glycine-Proline-Ile-thiazolidide (Gly-Pro-Ile-thiazolidide) and Proline-Ile-thiazolidide (Pro-Ile-thiazolidide). Termed pro-drugs, both have been demonstrated to remain inactive until cleaved by DP IV. The effect of this delayed action in vivo has not been established as yet. The relative effectiveness of these inhibitors as compared to Ile-thiazolidide in improving glucose tolerance has not been determined. Clearly, characterising these inhibitors using the fatty Zucker rat is of great interest as they may have therapeutic potential for the human state of NIDDM.

As mentioned previously, immunohistochemical techniques have shown high levels of DP IV to be present in the cells of the islets of Langerhans (Mentzel et al., 1996) and specifically the α-cell (Poulsen et al. 1993). Taking into consideration the role of the enzyme in incretin degradation, it is possible that DP IV is present also on the insulin secreting β cell, the target of circulating GIP and GLP-1. As this has yet to be determined, it is also unknown if there is a discrepancy in the distribution and relative expression levels of the enzyme on the β cell in lean and fatty Zucker rats. These questions formed the second part of this project, the rationale being a contribution to the understanding of the ability of enzyme inhibition to enhance expression of the enteroinsular axis.
CHAPTER 1: EFFECT OF ORALLY ADMINISTERED DP IV INHIBITORS ON GLUCOSE TOLERANCE IN ZUCKER RATS

PROJECT RATIONALE

It has been conclusively established that DP IV is the primary enzyme responsible for the cleavage of GIP$_{1-42}$ and GLP-$1_{7-36}$ amide to GIP$_{3-42}$ and GLP-$1_{9-36}$ amide, both of which lack insulinotropic activity. Ile-thiazolidide, Gly-Pro-Ile-thiazolidide and Pro-Ile-thiazolidide have all previously been shown to be effective inhibitors of DP IV activity. Studies with Ile-thiazolidide both in vitro and in vivo have reported prolonged half-lives of the two incretins and improved glucose tolerance in anaesthetised Wistar rats. It is of significant interest therefore to investigate the possible therapeutic role of DP IV inhibition on the enteroinsular axis using conscious animals which model a state of impaired glucose tolerance.

MATERIALS AND METHODS

Animals

A colony of Zucker rats was bred in the physiology department of the University of British Columbia. Obese (fa/fa) and lean (Fa/?) Zucker rats weighing 300-400 g were used. Wistar rats weighed 250-300 g. All experiments were carried out on overnight fasted, conscious unrestrained rats.

Oral Glucose Tolerance Tests (OGTT)

Obese or lean rats were administered oral glucose through a gavage tube (1 g/kg, 40 % wt/vol dextrose. This was taken as time 0 min and at 0, 5, 10, 20, 30 and 60 min following
the glucose challenge, blood samples were obtained from the tail vein using 250 µl heparinised blood collecting tubes (Fisher Scientific, Nepean, ON). The samples were stored on ice until centrifugation (10,000x g, 4 °C, 30 min) after which the plasma was collected and immediately assayed for DP IV activity. The remaining plasma was frozen at -20 °C until glucose and insulin analysis.

**Inhibition of DP IV**

A dose of 20 µmol per 300 g body wt of either Ile-thiazolidide, Gly-Pro-Ile-thiazolidide or Pro-Ile-thiazolidide, dissolved in distilled water (200 µl) was used for all experiments. All drugs were synthesised in and provided by the laboratory of Dr. H.-U. Demuth (Hans-Knöll Institute of Natural Product Research, Halle, Germany). For the protocol of simultaneous administration, the dissolved drug (or distilled water for control animals) was mixed with the glucose solution and given orally at time 0 min. Another protocol was also followed whereby animals were pre-treated with inhibitor prior to OGTT. In this case the drug solution or water was administered orally at -20 min followed by glucose challenge at 0 min; an additional blood sample was obtained at -20 min. Blood samples were processed as outlined previously.

For the experiment focusing on long term inhibition, rats were administered Ile-thiazolidide at 0 min with tail bleeds performed at 0 min and hourly thereafter for 8 hrs. Animals had free access to water throughout the experiment. Blood samples were processed as before.
**Plasma Analysis**

DP IV activity was measured by colorimetric assay in which Gly-Pro-4-nitroanilide is cleaved by the enzyme yielding Gly-Pro and the yellow product 4-nitroaniline, whose rate of appearance reflects DP IV activity and can be detected spectrophotometrically. A substrate solution was prepared consisting of 0.26 mM Gly-Pro-4-nitroanilide (Sigma, St. Louis, MO) in 0.04 M HEPES buffer, pH 7.6. The reaction was initiated with the addition of 30 µl of plasma to 270 µl of substrate. The assay was carried out in 96-well microtitre plates at room temperature. Progression of the reaction was monitored continuously over 30 min using a Dynatech MRX Microplate Reader (wavelength 405 nm) with DP IV activity expressed as change in optical density over 20 min.

Glucose analysis was performed using the glucose oxidase method (Beckman Glucose Analyzer 2, Fullerton, CA).

