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Reduced serum dipeptidyl peptidase-IV after metformin and pioglitazone treatments

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Abstract

Dipeptidyl peptidase-IV (DPP-IV) regulates metabolism by degrading incretins involved in nutritional regulation. Metformin and pioglitazone improve insulin sensitivity whereas glyburide promotes insulin secretion. Zucker diabetic rats were treated with these antidiabetic agents for 2 weeks and DPP-IV activity and expression were determined. Serum DPP-IV activity increased whereas tissue activity decreased as the rats aged. Treatment of rats with metformin, pioglitazone, and glyburide did not alter DPP-IV mRNA expression in liver or kidney. Metformin and pioglitazone significantly ($P < 0.05$) reduced serum DPP-IV activity and glycosylated hemoglobin. Glyburide did not lower DPP-IV activity or glycosylated hemoglobin. Regression analysis showed serum DPP-IV activity correlated with glycosylated hemoglobin ($r = 0.92$) and glucagon-like peptide-1 levels ($r = -0.49$). Metformin, pioglitazone, and glyburide had no effect on serum DPP-IV activity in vitro, indicating these are not competitive DPP-IV inhibitors. We propose the in vivo inhibitory effects observed with metformin and pioglitazone on serum DPP-IV activity results from reduced DPP-IV secretion.

Keywords: Diabetes; Dipeptidyl peptidase-IV; Glucagon-like peptide-1; Metformin; Pioglitazone; Glyburide

The discovery that the antidiabetic effects of the glitazones (rosiglitazone and pioglitazone) are due to ligand-induced peroxisome proliferator activated receptor γ (PPARγ) activation has allowed a better understanding of the mechanism of action of these agents [1]. These effects may be mediated by altered PPARγ-mediated gene expression that results in improved insulin sensitivity in peripheral tissue [2]. In contrast, glyburide is an antidiabetic agent that promotes insulin secretion [3]. Its mechanism of action requires binding to a high affinity sulfonylurea receptor, which results in closure of ATP-sensitive potassium channels in the β-cells of the pancreas leading to depolarization of the cells and insulin secretion [4]. Metformin belongs to the biguanide class of oral antidiabetic agents that improve glucose levels, in part, by reducing hepatic glucose output [5,6]. Unlike glyburide, metformin effects are glucose-dependent: metformin reduces glucose under hyperglycemic, but not euglycemic conditions [5].

Dipeptidyl peptidase IV (DPP-IV) is a serine protease that cleaves the penultimate proline or alanine at the N-terminus of several polypeptides, such as glucagon-like peptide 1 (GLP-1), gastric inhibitory peptide (GIP), enterostatin, procolipase, and other peptides [7]. Rapid inactivation of GLP-1 by DPP-IV limits its ability to enhance glucose-induced insulin secretion and improve glucose tolerance [8]. Glucose tolerance and GLP-1 stability are improved in DPP-IV deficient rodents [9] and rodents treated with DPP-IV inhibitors [10], supporting a role for this peptidase in metabolizing peptides involved in nutritional control.

The effects of PPARγ ligands and sulfonylureas on GLP-1 and DPP-IV have not been characterized.

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However, various studies suggest biguanides may increase GLP-1 secretion and decrease DPP-IV activity. For example, over a 2 week period, metformin treatment increased GLP-1 levels in obese nondiabetic subjects following an oral glucose load, but did not effect basal active GLP-1 concentrations [11]. Additionally, Yasuda et al. [12] found metformin treatment increased serum active GLP-1 levels in DPP IV deficient rats, but not DPP IV positive rats, suggesting biguanides enhance GLP-1 secretion. Mannucci et al. [11] reported that metformin incubation of intact GLP-1 in serum or buffer containing DPP-IV preserved intact GLP-1 and suggested metformin may inhibit serum DPP-IV activity in vitro. In contrast, Hinke et al. [13] and Yasuda et al. [12] reported that metformin is not a competitive inhibitor of DPP-IV in vitro. This indicates that the ability of metformin to improve oral glucose tolerance is related to increased GLP-1 levels and glucose-induced insulin secretion, although the mechanism remains unclear.

