Inhibition of Dipeptidyl Peptidase-4 by Vildagliptin during Glucagon-like Peptide-1 Infusion Increases Liver Glucose Uptake in the Conscious Dog

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ABSTRACT

**Objective.** This study investigated the acute effects of treatment with vildagliptin on dipeptidyl peptidase-4 (DPP-4) activity, glucagon-like peptide-1 (GLP-1) concentration, pancreatic hormone levels and glucose metabolism. The primary aims were to determine the effects of DPP-4 inhibition on GLP-1 clearance and hepatic glucose uptake.

**Research Design and Methods.** Fasted conscious dogs were studied in the presence (VIL, n=6) or absence (CON, n=6) of oral vildagliptin (1 mg/kg). In both groups, GLP-1 was infused into the portal vein (1 pmol/kg/min) for 240 min. During the same time, glucose was delivered into the portal vein at 4 mg/kg/min and into a peripheral vein at a variable rate to maintain the arterial plasma glucose level at 160 mg/dl.

**Results.** Vildagliptin fully inhibited DPP-4 over the 4h experimental period. GLP-1 concentrations were increased in the VIL group (50±3 versus 85±7 pM in the portal vein in CON and VIL, respectively; P<0.05) as a result of a 40% decrease in GLP-1 clearance (38±5 and 22±2 ml/kg/min, respectively; P<0.05). Although hepatic insulin and glucagon levels were not significantly altered, there was a tendency for plasma insulin to be greater (hepatic levels were 73±10 versus 88±15 µU/ml, respectively). During vildagliptin treatment net hepatic glucose uptake was 3-fold greater than in the CON group. This effect was greater than that predicted by the change in insulin.

**Conclusions.** Vildagliptin fully inhibited DPP-4 activity, reduced GLP-1 clearance by 40% and increased hepatic glucose disposal by means beyond GLP-1’s effects on insulin and glucagon secretion.
Glucagon-like peptide-1 (GLP-1) is a gut-derived hormone shown to enhance glucose-dependent insulin secretion, suppress inappropriately high glucagon secretion, slow gastric emptying and reduce food intake (1). In some type 2 diabetic patients (T2DM), GLP-1 levels are reduced, and elevation of GLP-1 by continuous infusion of the peptide leads to reductions in fasting glucose, post-prandial glucose excursions and hemoglobin A1C (2). The therapeutic potential of GLP-1 is limited, however, because it is rapidly inactivated by dipeptidyl peptidase-4 (DPP-4) (3; 4).

Vildagliptin is an orally effective selective DPP-4 inhibitor. In diabetic patients, vildagliptin improved glycemic control, increased the plasma insulin to glucagon molar ratio and reduced hemoglobin A1C levels (5; 6). During a meal tolerance test it augmented insulin secretion and decreased glucagon release, resulting in enhanced suppression of endogenous glucose production compared to placebo (7).

Ingested glucose and endogenously secreted GLP-1 are released from the gut, into the hepatic portal vein, which then perfuses the liver. Typically, studies have investigated the effects of DPP-4 inhibition following a meal, when GLP-1 secretion is increased. In the present study, GLP-1 and glucose were infused directly into the hepatic portal vein in the presence or absence of DPP-4 inhibition. The first aim was to examine the effect of vildagliptin on GLP-1 clearance under these carefully controlled conditions. In addition, although GLP-1 can increase glucose disposal by stimulation of insulin secretion, the hormone has been suggested to affect glucose metabolism by actions over and above its effects on the pancreas. Therefore, the second aim of this study was to investigate the effect of DPP-4 inhibition on glucose disposal, in particular by the liver.

RESEARCH DESIGN AND METHODS
Experiments were conducted on 12 healthy, conscious, 18h fasted dogs (20–27 kg). Prior to the study, they were fed a standard chow diet once a day, and water was provided ad libitum. The surgical facility met the standards published by the American Association for the Accreditation of Laboratory Animal Care, and the protocols were approved by the Lovelace Respiratory Research Institute Institutional Animal Care and Use Committee prior to the start of the study. All dogs underwent a laparotomy 3 weeks before the experiment in order to implant infusion catheters into the jejunal and splenic veins. Sampling catheters were implanted into the hepatic portal vein, the left hepatic vein and the left femoral artery. Ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY) were placed around the hepatic and right iliac arteries and the portal vein, as described elsewhere (8). Intraportal catheters (splenic and jejunal) were used for the infusion of glucose (Baxter Healthcare Corporation, Deerfield, IL) and GLP-1 (Bachem, King of Prussia, PA). Each animal was used only once.