Insulin was measured by radioimmunoassay (RIA) using either a rat insulin RIA kit (noted in figure legend; Linco, St. Charles, MO) or by the RIA method previously described by Pederson *et al.* (1982) which incorporates a rat insulin standard and guinea pig anti-human insulin antisera (GP01). Test samples were assayed by both methods and the results were comparable. Radioactivity was measured using an LKB Wallac 1277 Gammamaster Automatic Gamma Counter.

**Statistical Analysis**

Comparisons between inhibitor-treated and control animals were assessed with the unpaired, two-tailed Student's t-test. Integrated values were calculated using the 0 min insulin or glucose levels as basal. Comparisons between the three inhibitors were made using
a one-way analysis of variance with a Tukey post-test. P<0.05 was considered to be significant. Statistical analysis was carried out using software from GraphPad Prism (GraphPad Software Inc., San Diego, CA).

RESULTS

Oral Administration of Ile-thiazolidide to Wistar Rats

The protocol of orally administered Ile-thiazolidide was first carried out on Wistar rats to examine its efficacy as previous studies have investigated only the intravenous route. Significant inhibition of DP IV activity was observed after 5 min with 66.5 % maximum suppression occurring at 30 min (Fig.2A). While there was no difference in the insulin response between inhibitor-treated and control animals (Fig.2B), a lower peak glucose value was observed for the former group (232.6 ± 6.7 vs. 203.5 ± 5.8 mg/dl) (Fig.2C).

Effect of Ile-thiazolidide Administered with OGTT on DP IV Activity in Zucker Rats

Given the potency of oral Ile-thiazolidide in Wistar rats, similar studies were performed using the Zucker rat, a model of hyperinsulinemia and glucose intolerance. Figure 3 shows a representative DP IV response to Ile-thiazolidide administered orally. Effect on enzyme activity was comparable for obese and lean animals with significant inhibition after 5 min reaching a maximum of approximately 78 % at 20 min. Suppression of DP IV activity was sustained over the course of the experiment while control values remained unchanged.
Figure 2. Effect of oral Ile-thiazolidide on DP IV activity (A), and plasma insulin (B) and glucose (C) in response to OGTT in Wistar rats. Plasma was analysed following administration of Ile-thiazolidide + OGTT (▲) or water + OGTT (■) as outlined in Materials and Methods. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (*).
Effect of Ile-thiazolidide Administered with OGTT on Glucose Tolerance in Zucker Rats

Tolerance of an oral glucose challenge was investigated in the presence and absence of Ile-thiazolidide in obese and lean Zucker rats. The insulin response in obese animals was significantly greater than controls only at the 30 min point with both groups returning to near basal levels by 60 min (Fig.4A). However, the integrated insulin response to the OGTT was 1696.6 ± 80.1 μU in the inhibitor-treated rats, a 134 % increase over controls (Fig.4A inset). The lean Zucker rats receiving inhibitor demonstrated a significantly increased peak insulin value compared to controls (89.6 ± 7.3 vs 59.4 ± 2.6 μU/ml) which returned to fasting values by the end of the experiment (Fig. 4C). As with the obese rats, the treated lean animals showed a greater integrated response though it was only a 23 % increase over controls (Fig. 4C inset). The insulin profiles appeared much different for the lean and obese animals with
Figure 4. Effect of oral Ile-thiazolidide on plasma insulin (A, C) and glucose (B, D) in response to OGTT in obese (A, B) and lean (C, D) Zucker rats. Plasma was analysed following administration of Ile-thiazolidide + OGTT (▲) or water + OGTT (■) as outlined in Materials and Methods. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (*).
the former showing a notable peak response and return to fasting levels by 60 min in both control and treated groups. The obese animals however, were hyperinsulinemic in the fasted state compared to leans (73.4 ± 3.6 vs. 15.2 ± 2.8 μU/ml) and demonstrated a gradual and more sustained increase in plasma insulin.

The glucose profile for the Ile-thiazolidide-treated obese Zucker rats was one of improved glucose tolerance (Fig.4B). Not only did they display a lower peak response (292.2 ± 9.9 vs 346.0 ± 10.0 mg/dl) there was also an increased rate of clearance from the circulation to near basal values at 60 min. Conversely, untreated obese rats had a glucose response which appeared to plateau from 20 to 60 min. Additionally, the integrated values were significantly reduced in treated as compared to control animals (Fig.4B inset). With regard to the lean animals, they too demonstrated a decreased peak glucose value (Fig.4D) and reduced integrated response (Fig.4D inset) as compared to untreated leans.

In order to establish if Ile-thiazolidide was affecting glucose and insulin responses directly as opposed to acting through the enteroinsular axis, inhibitor (distilled water for controls) was administered orally in the absence of an OGTT. Suppression of DP IV activity was confirmed for both obese and lean Zucker rats (Fig.3). As shown in Figure 5A and the inset, there was no change in insulin values from basal and no difference in the integrated results for treated and control obese rats. A similar result was observed for the lean animals (Fig.5C and inset). The glucose profile in the obese rats remained unchanged following inhibitor, a result confirmed by the lack of difference in the integrated response (Fig.5B and inset). With regard to the lean Zucker rats, significantly greater glucose values were observed at all time points in the controls, though the two profiles parallel one another and the control group showed an elevated basal glucose level as compared to inhibitor-treated (Fig.5D). Therefore, the relative
Figure 5. Effect of oral Ile-thiazolidide alone on plasma insulin (A, C) and glucose (B, D) in obese (A, B) and lean (C, D) Zucker rats. Plasma was analysed following administration of Ile-thiazolidide (▲) or water (■) as outlined in Materials and Methods. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (*).
change in plasma glucose is comparable between the two groups, a result confirmed by the integrated response (Fig. 5D inset). Though there was a small increase in glucose values at the 5 min point compared to basal for both the obese and lean rats, this was likely due to stress from the oral feed.