The purpose of the current study was to investigate the effects of metformin, pioglitazone, and glyburide on DPP-IV in vivo using Zucker diabetic fatty (ZDF) rats. We report the novel observation that both biguanides and PPARγ agonists may inhibit serum DPP-IV activity in vivo and confirm these agents are not competitive inhibitors of DPP-IV in vitro. The in vivo effects are proposed to be due to reduced DPP-IV secretion.

Materials and methods

Chemicals

Metformin (1,1-dimethylbiguanide hydrochloride, Catalog # D5035) and glyburide (Catalog # G-0639) were obtained from Sigma (St. Louis, MO 63178) and pioglitazone, GW7845, and LAF237 were from GlaxoSmithKline chemical storage.

In vivo studies

Animals. All studies were conducted using age- and weight-matched male ZDF *fa/fa* rats (Genetic Models, Indianapolis, IN) or *db/db* mice (Jackson Labs) housed at 72 °F and 50% relative humidity with a 12 h light/dark cycle. Rats were fed PMI 5008 Formulab Diet (PMI Nutrition International, Saint Louis, MO) and mice were fed NIH R & M/Auto 6F-Ovals 5K67 (PMI Feeds, Richmond, IN). Rats at 6.5 and 8.5 weeks of age were dosed by oral gavage twice daily for two weeks with vehicle (0.5% methylcellulose with 0.1% Tween 80), 300 mg/kg metformin in vehicle, 10 mg/kg glyburide in vehicle or 30 mg/kg pioglitazone in vehicle. After 14 days, ad libitum fed rats were anesthetized with isofluorane for blood collection from the heart and serum chemistry analysis. Tissue samples (liver and kidney) were flash-frozen in liquid nitrogen at the time of necropsy for DPP-IV activity and mRNA analysis. In another study, rats were dosed by oral gavage twice daily for 1 week with vehicle (0.5% methylcellulose with 0.1% Tween 80) or 3 mg/kg of the PPARγ agonist, GW7845 [14], in vehicle. The rats were fasted overnight, given oral glucose (2 g/kg) in saline, and anesthetized with isofluorane for cardiac blood collection at 0, 5, and 15 min ($n = 6$), and serum chemistry analyses. In a third study, *db/db* mice (7 weeks of age) were dosed by oral gavage twice daily for 2 weeks with vehicle (0.5% methylcellulose with 0.1% Tween 80) or 30 mg/kg pioglitazone in vehicle. The mice were fasted for 10 h, given oral glucose (2 g/kg) in saline for 30–60 min, and anesthetized with isofluorane for cardiac blood collection ($n = 6$) and serum chemistry analyses. For GLP-1 measurements blood was collected in tubes containing 30 μM ile-thiazolidide and the samples were frozen to prevent ex vivo degradation by DPP-IV. Linco Research (St. Charles, MO) performed active GLP-1 measurements (http://www.lincoresearch.com/protocols/mendo-75k.html). Biochemistry measurements (e.g., glucose) of serum samples were obtained using an automated chemistry analyzer (Olympus Au640, Olympus America, Melville, NY). All research complied with the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and GlaxoSmithKline policy on animal use.