On the day of the study, intravenous catheters were placed into a leg vein for glucose delivery. Each experiment consisted of a basal period (−40 to 0 min) and an experimental period (0 to 240 min). Vildagliptin (1 mg/kg; VIL) or vehicle (sterile water; CON) were administered via stomach gavage at −20 min. Vildagliptin was well tolerated by all animals. At 0 min, constant portal infusions of glucose (4 mg/kg/min) and GLP-1 (1 pmol/kg/min) were started, and glucose was infused into a peripheral vein to maintain arterial plasma glucose at 160 mg/dl. Blood sampling and analytical procedures. Blood samples were collected from the femoral artery as well as the hepatic portal and hepatic veins. Hematocrit; plasma glucose, glucagon, insulin, cortisol and GLP-
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1; and blood alanine, lactate, and glycerol concentrations were determined as previously described (8). Hepatic blood flow was measured using ultrasonic flow probes and a Transit-time Perivascular Flow Meter (Model T403, Ithaca, NY) as described elsewhere (8). DPP-4 activity was measured in plasma samples by using 7-Amino-4-methylcoumarin (AMC; Bachem cat. # Q-1025) as standard and H-Gly-Pro-AMC.HBr (Bachem cat. # I-1225) as substrate. Five µl of plasma were incubated with 15 µl of 100 µM substrate for 20 minutes, and absorption was measured at excitation/emission wavelength of 360 nm/460 nm with a spectrophotometer. The DPP-4 activity is expressed as mU/ml where mU = nmol/min.

Data analysis. Net hepatic substrate balance (NHB) was calculated with the arterial-venous difference method as NHB = load_{out} – load_{in}, where load_{out} = H × HF and load_{in} = (A × AF) + (P × PF), in which H, A, and P are the substrate concentrations in the hepatic vein, femoral artery, and hepatic portal vein blood or plasma, respectively, and HF, AF, and PF are the blood or plasma flows in the hepatic vein, hepatic artery, and portal vein, as determined by the ultrasonic flow probes. Using this calculation, a positive value represents net output by the liver, and a negative value represents net hepatic uptake. For glucose balance calculations, glucose concentrations were converted from plasma to blood values using previously published correction factors (9). Blood glucose concentrations were used for the calculation of net glucose balance because the use of whole blood glucose ensures accurate balance measurements regardless of the characteristics of glucose entry into the erythrocyte. Non-hepatic glucose uptake was calculated as the glucose infusion rate plus net hepatic glucose balance, with changes in the glucose mass accounted for when deviations from steady-state were present (10-12). The approximate substrate levels in plasma entering the liver sinusoids were determined using the formula [A×(AF/HF) + P×(PF/HF)], where hormone concentrations and flow are abbreviated as previously. GLP-1 clearance was determined by dividing the hormone infusion rate by its arterial concentration after the basal GLP-1 level was subtracted.

Statistical analysis. Data are presented as means ± SEM. Between group differences were analyzed with two-way analysis of variance (ANOVA), and univariate F tests were used for post-hoc comparisons (SigmaStat; SPSS). One-way ANOVA was used for comparisons of mean data and AUC. Statistical significance was accepted at P < 0.05.

RESULTS
Following oral administration of vildagliptin, the arterial and portal vein plasma DPP-4 activities remained fully suppressed over the 4 hour experimental period (Fig 1; P<0.05). As a result of portal vein GLP-1 infusion, the plasma GLP-1 levels in the CON group increased from 2±1 to 30±3 pM in the artery and 3±1 to 50±3 pM in the portal vein (basal period to average of the last 3h of the experimental period; Fig 2). The rise in GLP-1 was greater in the VIL group, increasing in the artery and portal vein, respectively, from 3±1 to 51±5 pM and 4±2 to 85±7 pM (P<0.05). Whole body GLP-1 clearance rates were 38±5 and 22±2 ml/kg/min during the last 3h in the CON and VIL groups, respectively (P<0.05).

