Improved Glucose Tolerance in Rats Treated With the Dipeptidyl Peptidase IV (CD26) Inhibitor Ile-Thiazolidide

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The incretins glucose-dependent insulinotropic polypeptide (GIP_{1-42}) and truncated forms of glucagon-like peptide-1 (GLP-1) are hormones released from the gut in response to ingested nutrients, which act on the pancreas to potentiate glucose-induced insulin secretion. These hormones are rapidly inactivated by the circulating enzyme dipeptidyl peptidase IV ([DPIV] CD26). This study describes the effect on glucose tolerance and insulin secretion of inhibiting endogenous DPIV in the rat using Ile-thiazolidide, a specific DPIV inhibitor. High-performance liquid chromatography (HPLC) analysis of plasma following in vivo administration of ^{125}I-labeled peptides showed that inhibition of DPIV by about 70% prevented the degradation of 90.0% of injected ^{125}I-GLP-1_{7-36} after 5 minutes, while only 13.4% remained unhydrolyzed in rats not treated with the DPIV-inhibiting agent after only 2 minutes. Ile-thiazolidide treatment also increased the circulating half-life of intact GLP-1_{7-36} released in response to intraduodenal (ID) glucose (as measured by N-terminal specific radioimmunoassay [RIA]). In addition, inhibition of DPIV in vivo resulted in an earlier increase and peak of plasma insulin and a more rapid clearance of blood glucose in response to ID glucose challenge. When considered with the HPLC data, these results suggest that the altered insulin profile is an incretin-mediated response. DPIV inhibition resulting in improved glucose tolerance may have therapeutic potential for the management of type 2 diabetes mellitus.

NEURAL, HORMONAL, AND SUBSTRATE signals originating in the gut and stimulating hormone release from the endocrine pancreas comprise the enteroinsular axis.[1,2] Hormones that potentiate glucose-induced insulin secretion are called incretins. Glucose-dependent insulinotropic polypeptide (GIP_{1-42}) and glucagon-like peptide-1 (GLP-1) share considerable N-terminal sequence homology and are the most potent known incretins.[2] GIP_{1-42} and GLP-1_{7-36} were recently shown to be substrates of the circulating exopeptidase dipeptidyl peptidase IV ([DPIV] CD26), a highly specific protease preferentially hydrolyzing oligopeptides after aminoterminal penultimate proline or alanine residues.[3] Hydrolysis of GIP_{1-42} and GLP-1_{7-36} by DPIV yields the truncated oligopeptides GIP_{3-42} and GLP-1_{9-36} and the dipeptides Tyr-Ala and His-Ala, respectively.[3-6] Investigation by ourselves and others has demonstrated that N-terminally truncated forms of GIP_{1-42} and GLP-1_{7-36} are not insulinotropic,[7,8] and it has been speculated that DPIV-mediated hydrolysis is the primary mechanism of inactivation of these hormones in vivo.[3-6]

Competing substrates and specific DPIV inhibitors have been shown to prevent DPIV-mediated incretin degradation in vitro.[3-6] The present study was designed to investigate the physiological effects of DPIV inactivation in vivo on the enteroinsular axis. A protocol was developed for the inhibition of endogenous DPIV in the anesthetized rat using Ile-thiazolidide, a highly specific, reversible, competitive transition-state analog inhibitor of DPIV (I_i = 130 nmol/L).[6,9-11] Ile-thiazolidide has been shown not to inhibit the activity of pancreatic elastase, chymotrypsin, trypsin, prolyl endopeptidase, and dipeptidyl peptidase I (H.-U. Demuth, unpublished observations, July 1997). Figure 1 shows the structure of Ile-thiazolidide. The availability of this inhibitor allowed the investigation of DPIV inhibition with exogenously administered radiolabeled GLP-1_{7-36} and the effect on insulin secretion and glucose clearance in response to a glucose challenge. The availability of an N-terminal–specific radioimmunoassay (RIA) allowed the comparison of circulating levels of intact GLP-1_{7-36} in the presence or absence of DPIV inhibition.

