

Dipeptidyl peptidase-4 inhibition and the treatment of type 2 diabetes: Preclinical
biology and mechanisms of action

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Dipeptidyl peptidase-4 (DPP4) is a complex enzyme that exists as a membrane-anchored cell surface peptidase that transmits intracellular signals via a short intracellular tail, and a second smaller soluble form present in the circulation. DPP4 cleaves a large number of chemokines and peptide hormones *in vitro*, but comparatively fewer peptides have been identified as endogenous physiological substrates for DPP4 *in vivo*. Both glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are endogenous physiological substrates for DPP4 and chemical inhibition of DPP4 activity, or genetic inactivation of DPP4 in rodents, results in increased levels of intact bioactive GIP and GLP-1. Furthermore, mice and rats with genetic inactivation or inhibition of DPP4 exhibit improved glucose tolerance, elevated levels of GLP-1 and GIP and resistance to diet-induced obesity and hyperglycemia. Sustained DPP4 inhibition lowers blood glucose via stimulation of insulin and inhibition of glucagon secretion and is associated with preservation of β -cell mass in preclinical studies. Although DPP4 cleaves dozens of regulatory peptides and chemokines *in vitro*, studies of mice with genetic inactivation of incretin receptors demonstrate that GIP and GLP-1 receptor-dependent pathways represent the dominant mechanisms transducing the glucoregulatory actions of DPP4 inhibitors *in vivo*. The available preclinical data suggests that highly selective DPP4 inhibition represents an effective and safe strategy for the therapy of type 2 diabetes.

Dipeptidyl peptidase-4 (DPP4) is a widely expressed cell surface peptidase that exhibits a complex biology encompassing cell membrane-associated activation of intracellular signal transduction pathways, cell-cell interaction, and enzymatic activity exhibited by both the membrane-anchored

and soluble forms of the enzyme (1). DPP4, also originally known as the lymphocyte cell surface marker CD26, or as the adenosine deaminase (ADA)-binding protein, is a 766 amino acid serine protease that preferentially cleaves peptide hormones containing a position 2 alanine or proline. The human gene encoding DPP has been localized to chromosome 2 locus 2q24.3 (2). The majority of the DPP4 protein is extracellular, with a hydrophobic transmembrane sequence (amino acids 7-28) anchoring the protein in the cell membrane, followed by a very short 6 amino acid intracellular sequence. DPP4 is found on the cell surface as a glycosylated homodimer; however glycosylation does not appear to be essential for enzymatic activity or binding of adenosine deaminase (ADA). The catalytic region encompasses amino acids 511-766 and is also present in a soluble form of DPP4 (sDPP4), which is comprised of the majority of the extracellular DPP4 protein (amino acids 39-766) (3). sDPP4 is capable of exhibiting enzymatic activity and interacting with the mannose-6-phosphate/insulin-like growth factor-II receptor (M6P-IGFIIR) on specific cell types (4). The wide tissue distribution of DPP4 on numerous cell types and in different vascular beds and its presence as a soluble active enzyme in the circulation ensures that DPP4-mediated proteolysis is a common event in most tissue compartments.

DPP4 is a member of a complex gene family (Figure 1), many members of which also cleave structurally-related peptides (5; 6). The DPP4-related enzymes (Figure 1) include seprase, fibroblast activation protein alpha, DPP6, DPP8, DPP9, attractin, N-acetylated-alpha-linked-acidic dipeptidases I, II and L, quiescent cell proline dipeptidase, thymus-specific serine protease and DPP 4-beta (7). ADA immunoaffinity chromatography, which selectively binds and sequesters DPP4, removed the majority (95%) of DPP4-like enzymatic activity present in human plasma,

thereby identifying DPP4 as the predominant enzyme responsible for X-Pro or X-Ala cleavage in human serum (3). The multiple members of the DPP4 family mandates a careful assessment of the selectivity and specificity of any agent used to inhibit DPP4 activity (8).

DPP4 and the inactivation of incretin hormones

Circulating levels of DPP4 activity have been reported to be higher in some studies of subjects with chronic hyperglycemia and type 2 diabetes (9; 10); however whether circulating DPP4 activity correlates with the levels of active plasma GLP-1 in individual human subjects is not known. The observation that DPP4 was capable of cleaving the incretin peptides GIP and GLP-1 in human serum *in vitro*, together with the demonstration that chemical inhibitors of DPP4 prevented the degradation of GIP and GLP-1, firmly established the importance of DPP4 as a critical determinant of incretin inactivation (11). Subsequent studies demonstrated reduced cleavage of intact GLP-1(7-36)amide and GIP(1-42) in serum from DPP4-deficient rats *in vitro* or following infusion of the peptides into DPP4-deficient rats *in vivo*, providing complementary evidence for the importance of DPP4 in the control of incretin inactivation (12). Moreover, both GLP-1(7-36)amide and the N-terminal DPP4-generated metabolite GLP-1(9-36)amide were identified in plasma from both fasted and fed humans, and inhibitors of DPP4 prevented the conversion of GLP-1(7-36)amide to GLP-1(9-36)amide in human plasma *in vitro* (13). Similarly, the majority of circulating immunoreactive GIP in human plasma is the N-terminally cleaved GIP(3-42) peptide, accounting for over 70% of total plasma GIP immunoreactivity in the fasting state, and 58% of total GIP after meal ingestion (14). Furthermore, exogenous administration of either GIP or GLP-1 via the subcutaneous or intravenous routes was