Glucose was infused to increase the fasting arterial plasma glucose level from ~105 to ~160 mg/dl in both groups (Fig 3). The intraportal glucose infusion rate (GIR) was 4 mg/kg/min in both groups. To maintain the clamp, additional glucose had to be infused into a peripheral vein in both the CON (6.1±1.2 mg/kg/min) and VIL (8.6±1.5 mg/kg/min; P<0.05) groups (Fig 3).

In response to the rise in the plasma glucose level, in the CON group the plasma
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insulin increased from 7±1 to 26±4 µU/ml in the artery and from 17±3 to 73±10 µU/ml in the hepatic sinusoids (increases of 4.1 and 4.3 fold, respectively, from the basal period to the last 3h; Fig 4). The rise in insulin in the VIL group tended to be larger (6±1 to 36±8 and 17±3 to 88±15 µU/ml; 5.5 and 5.4 fold), although they were not significantly different from the changes in the control group. The elevations in the arterial plasma C-peptide levels were not significantly different between groups, increasing in the CON group from 0.45±0.04 to 1.27±0.09 ng/ml (2.9 fold) and in the VIL group from 0.39±0.04 to 1.40±20 ng/ml (3.6 fold) during the two periods, respectively (Fig 5). Hepatic sinusoidal plasma glucagon levels decreased to similar values in the two groups; from 59±11 to 23±4 pg/ml and 43±3 to 23±4 pg/ml in the CON and VIL groups, respectively (Fig 5). The hepatic plasma insulin to glucagon molar ratios were not significantly different (72±10 and 86±14 in the CON and VIL groups, respectively) during the last 3h of the study.

Following a meal, glucose uptake by the liver is stimulated by increased glucose and insulin levels in the blood. Therefore, in response to the increases in hepatic glucose load, portal vein insulin to glucagon molar ratio, and plasma GLP-1 level, there was a switch in net hepatic glucose balance (NHGB) in the CON group from net output (2.4±0.1 mg/kg/min during the basal period) to net uptake (-0.7±0.1 mg/kg/min during the last 3h hours of the experimental period; Fig 6). In the VIL group, there was a greater response to these stimuli such that the liver switched from net hepatic glucose output of 2.1±0.2 to net hepatic glucose uptake of -2.1±0.5 mg/kg/min (P<0.05). Non-hepatic glucose uptake (non-HGU; mg/kg/min) increased from basal by 6.8±1.2 in the CON group (2.3±0.2 during the basal period to 9.1±1.2 during the last 3h, respectively) and by 8.1±1.4 in the VIL group (from 2.3±0.3 to 10.4±1.4, respectively; Fig 6). Although the non-hepatic response was not significantly different between the two groups, the increases in the rate of glucose uptake by the liver and non-hepatic tissues were greater with vildagliptin treatment by about the same magnitude (~1.2 mg/kg/min). Following vildagliptin treatment, net hepatic glucose fractional extraction was 3-fold greater (0.02±0.01 vs. 0.06±0.01 in the CON and VIL groups, respectively; Fig 7; P<0.05) as was the ratio of net hepatic glucose uptake to the hepatic insulin level (0.01±0.00 vs. 0.03±0.01 in the two groups, respectively; Fig 7; P<0.05) during the last 3h of the study. The ratios of non-HGU to arterial insulin, on the other hand, were similar in the two groups (0.38±0.04 vs. 0.35±0.05, respectively; Fig 7).

No differences in plasma cortisol (data not shown) were observed between groups. Free fatty acid and glycerol levels were ~25% lower in the VIL group during the basal period and tended to remain lower during the study, but the levels were not significantly different between groups at the end of the experiment (Table 1). There was no treatment effect on the arterial level or net hepatic balance of blood lactate or alanine (Table 1).