DPP-IV assays. To determine DPP-IV activity from liver and kidney, frozen tissues were homogenized in 10 volumes (g/ml) of buffer (250 mM sucrose, 50 mM Tris, pH 8.0, and 5 mM EDTA) containing protease inhibitors 75 mg/ml TPCK, 1 mg/ml pepstatin A, and 10 mM iodoacetamide. Following homogenization, the samples were centrifuged at 1000*g* for 10 min. The supernatant was then centrifuged at 100,000*g* for 30 min. The supernatant representing the soluble protein fraction was removed for analysis. The pellet was suspended in homogenization buffer containing 0.5% Triton X-100 (Sigma Chemical, St. Louis MO), incubated on ice for 10 min, and centrifuged at 100,000*g* for 30 min. The supernatant representing the solubilized membrane fraction was removed for enzyme and protein analysis. Protein concentration was measured using the BCA protein assay (Pierce; Rockford, IL). Direct interaction of the compounds with DPP-IV was evaluated by measuring DPP-IV activity in rat serum incubated for 20 min with 10-fold serial dilutions of metformin, pioglitazone, glyburide, and LAF237 ranging from 1 pM to 1 mM. Serum dialysis studies were done using 10,000 molecular weight cutoff Slide-A-Lyzer cassettes (Pierce, Rockport, IL). The samples were dialyzed against three changes of 1000-fold volume Hanks' phosphate buffered saline overnight (Gibco-BRL, Gaithersburg, MD) at 4 °C to remove the drug. DPP-IV activity was measured using the fluorogenic substrate Gly-Pro-AMC according to the manufacturer's specifications (Enzyme System Products, Livermore, CA). The DPP-IV substrate (40 μL; 50 μM for serum and 500 μM for tissue homogenates) was mixed with serum (10 μL) or tissue homogenate in 50 mM Tris, pH 7.8. The product was monitored over 10 min incubation at 30 °C by fluorescence measurement using a Cytofluor spectrafluorimeter (360 nm excitation and 460 nm emission). While Gly-Pro-AMC is a substrate for other dipeptidases, DPP-IV represents over 95% of the serum dipeptidyl peptidase active at pH 7.8 [15].

RNA expression analysis. RNA was purified from kidney and liver using Trizol Reagent (Life Technologies), treated with Dnase I reagent (Ambion, Austin, TX), and quantified using Ribogreen fluorescent reagent (Molecular Probes, Eugene, OR) according to manufacturer's instructions. A standard reaction for TaqMan quantitative RT-PCR contained 25 ng RNA, 900 nM each of the forward and reverse primers, 100 nM of the oligonucleotide probe labeled at the 5′ end with 6-FAM and at the 3′ end with TAMRA fluorogenic dyes (Keystone Labs, Camarillo, CA), 300 μM dNTPs (Clontech), 10 U Rnase Inhibitor, 12.5 U MMLV reverse transcriptase, and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). cDNA syntheses were carried out at 48 °C for 30 min using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Following heat inactivation at 95 °C for 10 min, polymerase chain reaction was done (40 cycles of 94 °C 15 s, 59 °C 1 min). The forward and reverse primers were 5′-TGAAGACACCGTGGAAGGTTC-3′ and 5′-CTGGCAC GGTGATGATGGT-3′, respectively. The probe was 5′-FAM-ACAA GCGCAGCGACACCAAGCAGT-TAMRA-3′. The cycles performed for half maximal fluorescence for each reaction were calculated using the SDS Version 2.1 software (Applied Biosystems). The mRNA

level was normalized to 18S rRNA. RNA copies were quantified using the following equation: copies per 50 ng RNA $= 10^{[(C_t-40)/3.35]}$. Student's t test (unequal variance) was performed to determine significance between groups.

Results

Effects of treatment on serum DPP-IV activity, glucose, glycosylated hemoglobin, GLP-1, and insulin levels