associated with the rapid degradation of both peptides within minutes to the DPP4 metabolites GIP(3-42) and GLP-1(9-36)amide, respectively. Hence, DPP4 is a principal determinant of the circulating $t_{1/2}$ of intact bioactive GIP and GLP-1 (14; 15).

DPP4 inhibitors lower blood glucose

Related studies examined the effects of chemical inhibitors of DPP4 enzymatic activity on the structure and activity of GLP-1 in normal animals and in experimental models of diabetes. The non-selective DPP4 inhibitor valine pyrrolidide (VP) prevented the degradation of GLP-1 and GIP in anesthetized pigs and potentiated the incretin-mediated reduction of plasma glucose and stimulation of insulin secretion in response to an intravenous glucose challenge (16; 17). Similarly, VP acutely improved oral glucose tolerance in high fat-fed pigs, in association with increased levels of intact GLP-1 and increased levels of plasma insulin following oral glucose loading (18). A series of related studies then demonstrated that inhibition of DPP4 activity preserved levels of intact GLP-1, and improved glucose tolerance in normal and diabetic rats and mice (19-26), in association with enhanced glucose-stimulated insulin secretion in islets isolated from DPP4i-treated mice (25)

Verification that DPP4 was the dominant molecular target for the glucose lowering properties of NVP-DPP728 was illustrated in studies demonstrating that this compound acutely lowered blood glucose following oral glucose challenge in wildtype Wistar rats but not in Fischer 344 rats with an inactivating mutation in the DPP4 gene (27). Nevertheless, DPP4 inhibition is not capable of exerting significant anti-diabetic actions in all preclinical models, as acute VP administration increased plasma levels of intact GLP-1 in older db/db mice, but VP did not lower blood glucose in 24 week old severely hyperglycemic (fasting blood glucose of 29 mM) db/db mice (28).

DPP-4 inhibitors, β -cell mass and survival

DPP4 inhibitors exhibit favorable actions on islet and β -cell mass, morphology, and survival. Wistar rats treated with streptozotocin and twice daily P32/98 for 7 weeks exhibited increased body weight, lowered fed blood glucose, and increased levels of plasma insulin (29). Furthermore P32/98 improved glucose tolerance, enhanced glucose-stimulated insulin release in perfused pancreas experiments, and increased pancreatic insulin content. Histological analyses demonstrated an increased number of small islets and a greater proportion of β -cells within islets in rats treated with P32/98 (29).

The DPP4 inhibitor des-fluoro-sitagliptin (DFS) significantly reduced ambient and fed blood glucose, and levels of HbA1c in diabetic ICR mice, in association with decreased liver weight and reduced levels of hepatic and plasma triglycerides and plasma free fatty acids (30). Furthermore DFS-treated animals exhibited increased β -cell mass, and a reduction in the α -cell: β -cell ratio. A head to head comparison of glipizide vs. DFS for 10 weeks in diabetic mice demonstrated comparatively greater improvement of glycemia and HbA1c in DFS-treated mice, and improvements in pancreatic insulin content and relative β -cell area were observed in mice treated with DFS but not in glipizide-treated animals (30). Furthermore, islets isolated from DFS-treated mice exhibited improved insulin secretion in response to KCl or glucose and increased islet insulin content (30).

A comparative study of the DPP4 inhibitor Vildagliptin vs. the GLP-1R agonist Liraglutide was carried out in candy-fed rats for 12 weeks (31). Liraglutide reduced food intake and attenuated weight gain, however there were no major differences in glucose or HbA1c in the two treatment arms, whereas plasma insulin levels were significantly higher in rats treated with Vildagliptin (31).

Both Vildagliptin- and Liraglutide-treated rats exhibited a relative normalization of β -cell mass compared to vehicle-treated candy-fed rats.

The role of endogenous DPP4; studies in rats and mice with inactivating DPP4 mutations

The biological importance of DPP4 has been examined in rats with a naturally occurring loss of function mutation in the DPP4 gene and in mice with targeted genetic inactivation of DPP4. A strain of Fischer 344 (F344) rats originally identified in Japan harbor a Gly633-Arg mutation in the DPP4 gene within the active site of the enzyme. The mutant DPP4 protein is synthesized appropriately yet is not exported out of the endoplasmic reticulum and is rapidly degraded without being processed to the mature active enzyme (32-34). Subsequent studies identified heterogeneity in baseline levels of DPP4 activity in different inbred rat strains, emphasizing the importance of careful characterization of enzymatic activity in different rodent models (35). F344 rats exhibit improved glucose tolerance, and increased levels of plasma GLP-1 and insulin following oral glucose challenge. Furthermore, high fat-feeding of F344 rats for 7 weeks was associated with reduced weight gain, increased levels of intact GLP-1, improved glucose tolerance, and enhanced insulin sensitivity as assessed by homeostatic model assessment (HOMA) (36). Hence, loss of DPP4 activity in rats is associated with potentiation of endogenous GLP-1 action and improvement of glucose tolerance.