MATERIALS AND METHODS

Inhibition of Endogenous DPIV in the Rat

Overnight-fasted male Wistar rats (200 to 225 g) were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg). A jugular vein cannula (heparin-filled PE90 tubing) was used for intravenous (IV) injection of a 1.5-μmol loading dose of Ile-thiazolidide (Dr H.-U. Demuth, Hans Knöll Institute of Natural Product Research, Halle, Germany) in 0.9% saline (200 μL), followed by a 0.75-μmol/min infusion of the compound for 30 minutes at a rate of 33.3 μL/min using Syringe Infusion Pump 22 (Harvard Apparatus, South Natick, MA). Injection of the loading dose represented time 0 minutes, and blood samples were collected from the tail vein at 0, 5, 10, 20, 30, 40, 50, 60, 75, and 90 minutes using 250-μL heparinized microcapillary tubes. Samples were stored on ice and centrifuged, and the plasma was immediately assayed for DPIV activity.

DPIV Activity Assay

A colorimetric assay was used to assess rat plasma DPIV activity. Gly-Pro-4-nitroanilide, a chromogenic substrate of DPIV, is hydrolyzed into the dipeptide Gly-Pro and the yellow product 4-nitroaniline, whose rate of appearance can be monitored spectrophotometrically. A 1.11-mmol/L stock solution of Gly-Pro-4-nitroanilide (Sigma, St Louis, MO) was prepared in 0.1 mmol/L Tris buffer, pH 7.4. The assay mixture consisted of 100 μL plasma, 450 μL stock Gly-Pro-4-nitroanilide solution, and 450 μL 0.1-mmol/L Tris buffer, pH 7.4, resulting in a final Gly-Pro-4-nitroanilide concentration of 0.5 mmol/L. Formation of the yellow product was monitored spectrophotometrically at 405 nm.

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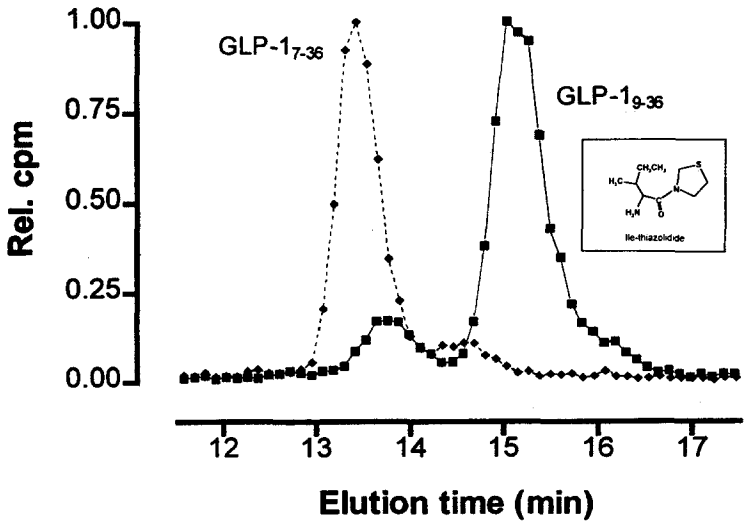

Fig 1. HPLC analysis of ^{125}I-GLP-1$_{7-36}$ metabolites following administration to rats in the presence and absence of Ile-thiazolidide. Purified ^{125}I-GLP-1$_{7-36}$ calculated to achieve a circulating concentration in the physiological range (50-100 pmol/L) was injected IV 20 minutes after initiation of Ile-thiazolidide or saline control infusion. Blood samples were collected, extracted, lyophilized, and analyzed by HPLC. Elution profiles for a drug-treated rat (◆) and control rat (■) were analyzed from blood samples collected 5 and 2 minutes after injection of radiolabeled hormone, respectively. Inset, chemical structure of Ile-thiazolidide.

Activity is expressed as the rate of 4-nitroaniline formation (micromoles per minute).

Radiolabeled GLP-1$_{7-36}$ Administration in the Presence of DPIV Inhibition In Vivo