ZDF rats represent an animal model of non-insulin-dependent diabetes that are characterized by profound insulin resistance, insulin hypersecretion, and obesity. To investigate whether altered glycemic control affects DPP-IV activity in vivo, ZDF rats at 6.5 and 8.5 weeks of age were treated with vehicle, glyburide, metformin or pioglitazone for 2 weeks. DPP-IV activity, glycosylated hemoglobin and glucose levels were 218%, 52%, and 84% greater ($P < 0.05$), respectively, in serum from the older compared to the younger ZDF rats (Table 1). As expected, pioglitazone and metformin significantly ($P < 0.05$) reduced serum glycosylated hemoglobin and glucose levels in rats at both ages (Table 1). However, glyburide treatment showed no significant effect on these parameters. Interestingly, treatment with pioglitazone or metformin treatment resulted in a significant ($P < 0.05$) reduction of DPP-IV activity in both the younger rats (57% and 31%, respectively), and older rats (71% and 34%, respectively) (Table 1). Consistent with the observed reduction in DPP-IV activity, pioglitazone treatment increased active GLP-1 (43%, $P < 0.05$) in younger rats. Glyburide treatment increased insulin (82%, $P < 0.05$) levels in the younger rats only. Multiple regression analysis was done using DPP IV activity as the dependent parameter and the other serum biochemical markers as independent parameters using all the rats ($n = 48$) after 2 weeks of treatment. Glycosylated hemoglobin ($r = 0.92$) and glucose ($r = 0.86$) levels were positively correlated with DPP-IV activity in serum, whereas GLP-1 ($r = -0.49$) and insulin ($r = -0.36$) levels were negatively correlated with DPP-IV activity in serum.

In vitro inhibition of DPP-IV activity

LAF237 is a competitive inhibitor of DPP-IV [16]. Direct interaction with DPP-IV was evaluated by measuring DPP-IV activity in serum incubated for 20 min with increasing concentrations of metformin, pioglitazone, glyburide, and LAF237. In contrast to LAF237, 1–1000 μM metformin, pioglitazone, and glyburide did not inhibit DPP-IV activity when mixed with rat serum (Fig. 1A). To determine if the in vivo effects of metformin and pioglitazone treatment on serum DPP-IV activity were reversible, serum from rats treated for two weeks with metformin or pioglitazone was dialyzed against phosphate buffered saline to remove the drugs. The dialysis experiments demonstrate the reversible inhibition of serum DPP-IV mixed with 100 μM LAF237 but irreversible inhibition of serum DPP-IV from metformin and pioglitazone treated ZDF rats (Fig. 1B). These observations show that metformin and pioglitazone treatments inhibit serum DPP-IV activity in vivo; however, they are not competitive inhibitors of serum DPP-IV activity in vitro.

Effects of metformin and pioglitazone on liver and kidney DPP-IV expression and activity in membranes

Given the reduced levels of serum DPP-IV activity observed with pioglitazone or metformin treatment, it was of interest to determine whether there were any changes in DPP-IV activity or mRNA expression in tissues. DPP-IV is expressed at very high levels in liver and kidney [17] where it is predominantly membrane bound but may be released from membranes by proteolysis. Quantification of DPP-IV mRNA levels by quantitative reverse transcriptase-polymerize chain reaction revealed DPP-IV mRNA expression in liver and kidney was not significantly ($P > 0.05$) different comparing metformin, pioglitazone or glyburide treated rats to vehicle treated

Table 1
Serum chemistry measurements after treating diabetic rats

Treatment	Serum DPP-IV activity (fluorescence units)	HbA1c (%)	Glucose (mmol/l)	Insulin (ng/ml)	GLP-1 (pM)
Treatment of ZDF rats aged 6.5–8.5 weeks					
Vehicle	1446 ± 104	6.6 ± 0.2	17.0 ± 3.3	3.3 ± 0.3	11.2 ± 1.0
Glyburide	1443 ± 168	6.5 ± 0.2	19.5 ± 2.3	6.0 ± 0.4**	9.3 ± 1.3
Metformin	998 ± 109**	5.7 ± 0.2**	9.7 ± 2.5*	3.2 ± 0.1	9.3 ± 1.5
Pioglitazone	618 ± 30**	5.3 ± 0.1**	8.0 ± 0.1*	0.6 ± 0.1**	16.0 ± 2.3*
Treatment of ZDF rats aged 8.5–10.5 weeks					
Vehicle	4602 ± 305	10.0 ± 0.4	31.2 ± 1.0	0.92 ± 0.18	7.5 ± 0.4
Glyburide	4150 ± 217	9.8 ± 0.3	34.0 ± 1.7	0.97 ± 0.22	8.0 ± 1.2
Metformin	3034 ± 275**	8.7 ± 0.5*	21.6 ± 2.8**	1.23 ± 0.32	7.0 ± 1.6
Pioglitazone	1322 ± 69**	7.1 ± 0.3*	10.6 ± 1.5**	0.90 ± 0.19	11.3 ± 2.2