Targeted inactivation of the DPP4 gene in mice also leads to increased plasma levels of GIP, GLP-1 and insulin, and reduced glycemic excursion following oral glucose challenge (37). Consistent with findings in DPP4 deficient rats, DPP4^{-/-} mice exhibit resistance to diet-induced obesity, reduced fat accumulation, decreased plasma levels of leptin, and reduced food intake yet increased

energy expenditure on a high fat diet (38). DPP4^{-/-} mice appear to be more insulin sensitive and fail to develop hyperinsulinemia, hepatic steatosis or islet hyperplasia after high fat feeding. Moreover, DPP4^{-/-} mice were resistant to the development of streptozotocin-induced diabetes following a single injection of streptozotocin (38).

The importance of DPP4 for control of immune function and behavior has also been examined in F344 rats and DPP4^{-/-} mice. DPP4-deficient rats exhibited increased pain sensitivity, reduced stress-like responses, and decreased susceptibility to the sedative effects of ethanol (39). Furthermore, splenocytes isolated from DPP4-deficient rats exhibited decreased NK cell-mediated tumor lysis using syngeneic MADB106 tumor cells as antigen (35). Modest yet detectable abnormalities in immune responses and behavior have also been described in DPP4^{-/-} mice, including changes in stress-associated mobility, curiosity, and exploratory behaviors (40). In separate studies, the relative number of CD4⁺ T cells was lower and NK cells was higher in spleen cells, and the numbers of circulating CD4⁺ NKT lymphocytes were reduced in DPP4^{-/-} mice (41). Furthermore, IL-4 production was significantly reduced and levels of IL-10 and interferon- γ were increased following stimulation with pokeweed mitogen in splenic DPP4^{-/-} lymphocytes. Following immunization with pokeweed mitogen, serum levels of total IgG, IgG1, IgG2, and IgE were significantly lower, accompanied by lower levels of plasma cytokines, in serum from DPP4^{-/-} mice (41). Analysis of nociceptive responses revealed reduced latencies to stimuli such as the hot plate or tail pinch test, in association with increased plasma levels of substance P. The abnormal latencies were abolished following treatment of DPP4^{-/-} mice with a substance P (neurokinin 1) receptor antagonist, and administration of two different DPP4 inhibitors reduced latencies in wildtype mice

(41). These findings implicate a role for DPP4 as a critical regulator of substance P-mediated inflammatory responses *in vivo*.

A role for DPP4 in the modulation of the inflammatory response is suggested by differences in the severity of experimental arthritis in wildtype vs. DPP4^{-/-} mice. Antigen-induced arthritis (AIA) and plasma levels of the proinflammatory chemokine stromal cell-derived factor-1 (SDF-1) were significantly increased in DPP4^{-/-} mice, in association with increased numbers of SDF-1 receptor (CXCR4)-positive cells infiltrating arthritic joints. Furthermore, plasma DPP4 activity was reduced in wildtype mice with AIA and in human subjects with rheumatoid arthritis (RA), and the levels of circulating DPP4 and DPP4 activity were inversely correlated with the severity of RA in affected subjects (42). Taken together, the data clearly implicate a role for DPP4 in the control of immune function, inflammatory responses, and behavior. However whether these phenotypes can be selectively ascribed to loss of the catalytic activity of the enzyme, or more generalized loss of DPP4 function independent of the catalytic activity, cannot yet be determined.

Importance of DPP4-selective inhibition

Although experimental results obtained using non-selective DPP4 inhibitors implicated a role for DPP4 in the control of immune regulation, transplantation biology, cancer cell growth and metastasis (43; 44), there is limited data for similar studies utilizing highly-selective DPP4-inhibitors that have been generated for the treatment of type 2 diabetes. More recent experiments comparing the actions of DPP4-selective vs. non-selective inhibitors suggests that preferential inhibition of DPP8/9 and QPP *in vivo* was associated with a species and tissue-specific profile of different toxicities. Inhibition of DPP8/9 produced alopecia, thrombocytopenia, splenomegaly, thrombocytopenia, and multiorgan pathology

leading to death in rats, and gastrointestinal toxicity in dogs. Moreover, similar toxicities were observed in wildtype and DPP4^{-/-} mice treated with DPP8/9 inhibitors (8). In contrast, inhibition of the related enzyme QPP produced reticulocytopenia in rats, whereas selective inhibition of DPP4 was not associated with detectable toxicity in rats or dogs (8). Similarly, inhibition of DPP8/9, but not DPP4, was associated with reduction of mitogen-stimulated proliferation of human mononuclear cells *in vitro* (8). Curiously, some but not all DPP4 inhibitors have been reported to produce skin lesions in monkey studies. The extent to which these findings reflect differential selectivity of specific agents for the money enzymes and whether the lesions are completely attributable to non-DPP4-dependent mechanisms, remains poorly understood. Collectively, these findings illustrate that data obtained using non-selective DPP inhibitors needs to be interpreted with caution in regard to the putative role of DPP-4 in the development of specific organ pathologies.