**Time Course of DP IV Inhibition by Ile-thiazolidide**

An eight hour experiment was carried out on both obese and lean Zucker rats in order to better characterise the time course of inhibition of DP IV by oral Ile-thiazolidide. As Figure 6 illustrates, enzyme activity was equally reduced for both groups at all time points save 240 min, with the obese animals showing 76 % maximal inhibition at 60 min. For both groups the trend toward increasing DP IV activity was observed over the course of the experiment with 43 % inhibition remaining at 480 min.

![Figure 6](image)

**Figure 6. Time course of DP IV inhibition by Ile-thiazolidide.** DP IV activity was analysed over the course of 480 min following oral administration of Ile-thiazolidide to obese (▲) or lean (■) Zucker rats. Enzyme activity was assessed as outlined in Materials and Methods. Data are presented as mean ± S.E.M. with n = 3 for obese and lean rats. P < 0.05 for significance (*).
Effect of Inhibitors Administered Prior to OGTT on DP IV Activity in Zucker Rats

Given that maximum inhibition of DP IV activity has been observed to occur 20 - 30 min following inhibitor treatment it was postulated that administering Ile-thiazolidide, Gly-Pro-Ile-thiazolidide, or Pro-Ile-thiazolidide 20 min prior to oral glucose would yield a more pronounced effect on the response to ingested nutrients. Enzyme activity was suppressed as before in both the obese and lean animals with an average nadir of 75.2 % (Fig.10A; data not shown for leans).

Effect of Inhibitors Administered Prior to OGTT on Glucose Tolerance in Zucker Rats

Ile-thiazolidide was given orally 20 min prior to OGTT to investigate its effect on glucose handling in obese and lean Zucker rats. As shown in Figure 7A inhibitor-treated obese rats demonstrated an 82 % increase over basal plasma insulin levels reaching a peak of 141.6 ± 13.6 μU/ml at 10 min followed by a plateau and then decline. A similar profile was observed for control animals with a maximal insulin response of 119.6 ± 15.4 μU/ml however, there was no significant difference between the two groups in either the insulin profile or integrated response (Fig.7A inset). The treated lean Zucker rats demonstrated a more pronounced rise in insulin to a peak value of 37.8 ± 4.0 μU/ml at 10 min, a 340 % increase over basal (Fig.7C). This was followed by a rapid return to near fasting levels. The untreated group showed a similar insulin profile reaching a peak of 29.9 ± 3.0 μU/ml at 20
Figure 7. Effect of oral Ile-thiazolidide administered prior to OGTT on plasma insulin (A, C) and glucose (B, D) in obese (A, B) and lean (C, D) Zucker rats. Plasma was analysed following administration of Ile-thiazolidide (▲) or water (■) 20 min prior to OGTT as outlined in Materials and Methods. The rat insulin assay kit was used for insulin analysis. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (*).
The resulting integrated response for the control animals was significantly lower than those receiving Ile-thiazolidide (Fig. 7C inset). Glucose handling of the OGTT in the obese rats was almost identical between the two groups with no difference in the integrated response (Fig. 7B and inset). In contrast, inhibitor-treated lean rats showed a significantly reduced and earlier peak plasma glucose concentration compared to controls (214.6 ± 7.1 mg/dl at 10 min vs. 267.9 ± 4.5 mg/dl at 30 min) (Fig. 7D). The glucose profiles remained significantly different for the duration of the experiment, and correspondingly, the integrated response was significantly reduced in the treated animals (3586.0 ± 289.8 vs. 5917.6 ± 243.2 mg; Fig. 7D inset).