$^{*}P < 0.05$, $^{**}P \leqslant 0.01$ relative to age-matched vehicle treated rats.

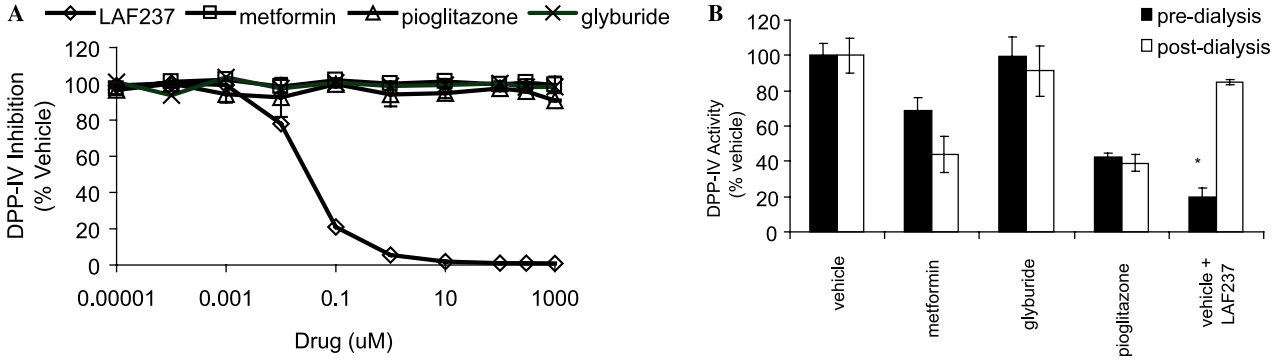

Fig. 1. Metformin, pioglitazone or glyburide in vitro does not inhibit DPP-IV. (A) Effect of addition of LAF237, metformin, pioglitazone, and glyburide on DPP-IV activity in vitro using rat serum. (B) DPP-IV activity before and following dialysis of serum taken from ZDF rats treated with metformin, pioglitazone, glyburide, and vehicle groups. As a control, the serum from vehicle treated rats was given 100 μM LAF237. Data are given as means ± SEM ($n = 6$). Significant differences before and after dialysis are indicated by $^*P < 0.05$.

rats from older rats (Table 2) and younger rats (data not shown). Furthermore, DPP-IV activity in solubilized liver membrane fractions was found to be similar in all treatment groups (Table 3). In kidney from the older rats, membrane-associated DPP-IV was not significantly ($P > 0.05$) different comparing pioglitazone, metformin or glyburide treated rats to vehicle treated rats (Table 3). Kidney membrane associated DPP-IV activity was slightly lower ($P = 0.03$) in glyburide treated younger rats, but not pioglitazone or metformin treated rats, when compared to the vehicle treated rats (Table 3). Kidney-membrane associated DPP-IV activity was significantly greater ($P = 0.017$) from the younger ZDF rats than the older ZDF rats treated with vehicle (Table 3).