DPP-4 Substrates and reduced DPP4 enzyme activity: Physiology versus Pharmacology

Numerous endocrine peptides, chemokines and neuropeptides contain an alanine or proline at position 2, and are putative DPP4 substrates (Table 1). An endogenous physiological DPP4 substrate is defined as a peptide whose endogenous circulating levels of intact vs. N-terminally cleaved forms are altered following reduction or elimination of DPP-4 activity *in vivo*. For the majority of peptides listed in Table 2, it is reasonable to assume that they may be pharmacological substrates, as DPP4 produces N-terminal cleavage of the peptide(s) *in vitro*. In contrast, there is limited evidence that the majority of these peptides are physiological substrates. Moreover, even small changes in the ratios of intact:cleaved peptide for physiological DPP4 substrates may not always be sufficient to

produce predicted biological changes in specific target tissues, as discussed below.

Both GIP and GLP-1 are physiological substrates for DPP4, as DPP4 inhibition is associated with increased circulating levels of intact GIP and GLP-1 *in vivo* (16; 17), and levels of intact GIP and GLP-1 are increased, relative to their N-terminally cleaved forms, in rats and mice with inactivating DPP4 gene mutations (37). Similarly, the chemokines SDF-1 α and SDF-1 β are cleaved by DPP4 at the N-terminus, and plasma levels of intact SDF-1 α (1-67) are increased in DPP4^{-/-} mice (42). Hence, endogenous levels of intact SDF-1 are clearly dependent on DPP-4 activity.

Substance P may also be a physiological substrate for DPP4. Levels of tissue DPP4 are reduced in nasal tissue of human subjects with chronic rhinosinusitis and the vasodilatory effects of substance P are attenuated by DPP4 *in vivo* (45). Conversely, DPP4 inhibition potentiates the vasodilatory effects of exogenous substance P, findings consistent with reports of nasopharyngitis in human subjects treated with DPP4 inhibitors (46; 47). Moreover, plasma levels of substance P were more than 2-fold higher in DPP4^{-/-} vs. DPP4^{+/+} mice (48). Hence, substance P fulfills the criteria for an endogenous substrate of DPP4. Whether clinically meaningful increases in the levels of SDF-1 or substance P occur in humans following partial reduction of DPP4 activity with selective DPP4 inhibitors remains uncertain.

Although the majority of peptide hormones listed in Table 1 may be cleaved by DPP4 *in vitro*, the endogenous levels of intact:cleaved peptide may not be significantly different in DPP4^{-/-} vs. DPP4^{+/+} mice or rats, or following administration of DPP4 inhibitors *in vivo*. For example, glucagon is cleaved by DPP4 *in vitro* to yield glucagon(3-29) and this cleavage is inhibited by the DPP4 inhibitor isoleucine thiazolidide (49) however increased plasma levels of intact vs. cleaved glucagon have not been reported following

administration of DPP-4 inhibitors *in vivo*, or in rats or mice with inactivating mutations of the DPP4 gene.

GLP-2(1-33) is cleaved by DPP4 at the position 2 alanine both *in vitro*, and following exogenous administration *in vivo* leading to the generation of GLP-2(1-33). (50; 51). Moreover, DPP4-resistant GLP-2 analogs exhibit much greater potency, compared to native GLP-2, *in vivo* (50). Nevertheless, although DPP-4 inhibition increased the plasma levels of intact nutrient-stimulated GLP-2(1-33) in rats, chronic administration of the DPP4 inhibitor VP alone had no effect on intestinal growth, a key biological readout of enhanced GLP-2 activity *in vivo* (52).

Growth hormone-releasing hormone (GHRH) was one of the first peptides demonstrated to be a substrate for DPP4 (53). Circulating levels of GHRH are low and difficult to measure in plasma. Nevertheless, increased levels of intact bioactive GHRH in the hypothalamic-pituitary axis would be predicted to stimulate growth hormone secretion leading to increased circulating levels of insulin-like growth factor-1 (IGF-1) and somatic growth. However DPP4^{-/-} mice and Fischer 344 mutant rats do not exhibit increased body size or organ growth. Furthermore, treatment of young pigs for 72 hours with a Sitagliptin analogue that produced 90% inhibition of plasma DPP4 activity was not associated with alterations in the circulating concentrations of IGF-1 (54). Similarly, 10 days of Sitagliptin administration to healthy non-diabetic male subjects did not produce significant elevations in IGF-1 or IGFBP-3 relative to placebo-treated control subjects (55). Hence, DPP4 inhibition may not always produce predictable changes in downstream biological pathways, despite altering the relative levels of intact:cleaved peptide substrates.