DISCUSSION

In this study, vildagliptin fully inhibited DPP-4 over a 4h experimental period. As a result GLP-1 clearance was reduced by 40% and the levels of the hormone in plasma were increased. Although hepatic insulin and glucagon levels were not significantly altered by treatment with the DPP-4 inhibitor, there was a tendency for plasma insulin to be greater. Hepatic glucose disposal was nevertheless increased by treatment, over and above effects attributable to a rise in insulin. No such effect was seen on non-hepatic glucose uptake.

GLP-1 levels in the circulation are regulated primarily by N-terminal cleavage at the position 2 alanine by DPP-4, and by renal elimination, but the kidneys appear to only
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account for 10-20% of the degradation of intact GLP-1 (13). To our knowledge, an effect of vildagliptin on renal GLP-1 clearance is not known. In this study, DPP-4 inhibition resulted in a 70% increase in arterial and portal vein GLP-1 concentrations, which resulted from a 40% decrease in GLP-1 whole body clearance. It should be noted that GLP-1 clearance was determined by dividing the rate of intraportal GLP-1 infusion by the change in plasma GLP-1. Although it is possible that a decrease in endogenous GLP-1 secretion may have occurred, the basal GLP-1 levels were low (2-3 pM), therefore any decrease during the clamp period would have had only a small effect on the estimate of clearance. In addition, these results are in line with previous findings in T2DM patients in which 4 weeks of treatment with vildagliptin resulted in a 2-fold or greater increase in the post-meal rise in active GLP-1 (6; 14).

GLP-1 has previously been shown to increase (1) or have no effect on plasma insulin levels (15; 16) and vildagliptin has been demonstrated to increase (17), have no effect (6), or decrease (14) the levels of insulin following a meal. Although the stimulatory effect of GLP-1 on insulin secretion is well established (1; 18), in some studies insulin levels increased with GLP-1 treatment as a result of reduced hepatic insulin clearance (19-22) while in others (23-26) clearance was not affected. In the present study the increases in arterial C-peptide levels were greater in the vildagliptin group compared to the control group by the same relative magnitude as the increases in insulin levels which occurred, although the differences were not statistically significant between groups. Thus, it appears that any elevation in plasma insulin levels during vildagliptin treatment was the result of a difference in insulin secretion, not a decrease in clearance.

The role of GLP-1 on canine insulin secretion is not well understood. An incretin effect has been clearly shown to be present in the dog (27), but the role of GLP-1 in this effect has not been clearly established. It is known that a pharmacological level of GLP-1 can induce insulin secretion in isolated canine pancreata (28) and islets (29). In addition, we have shown that a pharmacological dose of exendin-4 (a GLP-1R agonist) is able to induce insulin secretion (30) while an incretin effect has not been demonstrated in other studies in the dog when GLP-1 was infused intraportally in physiological amounts (16; 27; 31; 32). In the present study we once again did not observe a significant effect of elevated GLP-1 on insulin secretion, although it is possible that a greater difference in GLP-1 between groups would have revealed an incretin effect.

Patients with T2DM often exhibit inappropriately high postprandial glucagon levels, which can be suppressed by GLP-1 (33) and vildagliptin (7). In the present study, glucagon decreased to levels which were very similar in both groups. Since the animals studied were non-diabetic, pancreatic α-cell sensitivity to inhibition by hyperinsulinemia and hyperglycemia may have been maximal, even in the absence of drug. In addition, the elevated concentration of GLP-1 in the vehicle treated group may have been sufficient to create GLP-1’s maximal effect on α-cell suppression.

While some studies in the human have not revealed extrapancreatic effects of GLP-1 (34-37), others have demonstrated insulin independent effects, including inhibition of glucose production (38). Stimulation of liver glucose uptake by GLP-1 has been reported in the dog (39; 40) while non-hepatic effects on glucose clearance have been reported in both dog and man (16; 41-44). Recently, it was reported that during a meal tolerance test, patients with T2DM treated with vildagliptin showed greater suppression of endogenous glucose production (7). This effect was associated with increased plasma insulin and
reduced plasma glucagon concentrations. No significant differences in the glucose disappearance or metabolic clearance rates were noted. In other recent studies, exenatide (an incretin mimetic) was shown to increase splanchnic (T2DM; (45)) and hepatic glucose uptake (normal dog; (30)). In both studies the effect was associated with an increase in plasma insulin concentration. In another study (normal dog; (31)) exenatide reduced postprandial glycemia independent of islet hormones and slowing of gastric emptying.