Table 2
DPP-IV tissue expression after treating older diabetic rats

Treatment	Kidney DPP-IV mRNA copies per 50 ng total RNA ($\times 10^{-4}$) (P value vs vehicle)	Liver DPP-IV mRNA copies per 50 ng total RNA (P value vs vehicle)
Treatment of ZDF rats aged 8.5–10.5 weeks		
Vehicle	160 ± 67.3	24.9 ± 4.75
Glyburide	153 ± 38.3 (0.8)	35.0 ± 12.3 (0.11)
Metformin	113 ± 17.7 (0.16)	29.0 ± 6.64 (0.24)
Pioglitazone	153 ± 35.6 (0.8)	26.7 ± 6.73 (0.6)

Table 3
DPP-IV tissue activity after treating diabetic rats

Treatment	Kidney membrane DPP-IV (μmol/min/mg protein) (P value vs vehicle)	Liver membrane DPP-IV (μmol/min/mg protein) (P value vs vehicle)
Treatment of ZDF rats aged 6.5–8.5 weeks		
Vehicle	381 ± 44	11.37 ± 1.19
Glyburide	268 ± 16 (0.03)	11.78 ± 1.02 (0.1)
Metformin	322 ± 14 (0.24)	10.53 ± 1.11 (0.62)
Pioglitazone	303 ± 25 (0.53)	11.75 ± 2.24 (0.59)
Treatment of ZDF rats aged 8.5–10.5 weeks.		
Vehicle	245 ± 18	9.54 ± 0.13
Glyburide	233 ± 11 (0.62)	9.68 ± 1.07 (0.88)
Metformin	242 ± 15 (0.91)	9.91 ± 1.07 (0.74)
Pioglitazone	261 ± 29 (0.58)	8.79 ± 0.75 (0.42)

Oral glucose tolerance tests

To further support the effects of antidiabetic agents on serum DPP-IV activity in additional models, oral glucose tolerance tests were done using ZDF rats and *db*/*db* mice treated with the PPARγ agonists GW7845 (14) or pioglitazone, respectively. Compared to the vehicle group, GW7845 treatment significantly increased serum GLP1 levels and reduced serum glucose, insulin, and DPP-IV activity levels during the glucose tolerance test (Fig. 2), confirming that distinct PPARγ ligands (i.e., GW845 and pioglitazone) modulate DPP-IV activity in vivo. Likewise, in *db*/*db* mice pioglitazone treatment reduced glucose levels by 245–308 mg/dl ($P < 0.006$) and serum DPP-IV activity by 17–24% ($P < 0.02$) from 30 to 60 min after administering oral glucose, confirming pioglitazone is effective at lowering serum DPP-IV activity in alternative animal models of diabetes.

Discussion

While Mannucci et al. [11] reported that metformin inhibits serum DPP-IV in vitro, Yasuda et al. [12] and Hinke et al. [13] reported that metformin is not a competitive inhibitor of DPP-IV. Our results also demonstrate metformin and piogitazone are not competitive inhibitors of DPP-IV activity in vitro. Moreover, we show pioglitazone or metformin treatment lowered serum DPP-IV activity and glucose levels, whereas glyburide was ineffective. Although DPP-IV activity was reduced in serum after pioglitazone or metformin treatment, kidney and liver RNA levels, and membrane DPP-IV activity were unchanged. The observation that glyburide stimulated insulin secretion in younger rats