Inhibition of endogenous DPIV in overnight-fasted anesthetized male Wistar rats (200 to 225 g) was established as already outlined. A control animal was administered 0.9% saline in place of Ile-thiazolidide. At time 20 minutes, purified ^{125}I-GLP-1$_{7-36}$ (specific activity, ~1 mCi/pmol; Novo Nordisk, Copenhagen, Denmark) calculated to achieve a circulating concentration of 50 to 100 pmol/L was injected into the animals via a jugular vein cannula. Blood was collected from the tail vein 2 and 5 minutes after the injection of radiolabeled hormone and immediately placed on ice. The blood was centrifuged and the plasma was removed and immediately extracted. Radiolabeled peptide was purified from plasma using C_{18} SepPak cartridges (Waters, Nepean, Ontario, Canada) as previously described.[4] Extracted peptide was lyophilized and stored at −20°C until analysis by high-performance liquid chromatography (HPLC). Samples were reconstituted in 100 μL water and injected onto a μBondapak C_{18} column (Waters). Elution solvents, CH_3CN/trifluoroacetic acid (TFA) and HPLC-grade H_2O/TFA, were delivered to the column by one 110B and one 114M Solvent Delivery Module pump (Beckman, Mississauga, Ontario, Canada) and eluted as described previously.[4] Eluant fractions were collected during the gradient elution, and radioactivity per fraction was measured in a Wallac 1277 Gammamaster gamma-counter (Bromma, Sweden). The percentage of ^{125}I-GLP-1$_{7-36}$ and ^{125}I-GLP-1$_{9-36}$ present in each sample was calculated from the recovered radioactivity in the respective peptide peaks. These peaks have been previously characterized using a protein sequencer.[4]

Glucose Administration in the Presence of DPIV Inhibition In Vivo—Intraduodenal Glucose Administration

Overnight-fasted anesthetized male Wistar rats (200 to 225 g) were surgically prepared with a jugular vein cannula and externalization of the proximal duodenum via a midline abdominal incision (<2 cm). At time 0, a bolus intraduodenal (ID) injection of glucose (1 g/kg 50% dextrose) was delivered with a 1-mL syringe fitted with a 26-gauge needle. One rat group was given IV Ile-thiazolidide according to the inhibition protocol outlined earlier, while control rats received an equal volume of 0.9% saline without drug. Blood samples were collected from the tail vein at 0, 10, 20, 30, 45, 60, 75, and 90 minutes, and the plasma was prepared. Glucose measurements were made immediately on whole blood using a One Touch II Blood Glucose Meter (Lifescan, Burnaby, British Columbia, Canada). DPIV activity was determined on the same day as the experiment by the assay method already outlined. Plasma samples were frozen for subsequent insulin determination. In three drug-treated and three control experiments, plasma samples were collected at baseline, and at 10 and 30 minutes for measurement of GLP-1 levels by an assay specific for the N-terminus of the molecule. The rationale for this measurement was to assess the relative amount of intact GLP-1 circulating in the presence or absence of the DPIV inhibitor Ile-thiazolidide.

IV Glucose Administration

Overnight-fasted anesthetized rats were treated with the DPIV inhibitor Ile-thiazolidide as already described or an equal volume of 0.9% saline as a control. At 10 minutes, both groups of rats received an IV injection of glucose (0.5 g/kg 50% dextrose) via a jugular vein cannula. Blood samples were collected from the tail vein at 0, 10, 15, 30, 45, 60, 75, and 90 minutes as before. Blood glucose, plasma insulin, and plasma DPIV activity were assessed as described in the previous section.

Assays

Plasma insulin concentrations were analyzed by RIA.[12] The level of intact GLP-1$_{7-36}$ was measured by RIA using an antiserum with an absolute requirement for N-terminally intact biologically active GLP-1$_{7-36}$ (antiserum #93242; kindly supplied by Dr Jens Holst, Panum Institute, Copenhagen, Denmark). Assay conditions were as described elsewhere, including ethanol extraction of plasma.[13]

Statistical Analysis

Comparisons between drug-treated and control rats were made by unpaired two-tailed *t* tests ($P < .05$ for significance).

RESULTS

GLP-1$_{7-36}$ Metabolism in the Presence of Ile-Thiazolidide

To determine the effect of in vivo inhibition of DPIV on the metabolism of the incretins, IV ^{125}I-GLP-1$_{7-36}$ was administered to rats in the presence and absence of Ile-thiazolidide. Separation of SepPak-extracted plasma by HPLC demonstrated that in the absence of Ile-thiazolidide, only 13.4% of ^{125}I-GLP-1$_{7-36}$ remained unhydrolyzed by endogenous DPIV by 2 minutes.

However, 90.0% of labeled hormone administered in the presence of the DPIV inhibitor was still present in the intact form 5 minutes after injection (Fig 1).

In Vivo Inhibition of DPIV Activity by Ile-Thiazolidide

The combination of a 1.5-μmol IV loading dose of Ile-thiazolidide followed by 0.75-μmol/min IV infusion of the drug for 30 minutes proved effective in suppressing plasma DPIV activity 64.0% ± 4.2% by 30 minutes (Fig 2A and D).