Two other inhibitors Pro-Ile-thiazolidide and Gly-Pro-Ile-thiazolidide were also tested for their efficacy at suppressing DP IV activity when administered orally 20 min prior to OGTT. Figure 8A illustrates the insulin response of the obese Zucker rat treated with Pro-Ile-thiazolidide or water for controls. The experimental rats showed a rapid rise which peaked at 145.7 ± 17.4 μU/ml 10 min after OGTT while control animals demonstrated a significantly lower maximum insulin response of 98.9 ± 6.5 μU/ml at 5 min. While a plateau in insulin values was observed for both groups between 30 - 60 min, the overall integrated response was 173 % greater in the treated animals compared to controls (Fig. 8A inset). In contrast, the lean Zucker rats demonstrated no difference in insulin profiles with an average peak concentration of 58.3 μU/ml occurring at 10 min and subsequently, no difference in the integrated response (Fig. 8C and inset). Regarding the pattern of insulin release, again the lean animals demonstrated a marked peak and return to fasting levels by 60 min whereas the obese rats showed a more prolonged insulin profile. In keeping with the insulin response to Pro-Ile-thiazolidide, the obese Zucker rats demonstrated both a decreased peak glucose level.
Figure 8. Effect of oral Pro-Ile-thiazolidide administered prior to OGTT on plasma insulin (A, C) and glucose (B, D) in obese (A, B) and lean (C, D) Zucker rats. Plasma was analysed following administration of Ile-thiazolidide (△) or water (■) 20 min prior to OGTT as outlined in Materials and Methods. The rat insulin assay kit was used for insulin analysis. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (*).
(260.5 ± 6.2 vs. 311.8 ± 8.1 mg/dl) and integrated response (4170.6 ± 456.3 vs. 7056.1 ± 344.7 mg) as compared to the untreated group (Fig.8B and inset). A similar result was observed for the lean rats despite their insulin profiles. That is, the peak glucose and integrated response were lowered in the inhibitor-treated animals compared to (Fig.8D and inset). After reaching maximal glucose the treated and untreated groups of obese or lean Zucker rats displayed similar rates of glucose clearance from the circulation.

The second pro-drug, Gly-Pro-Ile-thiazolidide was similarly investigated and as Figure 9A shows the insulin response in obese, treated rats was significantly greater than the untreated group at all time points after the OGTT with a maximal concentration of 218.0 ± 22.5 μU/ml, 96.5 % greater than the peak response in controls. The integrated insulin value was similarly increased over untreated rats (Fig.9A inset). Interestingly, both groups demonstrated an increase in plasma insulin between 30 - 60 min. While the profile for the treated lean animals was not as striking, they did display a significantly higher peak response (66.0 ± 5.6 vs. 53.8 ± 2.8 μU/ml) though this did not translate to an increased integrated insulin value (Fig.9C and inset). The glucose profile for the obese Zucker rats treated with Gly-Pro-Ile-thiazolidide was very similar to that for the Pro-Ile-thiazolidide-treated animals except that the peak values for Gly-Pro-Ile-thiazolidide-treated rats and their controls (280.3 ± 6.6 vs. 331.2 ± 7.9 mg/dl; Fig.9B) were greater than in the previous experiment. The overall pattern of the response however, was alike, as was the observation of a decreased integrated glucose level in treated obese Zucker rats as illustrated in the inset of Figure 9B (4547.1 ± 432.5 vs. 7050.1 ± 522.8 mg). Likewise, the lean rats showed a similar pattern of glucose response to Gly-Pro-Ile-thiazolidide as Pro-Ile-thiazolidide with treated animals demonstrating a reduced peak value compared to the control group (248.0 ± 7.2 vs. 288.8 ±
Figure 9. Effect of oral Gly-Pro-Ile-thiazolidide administered prior to OGTT on plasma insulin (A, C) and glucose (B, D) in obese (A, B) and lean (C, D) Zucker rats. Plasma was analysed following administration of Ile-thiazolidide (▲) or water (■) 20 min prior to OGTT as outlined in Materials and Methods. The rat insulin assay kit was used for insulin analysis. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for control and treated rats. P < 0.05 for significance (†).
6.5 mg/dl; Fig. 9D). The integrated response was reduced by 67.5 % in the treated rats compared to controls (Fig. 9D inset).

A comparison of the effects of the three inhibitors given 20 min prior to OGTT on DP IV activity, insulin response and glucose tolerance in obese Zucker rats is illustrated in Figure 10. Although Ile-thiazolidide was slightly but significantly more effective than the pro-drugs in suppressing DP IV activity, with an average nadir of 75 %, all three were sufficiently potent to affect enzyme function. The insulin profile for Gly-Pro-Ile-thiazolidide-treated obese rats was greater and more prolonged compared to the two other inhibitors however, due to the elevated insulin level at 0 min in rats administered with this drug, the integrated responses were not different between the three groups. Finally, the glucose profiles and integrated responses were similar for the three different treatments.

**DISCUSSION**

DP IV is now well established as a primary enzyme responsible for the cleavage and inactivation of the incretin hormones GIP and GLP-1, therefore it is postulated to have a role in the regulation of expression of the enteroinsular axis. It has previously been reported that exogenous incretins are rapidly degraded *in vivo* but when incubated in the serum of DP IV-negative rats, even after twenty minutes a significant proportion of uncleaved hormones remained (Kieffer *et al.* 1995). In a subsequent study by ourselves it was found that incubation of the specific DP IV inhibitor Ile-thiazolidide with either GIP or GLP-1 in 20 % human serum completely prevented formation of the cleavage products, GIP$_{3-42}$ or GLP-1$_{9-36}$ (Pederson *et al.* 1998). Regarding the effect of DP IV inhibition on plasma insulin and
Figure 10. Comparison of the effects of Ile-thiazolidide (■), Pro-Ile-thiazolidide (▲) and Gly-Pro-Ile-thiazolidide (▼) on DP IV activity (A), and plasma insulin (B) and glucose (C) in obese Zucker rats. Inhibitors were administered 20 min prior to OGTT. Plasma was analysed as outlined in Materials and Methods. The rat insulin assay kit was used for insulin analysis. Integrated responses are shown in the insets. Data are presented as mean ± S.E.M. with n = 6 for all three groups. P < 0.05 for significance (* Gly-Pro-Ile-thiazolidide vs. Ile-thiazolidide; # Gly-Pro-Ile-thiazolidide vs. Pro-Ile-thiazolidide; $ Pro-Ile-thiazolidide vs. Ile-thiazolidide).
glucose responses to a glucose challenge in vivo, it was demonstrated that intraduodenal glucose followed immediately by infusion of Ile-thiazolidide resulted in an earlier insulin peak and improved glucose tolerance (Pauly et al. 1998). Based on these findings the aims of this project were to investigate the efficacy of orally administered Ile-thiazolidide, to compare this with other DP IV inhibitors, and to study their effects on glucose tolerance in the obese Zucker rat.