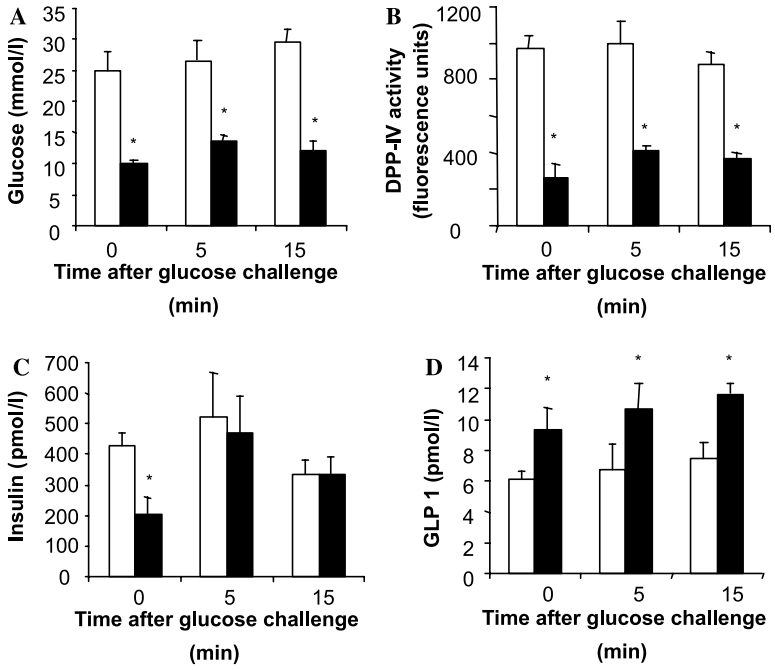

Fig. 2. GW7845 improves glucose tolerance and reduces serum DPP-IV activity in ZDF rats. ZDF rats were treated with the PPARγ agonist GW7845 for 7 days, fasted, and given an oral glucose solution. Serum was collected at various times after the oral glucose challenge. Glucose (A), DPP-IV (B), insulin (C), and GLP-1 (D) were measured as described in the methods. Open bars represent vehicle treatment and closed bars represent GW7845 treatment. Data are given as means ± SEM ($n = 6$) and significant differences between vehicle and drug treated groups are indicated by $^{*}P < 0.05$.

but did not alter serum DPP-IV activity or glucose suggests that increasing insulin secretion in the absence of improved glycemic control may not be sufficient to alter serum DPP-IV activity.

One hypothesis is decreased serum DPP-IV activity may be secondary to improved glycemic control. Consistent with this hypothesis, we show serum DPP-IV activity is elevated in older diabetic rats and positively correlated with glycosylated hemoglobin and glucose. Serum DPP-IV activity also is greater in subjects with type 2 diabetes than control subjects [18] and serum DPP-IV activity positively correlates ($r = 0.989$) with the duration of type 2 diabetes [19]. In particular, Toft-Nielsen et al. [20] show DPP-IV activity increases with increasing fasting glucose levels in 54 heterogeneous type 2 diabetic patients treated with diet, biguanide or sulfonylurea. DPP-IV activity also increases in urine from diabetic patients with nephropathy [21] and treatment of ZDF rats with the PPARγ-agonist GW7845 reduces DPP-IV activity in urine [22]. Although these observations suggest the possibility that glucose levels influence DPP-IV activity, possibly by affecting secondary organ lesions [18,21], alternate explanations, such as altered lipid metabolism, cannot be ruled out.

Several observations suggest that DPP-IV secretion may be regulated. DPP-IV immunoreactivity occurs most at the secretory or absorptive apex (e.g., luminal membrane) of epithelial cells, suggesting that DPP-IV function is related to secretory and/or absorptive processes [17]. In seminal vesicles of the prostate, DPP-IV is mainly secreted in particles called prostasomes [23]. Additionally, in subjects with rheumatoid arthritis, DPP-IV activity increases in T cells but decreases in serum [24] and within pancreatic islet A-cells soluble DPP-IV and glucagon are sorted to secretory granules [25]. In the study presented here, older diabetic rats had significantly greater DPP-IV activity in serum ($P < 0.001$), but lower activity in kidney tissue ($P < 0.05$) than younger rats. While the molecular mechanisms regulating DPP-IV secretion are unknown, it would be of interest to determine if glycemic control regulates the release of DPP-IV from T-cells, endothelial cells, pancreatic islet A-cells or other cells types and determine the extent these cells contribute to the pool of soluble DPP-IV activity.

In conclusion, this study indicates that pharmacologically distinct agents that improve glycemic control inhibit soluble DPP-IV activity in vivo but not in vitro. This may occur as a result of decreased DPP-IV secretion into serum. This raises the interesting possibility for a distinct pathway (i.e., reduced DPP-IV in serum and improved glucose tolerance) by which biguanides and glitazones may affect metabolism. However, the consequence of altering the soluble pool of DPP IV activity is unknown.

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