Neuropeptide Y (NPY) and peptide YY (PYY) exert opposing actions on control of food intake, and both peptides are cleaved by

DPP4 *in vitro*, resulting in the generation of N-terminally truncated peptides with different receptor affinities. Inhibition of DPP4 activity prevents the generation of the anorectic PYY(3-36) from PYY(1-36), and reduced levels of PYY(3-36) have been detected following infusion of PYY(1-36) into rats treated with a DPP4 inhibitor (56). Although DPP4 is clearly important for cleavage of exogenous PYY(1-36), biologically significant alterations in the levels of endogenous PYY(1-36):PYY(3-36) have not yet been described in rodents or humans with reductions in DPP4 activity. DPP4 cleavage of NPY(1-36) *in vitro* leads to the generation of NPY(3-36) which exhibits a markedly reduced affinity for the orexigenic Y1 receptor but interacts with the Y2/Y5 receptor. Exogenous administration of NPY also exert effects on vasomotor activity, angiogenesis and vascular remodeling, however the importance of endogenous basal levels of NPY(1-36) and NPY(3-36) for control of these activities remains uncertain. Although administration of NPY produces potent anxiolytic and sedative-like effects in DPP4-deficient F344 rats (57), there is little evidence that endogenous circulating or tissue levels of NPY(1-36) vs. NPY(3-36) are significantly altered following reduction of DPP4 activity *in vivo*.

Biological activities of DPP4 not related to control of glucose homeostasis

DPP4 has been implicated in the control of lymphocyte and immune function, cell migration, viral entry, cancer metastasis, and inflammation (reviewed in (1; 58)). DPP4 expression often varies with the state of cellular differentiation, and loss of DPP4 expression has been associated with changes in tumor growth and enhanced metastatic or invasive behavior (59-61). The importance of DPP4 for retention of chemotherapy sensitivity and topoisomerase-2 expression has been mapped, using site-directed mutagenesis, to a region of the protein

essential for its enzymatic activity (62; 63). DPP4/CD26 is expressed at low levels on resting T cells, however DPP4 expression increases following T cell activation. DPP4 functions as a T cell co-stimulatory molecule that enhances antigen-specific T cell proliferation (64) and sDPP4 enhances T cell transendothelial migration *in vitro*, actions which require the catalytic activity of the DPP4 enzyme and a functional M6P-IGFIIR (4).

DPP4 also regulates migration of human cord blood CD34⁺ progenitor cells and the homing and engraftment of hematopoietic stem cells. Inhibition of DPP4 enzymatic activity promotes human hematopoietic stem cell migration and bone marrow engraftment via potentiation of the levels of intact CXCL12/SDF-1 α , a physiological substrate for DPP4 activity (Table 1) (65; 66). Furthermore, inhibition of DPP4 activity enhanced homing and engraftment of bone marrow cells or enriched hematopoietic stem cells in the liver of allogeneic fetal mice following *in utero* hematopoietic-cell transplantation (67), likely due to potentiation of SDF-1 α interaction with the CXCR4 receptor.

The importance of the interaction of human DPP4 with ADA remains incompletely understood. Adenosine exerts acute anti-inflammatory effects during tissue hypoxia, however chronically elevated levels of adenosine may be deleterious in experimental models of inflammation. Experimental hypoxia induces the cell surface expression of both ADA and DPP4 on vascular endothelial cells, and ADA activity is also increased in plasma from hypoxic human subjects (68). However there does not seem to be a correlation between the ability of ADA to bind DPP4, and the development of immunodeficiency in human subjects with ADA mutations (69). Hence, the functional importance, if any, of selective DPP4

inhibition on ADA binding and activity remains obscure.

Biological importance of GLP-1(9-36)amide and GIP(3-42)

Intact GLP-1 and GIP are rapidly cleaved by DPP4 to yield GLP-1(9-36)amide and GIP(3-42) (Figure 2) and the levels of N-terminally truncated incretins are greater than the levels of intact GIP and GLP-1 in both the fasting and postprandial states (13; 14). Following sustained inhibition of DPP4 activity, plasma levels of GLP-1(7-36)amide and GIP(1-42) are increased (70) whereas levels of GLP-1(9-36)amide and GIP(3-42) are substantially decreased. Hence, it seems reasonable to consider whether differences in the ratios of intact:cleaved incretin peptides have biological implications. Although GIP(3-42) may be a weak GIP receptor antagonist *in vitro*, it does not exert glucoregulatory actions *in vivo* (71; 72).