Glucose Clearance and Insulin Secretion in the Presence of Ile-Thiazolidide

ID glucose was administered to anesthetized rats to stimulate the release of endogenous incretins in the presence and absence of Ile-thiazolidide, to assess the effect of endogenous inhibition of DPIV on the enteroinsular axis. Figure 2A, B, and C summarizes the results from these experiments. Similar to the previous experiments, plasma DPIV activity was maximally suppressed by 71.4% ± 2.2% at 30 minutes. By the termination of the experiment at 90 minutes, the effects of Ile-thiazolidide were still evident, with 59.9% ± 4.4% inhibition of plasma DPIV activity (Fig 2A).

In animals that did not receive Ile-thiazolidide, plasma insulin reached a peak level of 433.2 ± 87.6 pmol/L by 30 minutes before returning to near-basal levels by 75 minutes. Ile-thiazolidide–treated animals attained a similar plasma insulin peak of 477.0 ± 153.0 pmol/L, but they reached this concentration by 20 minutes. Although the peak insulin response to ID glucose was unaffected by Ile-thiazolidide, the peak occurred 10 minutes earlier in drug-treated rats versus control animals. In control animals, blood glucose increased and remained elevated for the majority of the time of blood

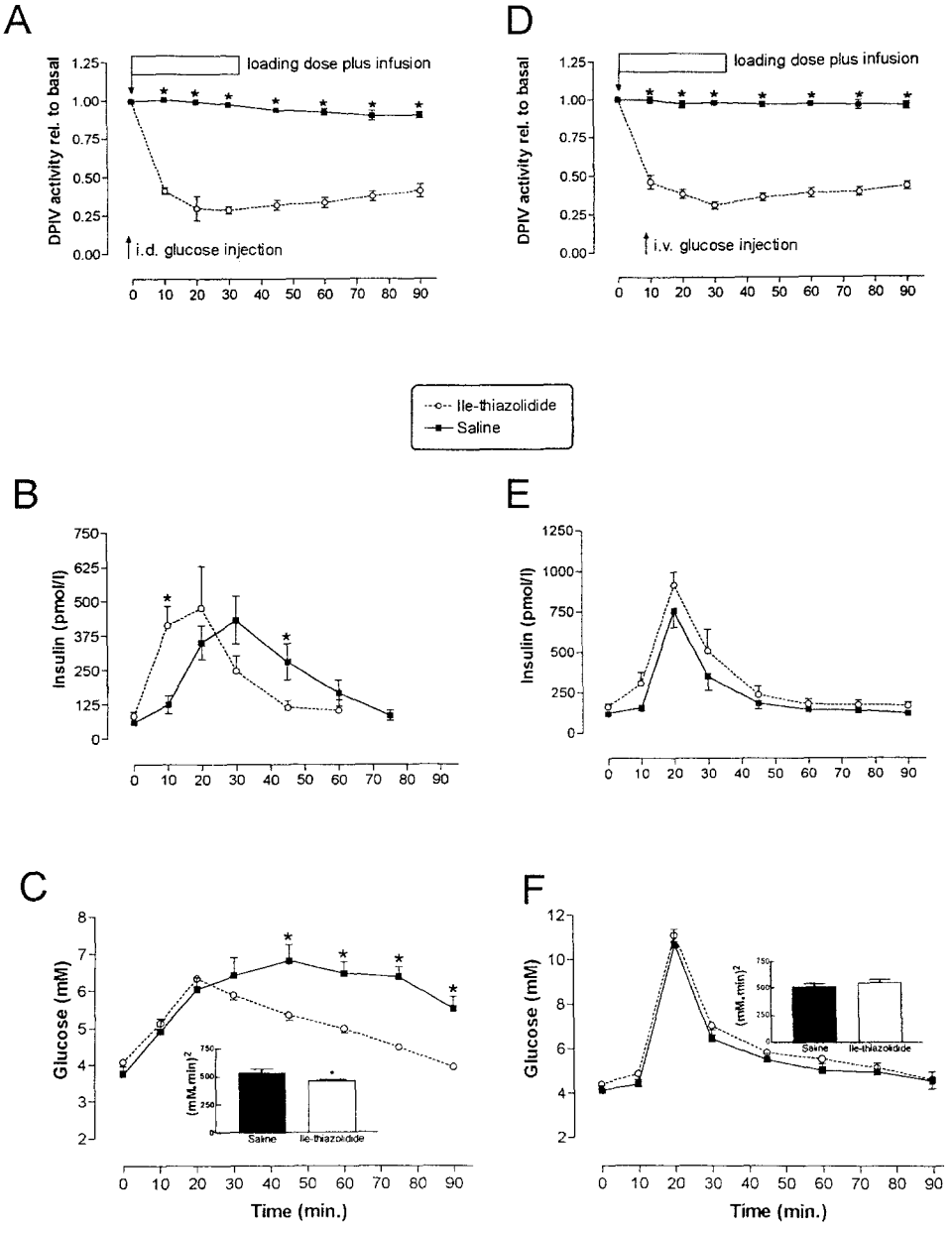

Fig 2. Effect of endogenous DPIV inhibition on blood glucose and plasma insulin in response to a glucose challenge. Plasma insulin and blood glucose were assessed in response to ID (A, B, and C) and IV (D, E, and F) glucose in the presence and absence of Ile-thiazolidide. Data are the mean ± SE for 5 or 6 rats in each drug-treated or control group. Significance was determined by unpaired 2-tailed *t* tests: *$P < .05$. Inset, area under the curve for glucose data in C and F.

sampling (90 minutes). Rats treated with Ile-thiazolidide exhibited a comparable increase in blood glucose, reaching a peak by 20 minutes, followed by a steady rate of blood glucose clearance. Glucose levels from 45 minutes onward were significantly lower in the drug-treated group compared with the control animals. In experiments designed to determine whether Ile-thiazolidide had any incretin-independent effects on insulin secretion and glucose clearance, IV glucose was administered to bypass the stimulation of endogenous incretin secretion from the gut. Plasma insulin peaked at 15 minutes, with no significant difference in insulin or glucose values between control and drug-treated rats, respectively (Fig 2E and F).