Initial experiments using a protocol of oral Ile-thiazolidide were carried out using Wistar rats in order to establish if this method was effective in suppressing DP IV activity. Inhibition was rapid and significant, resulting in a lowered peak glucose value (Fig.2). Based on these results further studies were performed using Zucker rats. The colony from which the obese animals were taken displayed not only fasting and stimulated hyperinsulinemia (Figs.4A & C), but also hyperglycemia and impaired glucose tolerance (Figs.4B & D) compared to leans, symptoms of the development of NIDDM. Oral treatment with Ile-thiazolidide resulted in immediate inhibition of enzyme activity in both obese and lean rats and was accompanied by enhanced insulin responses to oral glucose (Figs.4A & C). The insulin profile of inhibitor-treated lean Zucker rats was greater at 10 and 20 min compared to controls and returned to fasting values by 60 min, while the insulin response of the obese animals was more modest in its increase from basal and was significantly more prolonged compared to leans. The decrease in insulin observed after 10 min in the lean group despite the prediction of elevated levels of biologically active incretin suggests the action of a regulatory mechanism preventing inappropriate insulin secretion under such conditions. A falling circulating glucose concentration is likely involved in such a mechanism given the glucose-dependency of the insulinotropic action of GIP and GLP-1 (Jia et al. 1995a; Pederson and Brown 1976; Nathan
et al. 1992). The prolonged insulin response in obese rats may be accounted for by the absence of a glucose threshold for incretin action in these animals (Chan et al. 1984; Chan et al. 1985; Jia et al. 1995b). The corresponding plasma glucose responses demonstrated lowered peak values (improved glucose tolerance) for both inhibitor-treated rat phenotypes and increased glucose clearance in the obese group such that the glucose profile resembled that of the lean group (Figs.4B & D). The significant increase in the integrated insulin response to oral glucose in Ile-thiazolidide-treated obese rats, together with the extrapancreatic actions of the incretins could explain the improvement in glucose tolerance following DP IV inhibition (increased incretin circulating half-life). GLP-1 is known to suppress glucagon release, an important factor in the hyperglucagonemic obese Zucker rat, and may increase glucose uptake by adipocytes (Oben et al. 1991), hepatocytes (Valverde et al. 1994) and myocytes (O'Harte et al. 1997). GIP is thought to have a similar effect on adipocytes (Knapper et al. 1995b) and myocytes (O'Harte et al. 1998), all of which likely contributed to the observed glucose response.

It was apparent therefore, that inhibition of DP IV activity had pronounced effects on the insulin and glucose responses to an OGTT and improved glucose tolerance significantly in the hyperglycemic, insulin resistant obese Zucker rat. It was important then to determine if Ile-thiazolidide was affecting these changes directly, e.g. by directly stimulating insulin secretion, or secondarily via its action on the enteroinsular axis. Experiments were performed as before with the exception that inhibitor or water was orally administered in the absence of an OGTT. Suppression of enzyme activity was observed as before however, there was no change in the insulin or glucose responses or in the integrated values between the treated and
control groups, lending support to the hypothesis that inhibition of DP IV catalytic activity enhances expression of the enteroinsular axis via an incretin-mediated process (Fig. 5).

It was consistently found that inhibition of DP IV by Ile-thiazolidide was sustained for the duration of the experiment, therefore it was of interest to establish a time course of DP IV suppression over several hours. The duration of suppression of DP IV activity by a single oral dose of inhibitor is also important in the consideration of Ile-thiazolidide as an anti-diabetogenic drug. Circulating enzyme activity recovered slowly such that at 8 hrs, DP IV inhibition in both obese and lean Zucker rats had decreased to 43 %, from 74 %, reflecting the reversible nature of the action of Ile-thiazolidide (Fig. 6).