The present study extends these observations by demonstrating that DPP-4 inhibition, accompanied by increased GLP-1 levels, can increase hepatic glucose disposal. This occurred without a significant difference in the insulin to glucagon molar ratio, although the average hepatic insulin level in the VIL group was 15 µU/ml greater than in the control group. Even though this difference was not statistically significant, small differences in insulin can affect hepatic glucose production. Based on previous insulin dose response experiments (with intraportal glucose and insulin infusions), a 15 µU/ml difference would have increased net hepatic glucose uptake by ~0.4 mg/kg/min (46). Thus, net hepatic glucose uptake was ~1 mg/kg/min greater during vildagliptin treatment than would have been predicted from the difference in plasma insulin levels. Although hepatic glucose production was most likely fully inhibited in both groups due to the elevations in glucose and insulin, a difference in suppression of glucose production could also account for some of the difference in net hepatic glucose uptake between groups. Nevertheless, net hepatic glucose fractional extraction and the ratio of net hepatic glucose uptake to the insulin level at the liver were 3-fold greater during DPP-4 inhibition (Fig 7). Conversely, the tendency for non-hepatic glucose uptake to be increased was solely attributable to the prevailing arterial insulin levels (Fig 7).

DPP-4 inhibition also extends the half-life of GIP (gastric inhibitory polypeptide). However, since there was no stimulus for incretin secretion in the present study GIP levels presumably remained close to basal. Additional experiments would have to be performed, however, to definitively rule out the possibility that the effects observed in this study were due to differences in GLP-1 levels, not to changes in the activity of another substrate of DPP-4. The likelihood that they were attributable to GLP-1 is supported by our earlier studies. In one experiment, which used a similar design as the present study (no pancreatic clamp and glucose clamped at 160 mg/dl), when saline or GLP-1 were infused into the liver via the portal vein or hepatic artery, plasma insulin and glucagon levels were not affected by GLP-1 infusion (16). Nevertheless, the change from basal net hepatic glucose uptake was greater with GLP-1 vs. saline infusion by 1 to 1.5 mg/kg/min, although these differences were not statistically significant. In other studies in which hepatic insulin and glucagon levels were clamped at similar levels between groups, there was a linear relationship between net hepatic glucose uptake and the plasma concentration of GLP-1 (39). Thus, those studies support the present data which suggest a dose dependant increase in net hepatic glucose uptake.

The effect of vildagliptin treatment on liver glucose disposal was presumably mediated by the difference in GLP-1 concentrations between groups. The GLP-1 receptor has been identified in the hepatic portal vein (47) and the liver (48; 49) and could exert its effect through binding at either site, although it appears that portal receptors may not be necessary for the effect (39). Glucose entering the liver may be directed into glycogen since GLP-1 has been shown to increase glycogen storage in rat hepatocytes (50). Although DPP-4 was maximally inhibited by the start of the experimental
period, and the maximal difference in GLP-1 was present by the first sampling point in that period, the maximal difference in response of the liver was not apparent until 90 minutes. This slow onset of action may reflect the time required for the synthesis of gluco-regulatory proteins in the liver.

In summary, in the non-diabetic, overnight fasted dog, inhibition of DPP-4 by vildagliptin increased plasma GLP-1 levels by reducing its clearance by about 40%. In addition, the rise in GLP-1 associated with DPP-4 inhibition produced an augmentation of hepatic glucose utilization which was not accounted for by the hormone’s effects on the pancreas.

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**Figure 1.** Arterial and portal plasma DPP-4 activity in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group; * P<0.05).

**Figure 2.** Arterial and portal plasma GLP-1 levels in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group; * P<0.05).
Figure 3. Arterial plasma glucose level and peripheral glucose infusion rate in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group; * P<0.05). Glucose was infused into the portal vein at 4 mg/kg/min in both groups during the experimental period.

Figure 4. Arterial and hepatic sinusoidal plasma insulin levels in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group).
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Figure 5. Arterial plasma C-peptide and hepatic sinusoidal plasma glucagon levels in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group).