There is considerable evidence that GLP-1(9-36) amide has biological actions *in vivo* (Figure 2). GLP-1(9-36)amide modestly enhances glucose clearance independent of changes in insulin secretion in pigs (73), whereas studies in mice showed no effect of GLP-1(9-36)amide on insulin secretion or glucose clearance (74). GLP-1(9-36)amide had no effect on glucose clearance or insulin secretion in healthy human volunteers following intravenous glucose infusion (75). In contrast, acute infusion of GLP-1(9-36)amide lowered postprandial glucose following meal ingestion independent of changes in levels of insulin or glucagon or gastric emptying in human subjects (76). Although substantial amounts of GLP-1(9-36) are generated following meal ingestion, GLP-1(9-36)amide does not appear to antagonize the glucose-lowering properties of GLP-1(7-36) amide in diabetic human subjects (77). Remarkably, GLP-1(9-36)amide increased myocardial glucose uptake and improved left ventricular function in dogs with pacing-

induced dilated cardiomyopathy (78). The mechanisms through which GLP-1(9-36)amide mediates its emerging biological actions are currently poorly understood and the subject of active investigation.

DPP4 inhibition and reduction of blood glucose: Mechanisms of action

A considerable number of glucoregulatory peptides, in addition to GLP-1 and GIP, have been identified as exogenous substrates susceptible to DPP4 cleavage (Table 1). For example, DPP4 cleaves vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), oxyntomodulin and gastrin-releasing peptide (GRP) (79; 80), and differential metabolism of exogenously infused PACAP38 was observed in wildtype vs. DPP4^{-/-} mice (80). Furthermore, DPP4 inhibition potentiates the insulinotropic response to exogenous PACAP and GRP in mice *in vivo* (81). Hence it is reasonable to postulate that one or more of these peptides, together with GLP-1 and GIP, contribute to the reduction in glycemia observed following acute or chronic DPP4 inhibition.

In contrast, studies in mice with disruption of single incretin receptors, or analysis of mice with combined genetic disruption of both the GIP and GLP-1 receptors (double incretin receptor knockout or DIRKO mice) strongly suggest that GIP and GLP-1 are the principal peptide substrates responsible for transducing the glucose-lowering actions of DPP4 inhibitors (Figure 3). Although DPP4 inhibitors lower blood glucose and stimulate insulin secretion in Gipr^{-/-} or Glp1r^{-/-} mice (37; 82), four different DPP4 inhibitors (DPPi) failed to reduce blood glucose following acute oral glucose challenge in normoglycemic DIRKO mice (82) (Figure 3). To determine the importance of GIP and GLP-1 receptor signaling for the chronic glucoregulatory actions of DPP4 inhibitors, high fat-fed DIRKO mice were treated with Vildagliptin continuously in the drinking water for 8 weeks. Although Vildagliptin

improved insulin secretion and lowered blood glucose in wildtype mice, no effect of Vildagliptin on glucose control or insulin secretion was observed in DIRKO mice (83). Hence the available preclinical data strongly supports the essential importance of the GIP and GLP-1 receptors as dominant mediators for the anti-diabetic actions of DPP4 inhibitors.

DPP4 inhibitors: Current concepts and major unanswered questions

A large number of actions ascribed to inhibition of DPP4 activity were originally delineated in experiments using non-selective DPP4 inhibitors. However many of these inhibitors were subsequently shown to exhibit inhibitory “off target” actions on related proteases in the absence of DPP4 activity (8; 84). Hence the available literature on the pleiotropic effects of DPP4 inhibition using first generation non-selective inhibitors must be interpreted with caution, pending analysis of data from confirmatory experiments carried out using highly selective inhibitors of the DPP4 enzyme. Similarly, although intriguing metabolic, behavioral and immunologic phenotypes have been described in rodents with inactivating mutations in the DPP4 gene, Fischer 344 rats and DPP4^{-/-} mice exhibit a complete loss of DPP4 activity. In contrast, there is little data on these parameters following administration of highly selective DPP4 inhibitors that produce a 50-80% reduction in enzymatic activity. Thus, whether or not biological results obtained with selective DPP4 enzyme inhibitors will be identical to data obtained in studies of rodents with complete absence, during both development and adult life, of a multifunctional DPP4 protein, requires more careful investigation. Furthermore, it will be important to monitor DPP4-treated human subjects carefully for the development of inflammatory conditions, angioedema, rhinitis, and urticaria, given the potential importance

of SDF-1 and/or substance P as DPP4 substrates.

Equally compelling questions arise from attempts to understand how DPP4 inhibitors lower blood glucose in diabetic subjects. The major actions of DPP4 inhibitors *in vivo* include suppression of glucagon secretion and enhancement of insulin secretion, consistent with the known actions of GLP-1 and GIP. Preclinical data in rodents with loss of incretin receptor signaling supports a critical role for the GLP-1 and GIP receptors for transduction of the anti-diabetic actions of DPP4 inhibitors (82; 83). Nevertheless, prolonged DPP4 inhibition in diabetic human subjects may recruit additional as yet unidentified mechanisms that promote glucose lowering. Moreover, the long term consequences of DPP4 inhibition on β -cell function, and the durability of glucose lowering achieved with sustained DPP4 inhibition require careful clinical assessment. Taken together, it seems prudent to pursue additional detailed studies of the biological role(s) of DPP4 and the consequences and

safety of highly selective DPP4 inhibition in experimental and clinical models of diabetes.