Since maximum inhibition of DP IV activity occurs between 20 - 30 min after oral inhibitor, it was postulated that administering the enzyme inhibitor 20 min prior to OGTT would allow for a more immediate and enhanced response of the enteroinsular axis to the subsequent glucose load. The initial investigation was performed administering Ile-thiazolidide 20 min prior to glucose. DP IV activity was inhibited to the same degree as in previous studies (Ile-thiazolidide and glucose administered simultaneously; Fig. 10A vs. Fig. 3). The insulin profile and integrated responses were not different between treated and control obese Zucker rats (Fig. 7A). This was largely due to elevated values in the control group, although the trend was towards enhanced insulin secretion following inhibitor administration. Elevated insulin levels in inhibitor-treated animals however, did not result in improved glucose handling for reasons which are not entirely clear (Fig. 7B). Experimental error and the relatively small number of animals may have concealed an increased insulin response in the obese rats. Conversely in the lean group, the small but significant increase in the integrated response resulted in lowered glucose values (Figs. 7C & D). Undoubtedly
however, the insulin-independent extrapancreatic actions of the incretins also contributed to the observed glucose effect.

Similar to Ile-thiazolidide, administration of Pro-Ile-thiazolidide both inhibited DP IV activity and had a more pronounced effect on plasma insulin in obese compared to lean animals (Figs.10A, 8A & C). The insulin response to the OGTT was relatively rapid and significantly greater in inhibitor-treated versus controls, accounting for the improved glucose tolerance in this group (Fig.8B). There was no change in the insulin profile of treated lean animals however, while the corresponding glucose response was improved. Again, the insulin-independent actions of GIP and GLP-1 may account for these results (Fig.8D).

The effects of Gly-Pro-Ile-thiazolidide were comparable to those of Pro-Ile-thiazolidide. Again, inhibition of DP IV was rapid and significant (Fig.10A). The insulin response in inhibitor-treated obese Zucker rats was greater than in control animals, resulting in lowered plasma glucose values (Figs.9A & B). The treated lean rats demonstrated a small increase in peak insulin values compared to controls, which probably acted in concert with the extrapancreatic actions of GIP and GLP-1 to affect the change in plasma glucose following the OGTT.

The insulin responses of obese Zucker rats to pre-treatment with DP IV inhibitor were uniformly more rapid than when the inhibitor was administered at 0 min. This was presumably due to a “priming” effect of the enteroinsular axis. Because maximum inhibition of DP IV activity has been observed to occur between 20 - 30 min it was postulated that administering enzyme inhibitor prior to OGTT would allow for a more immediate and enhanced response of the enteroinsular axis to the ingested nutrients. At the time of glucose loading, DP IV activity had reached its nadir such that incretins released post-prandially
would be less prone to degradation and a greater portion would retain insulinotropic action. Thus, after nutrient ingestion there would be a decreased time lag between enzyme inhibition and peak insulin response. As with the 0 min protocol however, the insulin profile of the obese rats was generally more prolonged than that of the lean animals for reasons postulated previously.

Although the results indicate a significant difference in DP IV inhibition between the three drugs, when considered in terms of enzyme activity relative to basal, the results are nearly the same. The insulin profile showed Gly-Pro-Ile-thiazolidide to enhance secretion to a greater extent than either Pro-Ile-thiazolidide or Ile-thiazolidide. Taken as change in insulin secretion compared to basal, the difference is not as pronounced. This is supported by the integrated responses which were not different between treatment groups as well as the similarity in glucose responses and integrated glucose levels.

The rationale for synthesising the pro-drugs was the hypothesis that DP IV activity may vary throughout the day. Because these drugs remain inactive until cleavage of the N-terminal peptide or dipeptide, these inhibitors would become active only when DP IV levels increased. Therefore, DP IV would essentially regulate its own activity throughout the day by cleaving and activating an appropriate amount of inhibitor. DP IV expression over 24 hrs has not been investigated but would provide useful information into the value of the pro-drugs as potential therapeutic agents in the treatment of NIDDM.
CHAPTER 2: DP IV EXPRESSION ON PANCREATIC β CELLS

PROJECT RATIONALE

Reports of the presence of DP IV within the pancreatic islet and specifically within the α cell led to the investigation of the β cell as another possible location of DP IV considering the role of the enzyme in incretin degradation and the β cell as the target of these hormones. Since NIDDM is often associated with obesity, as well as abnormal secretion and impaired action of incretins, the effect of obesity on DP IV expression was also investigated comparing obese and lean Zucker rats.

MATERIALS AND METHODS

Tissue Isolation and Preparation

Fed obese and lean Zucker rats were anesthetised by an intraperitoneal injection of sodium pentobarbitol (65 mg/kg). The pancreas was surgically removed and immediately washed in ice cold phosphate buffered saline (PBS) pH 7.4. For studies on the effect of DP IV inhibitors, the drug was administered orally to both obese and lean rats according to the previous protocol at time -20 min. The animal was then anaesthetised, the pancreas removed at 0 min and washed. The pancreas was then fixed for 30 min in 4 % paraformaldehyde (0.1 M phosphate buffer, pH 7.4) followed by immersion in 20 % sucrose in PBS. Each pancreas was placed in OCT compound (Fisher Scientific), frozen in liquid nitrogen-chilled isopentane (~ -60 °C) and stored at -70 °C for immunohistochemical analysis.
**Immunohistochemistry (IHC)**

Cryostat sections (20 μm) were mounted on 3-aminopropyl-triethoxysilane (APES; Sigma) coated slides and the slides heated overnight at 36 °C. The slides were washed 3 x 10 min in PBS followed by 30 min immersion in PBS with 50 mM NH₄Cl and 50 mM glycine. After rinsing with PBS, the slides were incubated in PBS containing 10 % horse serum for 60 min at 4 °C, rinsed again in PBS and incubated overnight with anti-DP IV immunoglobulins (IgG) at 4 °C (see Table 1). The slides were incubated with a fluorochrome conjugated anti-IgG antibody for 60 min at room temperature in order to localise bound primary antibodies. Sections were then double labelled with an anti-insulin IgG. Bound insulin antibodies were localised using a fluorochrome conjugated anti-guinea pig IgG. Details of the antibodies used are outlined in Table 1. After the final incubation, slides were again washed in PBS and glass coverslips applied with PBS:glycine (1:9).