Figure 6. Net hepatic glucose balance and non-hepatic glucose uptake in conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min) treated with vehicle (○) or vildagliptin (■) (mean ± SEM; n=6 per group).
Figure 7. Hepatic plasma insulin to glucagon molar ratio, net hepatic glucose uptake to hepatic sinusoidal plasma insulin ratio and non-hepatic glucose uptake to arterial plasma insulin ratio during the last 3h of the experimental period (60-240 min) in conscious dogs treated with vehicle (□) or vildagliptin (■) (mean ± SEM; n=6 per group).
TABLE 1. Arterial blood lactate, alanine, and glycerol levels and net hepatic balance and plasma free fatty acid levels in 18h-fasted conscious dogs during the basal (-40 to 0 min) and experimental periods (0-240 min).

|                     | Basal Period | Experimental Period (min) |
|---------------------|--------------|---------------------------|
|                     | -40 to 0     | 60                        | 120 | 180 | 210 | 240 |
| Plasma Free Fatty Acid Level (µmol/l) |              |                           |     |     |     |     |
| Control             | 924 ± 69     | 245 ± 54                  | 117 ± 23 | 115 ± 27 | 147 ± 48 | 124 ± 39 |
| Vildagliptin        | 736 ± 118*   | 111 ± 23                  | 88 ± 26 | 71 ± 25 | 82 ± 31 | 66 ± 15 |
| Blood Glycerol Level (µmol/l) |              |                           |     |     |     |     |
| Control             | 97 ± 6       | 42 ± 9                    | 28 ± 4 | 31 ± 5 | 35 ± 6 | 32 ± 6 |
| Vildagliptin        | 70 ± 9*      | 21 ± 4*                   | 24 ± 4 | 20 ± 5 | 19 ± 4 | 16 ± 2 |
| Net Hepatic Glycerol Uptake (µmol/kg/min) |              |                           |     |     |     |     |
| Control             | 1.7 ± 0.1    | 0.8 ± 0.2                 | 0.5 ± 0.1 | 0.6 ± 0.2 | 0.6 ± 0.2 | 0.6 ± 0.2 |
| Vildagliptin        | 1.6 ± 0.3    | 0.5 ± 0.2                 | 0.5 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.2 | 0.3 ± 0.1 |
| Blood Lactate Level (µmol/l) |              |                           |     |     |     |     |
| Control             | 482 ± 81     | 534 ± 71                  | 536 ± 89 | 545 ± 66 | 593 ± 72 | 564 ± 59 |
| Vildagliptin        | 433 ± 87     | 571 ± 82                  | 535 ± 83 | 555 ± 63 | 543 ± 49 | 551 ± 68 |
| Net Hepatic Lactate Balance (µmol/kg/min) |              |                           |     |     |     |     |
| Control             | -4.5 ± 0.7   | 2.4 ± 1.7                 | 1.1 ± 0.8 | 0.3 ± 1.2 | 0.7 ± 0.9 | 1.5 ± 1.1 |
| Vildagliptin        | -2.3 ± 2.7   | 5.4 ± 2.4                 | 2.1 ± 1.6 | 3.2 ± 1.7 | 2.5 ± 1.7 | 3.2 ± 1.9 |
| Blood Alanine Level (µmol/l) |              |                           |     |     |     |     |
| Control             | 289 ± 24     | 261 ± 23                  | 239 ± 16 | 230 ± 14 | 235 ± 16 | 225 ± 15 |
| Vildagliptin        | 300 ± 33     | 275 ± 24                  | 245 ± 13 | 217 ± 18 | 221 ± 21 | 219 ± 24 |
| Net Hepatic Alanine Uptake (µmol/kg/min) |              |                           |     |     |     |     |
| Control             | 2.2 ± 0.2    | 1.5 ± 0.2                 | 1.7 ± 0.1 | 2.1 ± 0.2 | 2.0 ± 0.1 | 2.2 ± 0.3 |
| Vildagliptin        | 2.3 ± 0.4    | 1.8 ± 0.2                 | 2.0 ± 0.5 | 2.2 ± 0.2 | 2.4 ± 0.2 | 2.5 ± 0.3 |

Mean ± SEM; n=6 per group; * P<0.05.
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