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Figure 1

The family of DPP4-related proteases and their substrate specificities is shown. For the majority of enzymes, the biological roles and identity of endogenous substrates remains poorly understood. DPP = dipeptidyl peptidase, FAP = fibroblast activation protein; PEP = Prolyl endopeptidase; QPP = quiescent cell proline dipeptidase; APP = Aminopeptidase P

The DPP4 Protease Family

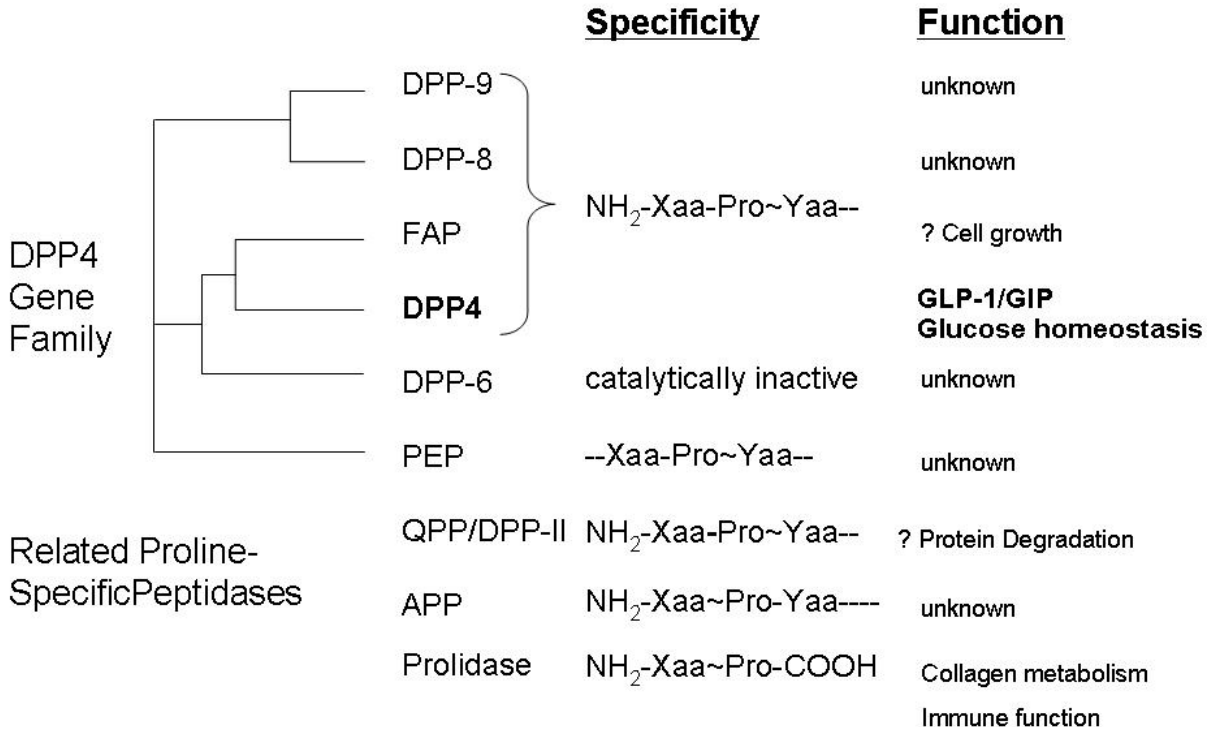


Figure 2

The principal biological actions of the active incretin hormones GLP-1(7-36)amide and GIP (1-42), and the actions of the peptides GLP-1(9-36)amide and GIP(3-42), generated following cleavage by DPP4, are depicted.