Glucose-Stimulated GLP-1 Levels in the Presence of Ile-Thiazolidide

The results in Table 1 indicate that Ile-thiazolidide treatment increased the circulating level of intact GLP-$1_{7\text{-}36}$ following ID glucose when compared with saline treatment.

DISCUSSION

Matrix-assisted laser desorption ionization time of flight mass spectrometry has been used by our group to investigate the effect of Ile-thiazolidide on the in vitro degradation of $GIP_{1\text{-}42}$ and GLP-$1_{7\text{-}36}$ after incubation in human serum.[6] In the same study, it was shown that DPIV is the principal serum protease responsible for the degradation of $GIP_{1\text{-}42}$ and GLP-$1_{7\text{-}36}$ into the inactive peptides $GIP_{3\text{-}42}$ and GLP-$1_{9\text{-}36}$, since Ile-thiazolidide, a highly specific inhibitor of DPIV, was able to completely block the formation of DPIV reaction products. Because of the importance of both $GIP_{1\text{-}42}$ and GLP-$1_{7\text{-}36}$ in the incretin response, it was of interest to determine the effect of blocking endogenous DPIV activity and to characterize the effect of this inhibition on the enteroinsular axis. The protocol for inhibiting DPIV activity in vivo as described in the present study consistently resulted in the suppression of plasma DPIV activity by 65% to 70% (Fig 2A and D).

To test the effectiveness of the inhibition protocol on incretin degradation, ^{125}I-GLP-$1_{7\text{-}36}$ was administered to rats in the presence and absence of Ile-thiazolidide. Virtually all of the ^{125}I-GLP-$1_{7\text{-}36}$ (calculated to achieve a physiological concentration of 50 to 100 pmol/L) coadministered with Ile-thiazolidide remained in its intact form 5 minutes after administration of the radiolabeled hormone, whereas the majority of the peptide administered to animals not receiving Ile-thiazolidide was converted to the DPIV reaction product by 2 minutes after label administration. This indicates that the in vivo DPIV inhibition protocol described in this study was effective to inhibit the degradation of exogenously administered circulating incretin.

Table 1. Plasma GLP-1 Levels (pmol/L, mean ± SEM) Measured by an NH_2-Terminally Directed RIA Following ID Glucose in the Presence of IV Saline or Ile-Thiazolidide (n = 3)

Time	Saline	Ile-Thiazolidide
Basal	Nondetectable	Nondetectable
10 min	51 ± 3.8	85 ± 6.1*
30 min	24 ± 2.1	35 ± 2.7*

*$P < .05$, saline *v* Ile-thiazolidide.