**Fluorescence Microscopy**

All slides were viewed under a Zeiss axiophot microscope using a 488 nm excitation filter to visualise FITC-conjugated antibodies, and 568 nm filter for Cy3 and rhodamine-conjugated antibodies. Images were then imported into Adobe Photoshop (Adobe Systems, Inc., San Jose CA) for colourisation.
Table 1. Details of primary and secondary antibodies

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PharMingen, Pharmingen Canada; Santa Cruz, Santa Cruz Biotechnology; RPG, Regulatory Peptides Group; Cy3, indocarbocyanine/cyanine dye 3; Jackson, Jackson Immunoresearch Laboratories; FITC, fluorescein isothiocyanate; Vector, Vector Laboratories.

RESULTS

Since GIP and GLP-1 are subject to DP IV cleavage, experiments were carried out to determine if DP IV was synthesised by the pancreatic β cell, a major target of incretins. In both obese and lean Zucker rats co-localisation of DP IV and insulin was observed in the β cell with the pattern of staining indicating a cytoplasmic location (Fig.11). The exocrine pancreas did not exhibit DP IV-immunoreactivity (IR). There was no difference between the two rat phenotypes with regard to the localisation of DP IV and insulin or the intensity of staining, and virtually all islets demonstrated co-localisation of DP IV and insulin. Pre-incubation of the polyclonal anti-DP IV IgG with pure DP IV entirely eliminated DP IV-IR but did not affect insulin-IR (data not shown).

Zucker rat islets were also immunostained using a mouse monoclonal antibody directed against DP IV. Results with this antibody were comparable to those obtained using
Figure 11. Co-localisation of DP IV and insulin within the same islet of a Zucker rat. Photomicrographs of an islet stained simultaneously for DP IV (A) using a polyclonal antibody (C-19) and insulin (B) using guinea pig antisera (GP01). Superimposition (C) shows co-localisation of DP IV and insulin in yellow.
the goat polyclonal antibody. Again there was no difference in staining pattern between obese and lean animals (Fig. 12). A small percentage of β cells did not contain DP IV, though DP IV-IR was not observed in the absence of insulin staining, suggesting a lack of DP IV in α and δ cells. While all islets showed the presence of insulin, slightly fewer contained DP IV. Pre-incubation of the monoclonal antibody with purified DP IV, as before, completely abolished DP IV staining but had no effect on insulin-IR (data not shown). As a negative control, pancreata from DP IV-negative rats were obtained and investigated using IHC. While the islet showed the presence of insulin, no specific staining for DP IV in the β cell was observed (Fig. 13).

**DISCUSSION**

DP IV is known to have ubiquitous distribution both as a membrane bound molecule on T lymphocytes and the brush border membranes of enterocytes for example, and also in soluble form in urine and plasma. A study by Mentzel *et al.* (1996) localised DP IV to the islets of Langerhans but did not identify the specific cell. It was hypothesised that since the β cell is a major target of incretins, DP IV may be located there as part of the incretin recognition and regulation process. Indeed, it was demonstrated using two separate antibodies directed towards the enzyme, that DP IV was co-localised with insulin in the β cell (Figs. 11 & 12). In order to determine the precise intracellular location of the enzyme, electron microscopy would have to be done. Using this technique, Poulsen *et al.* (1993) reported the presence of DP IV within the secretory granules of the glucagon secreting cell of pig. These apparently conflicting results may be accounted for by species differences in DP IV expression within the islet. Because the enzyme was observed to be present within
Figure 12: Co-localisation of DP IV and insulin within the same islet of a Zucker rat. Photomicrographs of an islet stained simultaneously for DP IV (A) using a monoclonal antibody (M-A261) and insulin (B) using guinea pig antiserum (GP01). Superimposition (C) shows co-localisation of DP IV and insulin in yellow.
Figure 13. Co-localisation of DP IV and insulin within the same islet of a DP IV-negative rat. Photomicrographs of an islet stained simultaneously for DP IV (A) using a monoclonal antibody (M-A261) and insulin (B) using guinea pig antisera (GP01). Arrows indicate individual β cells.
the β cell and not solely membrane bound, the question of how it interacts with GIP and GLP-1 to attenuate insulin release is raised. DP IV may be synthesised by the cell and transported to the plasma membrane where it could cleave and inactivate the incretins. More likely however, is the possibility that, as in the α cell, DP IV is localised to the secretory granules and co-released with insulin. In the circulation, it could degrade the two hormones thereby preventing inappropriate levels of insulin secretion.
Future Directions

Alternative therapies for NIDDM have recently received increasing interest, and manipulating the interaction of DP IV and incretins may have an important role. Results from this thesis and the work of others, point to a variety of research directions.