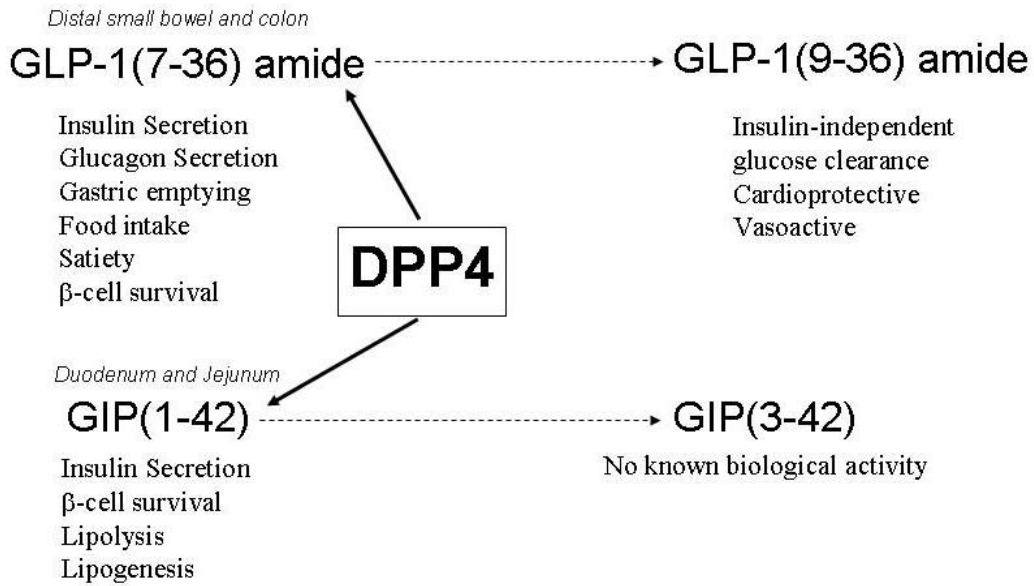


Figure 3

Four different DPP-4 inhibitors (LAF237/Vildagliptin, VP, Syrrx106124, and TP8211) acutely lower blood glucose and enhance glucose-stimulated insulin secretion in wildtype mice, and in mice with targeted disruption of single incretin receptors (*Glp1r*^{-/-} and *Gipr*^{-/-}). In contrast, none of the DPP4 inhibitors lowers blood glucose or stimulates insulin secretion in mice with genetic disruption of both the GIP and GLP-1 receptors (dual incretin receptor knockout or DIRKO). Data shown is from experiments using VP, however identical results were obtained with the 3 other DPP4 inhibitors, as described in (82).

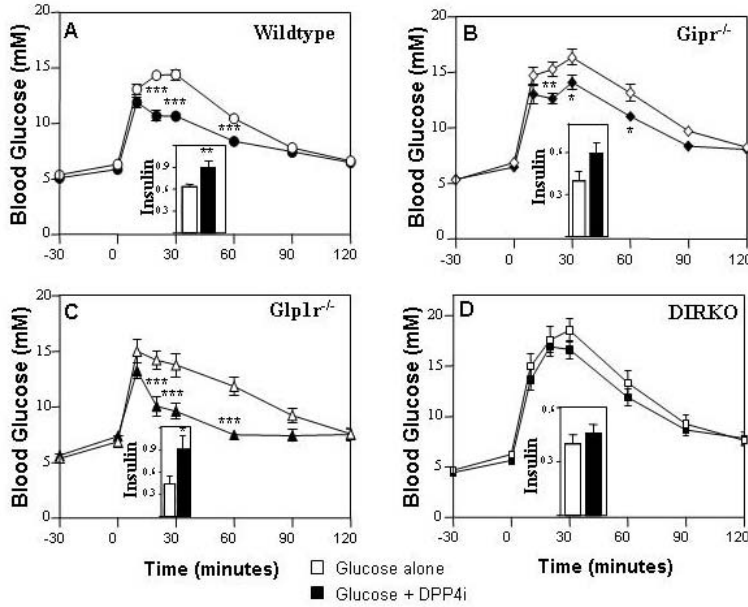


Table 1

Peptides that are cleaved by DPP4 may be Pharmacological or Physiological Substrates. Physiological peptide substrates are defined as those peptides whose endogenous levels of intact to cleaved forms are significantly different following genetic inactivation or chemical inhibition of DPP4 activity in vivo.

CG = chromogranin; CLIP = corticotropin-like intermediate lobe peptide; GCP-2 = granulocyte chemotactic protein-2; GHRH = growth hormone-releasing hormone; GRP = gastrin-releasing peptide; IGF-1 = insulin-like growth factor 1; IL-2 = interleukin-2; IL-1 β = interleukin-1 β ; IP-10 = interferon γ -inducible protein 10, also known as CXCL10, or chemokine (C-X-C motif) ligand 10; MDC = Macrophage-derived chemokine; MCP = monocyte chemotactic protein; NPY = neuropeptide Y; PHM = peptide histidine methionine; PYY = peptide YY, RANTES = regulated on activation normal T cell expressed and secreted; GLP = glucagon-like peptide; GIP = glucose-dependent insulinotropic polypeptide; SDF = Stromal cell-derived factor.

DPP4 Peptide Substrates

	Pharmacological	Physiological
Aprotinin	IP-10	GLP-1
Bradykinin	MDC	GLP-2
β -casomorphin-2	MCP-1	GIP
CG	MCP-2	SDF-1 α/β
CLIP	MCP-3	Substance P
Endomorphin-2	Tyr-melanostatin	
Enterostatin	α 1-microglobulin	
Eotaxin	NPY	
GCP-2	PHM	
GHRH	Prolactin	
GRP	PYY	
IGF-1	RANTES	
IL-2	Trypsinogen	
II-1 β	Trypsinogen pro-peptide Colipase	