Since the kinetics of DPIV-catalyzed GLP-$1_{7\text{-}36}$ and $GIP_{1\text{-}42}$ hydrolysis are comparable,[6] similar results would be expected with ^{125}I-$GIP_{1\text{-}42}$. Circulating levels of N-terminally intact GLP-1 were determined after ID glucose administration in the presence or absence of Ile-thiazolidide. The results in Table 1 indicate a 40% increase in intact (biologically active) GLP-1 at time 10 minutes in the Ile-thiazolidide versus control experiments, indicating that Ile-thiazolidide inhibits the breakdown of endogenously released incretins. Unfortunately, a RIA specific for N-terminally intact GIP has not yet been developed.

To assess the effect of DPIV inhibition on the gut-pancreas axis, secretion of endogenous $GIP_{1\text{-}42}$ and GLP-$1_{7\text{-}36}$ was stimulated by administration of ID glucose in the presence and absence of Ile-thiazolidide. Analysis of plasma insulin showed that the peak insulin response to ID glucose was unchanged, but it occurred 10 minutes earlier than in rats not treated with Ile-thiazolidide. The finding that insulin levels do not remain elevated in DPIV-inhibited rats, despite the prediction that the half-life of endogenously released incretins is prolonged, implies the existence of a mechanism that prevents the secretion of inappropriate amounts of insulin even in the presence of elevated incretin concentrations. Part of the reason for decreased insulin levels in the presence of elevated incretin levels is undoubtedly the reduction in plasma glucose, as both incretins stimulate insulin in a glucose-dependent manner. A possible explanation for the prolonged glucose-lowering effects of Ile-thiazolidide infusion despite a rapid insulin response could be the insulin-independent glucose-lowering actions of intact circulating GLP-1. These include suppression of glucagon secretion[14,15] and insulin-like actions on peripheral glucose uptake.[16,17] The importance of a rapid incretin-mediated insulin response has been highlighted in studies by D'Alessio et al.[18] Blocking the actions of GLP-1 in baboons with exendin-9 led to the elimination of the early insulin response to an oral glucose tolerance test, which resulted in impaired glucose tolerance. In experiments designed to determine whether Ile-thiazolidide has a non–incretin-dependent effect on insulin secretion and glucose clearance, glucose was administered intravenously to bypass the stimulation of endogenous $GIP_{1\text{-}42}$ and GLP-$1_{7\text{-}36}$ from the gut. No difference was observed in plasma insulin or blood glucose responses between inhibitor-treated and control rats, implying that Ile-thiazolidide had no direct insulin-releasing action on islet β cells or insulin-like effects on peripheral glucose uptake. The data presented in this study are supported by recent findings that DPIV inhibition in anesthetized pigs increased the half-life and insulinotropic activity of exogenously administered GLP-1.[19]

Based on these observations, it is concluded that inhibition of DPIV activity in the rat improved the glucose tolerance by an incretin-mediated mechanism. The incretin GLP-1 has received considerable attention as an antidiabetogenic.[20] Interest in the potential clinical application of incretin therapy was generated by reports that exogenously administered GLP-$1_{7\text{-}36}$ and GLP-$1_{7\text{-}37}$ before and during a test meal in healthy and type 2 diabetic subjects produced a rapid normalization of postprandial hyperglycemia.[21,22] Since both incretins demonstrate a glucose dependence for their insulinotropic effects,[1,2] a significant advantage

of incretin therapy over traditional oral hypoglycemic or insulin therapy is that the drug-induced hypoglycemia common to type 2 diabetes may be avoided. DPIV also plays a role in the immune system, where it acts as the cell differentiation antigen (CD26) on the surface of T lymphocytes.[9] In addition, DPIV is involved in the inactivation of regulatory peptides (other than the incretins) that possess proline or alanine in the penultimate N-terminal position: examples are growth hormone–releasing hormone, neuropeptide Y, peptide YY, and prolactin.[3] The effect of short-term inhibition of circulating DPIV activity on the actions of these peptides is unknown. It seems likely that manipulation of plasma incretin concentrations by acute inhibition of DPIV could be a therapeutic approach for improving glucose tolerance and possibly provides an alternative therapy to currently prescribed medications such as sulfonylureas and biguanides.

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