Cellular location of DP IV

Poulsen and colleagues (1993) reported the presence of DP IV in the secretory granules of the α cell. While results presented here show the enzyme to be localised within the β cell, the specific cellular location remains unknown. It is of interest then to investigate the β cell further using electron microscopy which may enable conclusions to be made regarding the interaction of DP IV and incretins at their target cell.

DP IV and incretin binding

Although DP IV was observed within the β cell it may be sorted to the plasma membrane and could potentially act there to influence incretin binding and signal transduction. Okamoto an co-workers (1994) studied such an interaction between the substance P receptor (SPR) and neural endopeptidase 24.11 (NEP 24.11), one of the enzymes responsible for degradation of SP. The group showed that when the receptor and enzyme were expressed in the same cell, the rate of SP cleavage was significantly increased, while receptor binding and increase in intracellular calcium were markedly decreased. This was compared to SPR and NEP 24.11 expression on separate cells, expression of SPR alone
and use of an NEP 24.11 inhibitor. Of interest is the fact that DP IV is also known to degrade circulating SP (Ahmad et al., 1992) and may therefore act in a similar manner at the β cell to cleave and prevent the binding and signal transduction of GIP and GLP-1. For example, changes in GIP receptor binding and signalling following co-expression of DP IV and GIP receptor in a cell line may indicate a DP IV/GIP receptor interaction.

**Side effects of DP IV inhibitors**

As mentioned previously DP IV is known to cleave a number of biologically active peptides including substance P, neuropeptide Y and GHRH. The effect of short term enzyme inhibition on the actions of these peptides remains unknown. Using a protocol of repeated daily doses of Ile-thiazolidide to obese Zucker rats, no outward manifestations of malaise were observed by ourselves however, any physiological changes would have been undetected. Investigating the effects of sustained inhibition of DP IV on these peptides and the biological processes in which they are involved would provide further information regarding the overall value of this enzyme as a potential therapeutic agent.

**Inhibition of DP IV after a meal**

Studies in this thesis focused on improvements in glucose tolerance following administration of DP IV inhibitor and oral glucose. Of interest is whether the results would be similar following oral nutrients or a mixed meal since both are physiological stimuli of incretin release. Additionally, the effect on glucose handling following a dose of inhibitor and glucose or nutrients to fed rats would provide data regarding the time of inhibitor administration relative to eating which produces the greatest improvement in glucose
tolerance. Changes in DP IV levels throughout the day also would provide useful information as to the value of the pro-drugs, Gly-Pro-Ile-thiazolidide and Pro-Ile-thiazolidide, as a potential therapy in the treatment of NIDDM.

**DP IV-resistant incretin analogues**

A major disadvantage to administering exogenous incretins in their native form as a potential therapy is the rapidity with which they are degraded in the circulation and the ineffectiveness of oral administration. Because of the specificity of the action of DP IV- it requires a free, unprotonated N-terminus with the two terminal amino acids to be in the L-confirmation- interest has recently focused on amino acid substitutions which would render the peptide DP IV-resistant. Frohman *et al.* (1989) originally reported that D-Ala\(^2\)-GHRH\(_{1-29}\), D-Tyr\(^1\)-GHRH\(_{1-29}\) and desNH\(_2\)Tyr\(^1\)-GHRH\(_{1-44}\) were completely resistant to DP IV catalysis. Studies in this lab have shown D-Ala\(^2\)-GIP\(_{1-42}\) to remain intact following incubation with purified porcine kidney DP IV or human serum. Furthermore, GLP\(_{7-37}\)-I-V (an analogue of GLP-1 with valine substituted for alanine at position 8), D-Ala\(^9\)-GLP-1\(_{7-36}\) amide and D-Ala\(^2\)-GIP\(_{1-30}\) amide have been observed to increase glucose clearance compared to the native peptide (unpublished observations). Similar observations have been reported for modified GLP-1 analogues which retained biological activity but were resistant to DP IV activity resulting in prolonged metabolic stability (Deacon *et al.* 1998b). Additional experiments are required to establish the administration protocol most effective in improving glucose tolerance.
Summary

The studies in this project were performed to gain insight into the effects of DP IV inhibition on glucose tolerance in an animal model of NIDDM, and to investigate the islet distribution of DP IV. Administration of oral inhibitor both prior to and with glucose suppressed enzyme activity, enhanced in the insulin response and improved glucose tolerance in obese Zucker rats (with the exception of Ile-thiazolidide given at -20 min). The protocol of inhibitor administration prior to glucose resulted in a more rapid insulin response in the obese Zucker rat, presumably due to a "priming" effect of the enteroinsular axis. Using IHC, DP IV was found to be co-localised with insulin in the β cell. These results indicate that inhibition of DP IV significantly improves glucose handling in diabetic animals and may therefore have a role in NIDDM therapy. The presence of DP IV in the β cell may be more important in the euglycemic state when it may act in part to degrade incretins, thereby preventing inappropriate levels of insulin secretion post-prandially.
REFERENCES


