Dipeptidyl Peptidase IV Regulates Proliferation of Preglomerular Vascular Smooth Muscle and Mesangial Cells

Edwin K. Jackson, Stanton J. Kochanek, Delbert G. Gillespie

Abstract—The purpose of this study was to investigate the role of dipeptidyl peptidase IV in regulating the effects of 2 of its substrates, neuropeptide Y1-36 and peptide YY1-36, on proliferation of and collagen production by preglomerular vascular smooth muscle and glomerular mesangial cells from spontaneously hypertensive and normotensive rats. In cells from hypertensive rats, neuropeptide Y1-36 and peptide YY1-36 stimulated [3H]-thymidine incorporation (cell proliferation index), cell number, and [3H]-proline incorporation (index of collagen synthesis); and sitagliptin (dipeptidyl peptidase IV inhibitor) significantly enhanced most of these effects. Neuropeptide Y3-36 and peptide YY3-36 (products of dipeptidyl peptidase IV) had little effect on [3H]-thymidine incorporation, and sitagliptin did not enhance the effects of either peptide. BIBP3226 (Y1 receptor antagonist) blocked the effects of neuropeptide Y1-36 and peptide YY1-36 on [3H]-thymidine incorporation and cell number in cells from normotensive rats; however, the effects were weak and mostly not affected by sitagliptin. Real-time PCR and Western blotting showed similar dipeptidyl peptidase IV mRNA and protein levels in cells from hypertensive versus normotensive rats, with greater levels in smooth muscle versus mesangial cells. Both cell types converted peptide YY1-36 to peptide YY3-36 in a concentration-dependent manner that was attenuated by sitagliptin, and dipeptidyl peptidase IV activity was greater in smooth muscle versus mesangial cells. In conclusion, dipeptidyl peptidase IV inhibitors might entail a risk of renal dysfunction because of abnormal proliferation of cells in the preglomerular microcirculation and glomeruli. (Hypertension. 2012;60:757-764.) ● Online Data Supplement

Key Words: dipeptidyl peptidase IV ■ peptide YY1-36 ■ neuropeptide Y1-36 ■ preglomerular vascular smooth muscle cells ■ mesangial cells ■ spontaneously hypertensive rats ■ cell proliferation

Inhibitors of dipeptidyl peptidase IV (DPPIV), for example, sitagliptin, represent a novel class of antidiabetic drugs for treatment of type 2 diabetes mellitus that afford sustained reductions in hemoglobin A1c with a low risk of hypoglycemia and little effect on body weight.1 Because millions of patients will be taking DPPIV inhibitors for life, there is some urgency to more fully understand the long-term risks associated with DPPIV inhibition. The long-term risks of DPPIV inhibitors in the setting of hypertension are of particular interest, because frequently this condition is a comorbidity in type 2 diabetics.2

DPPIV metabolizes incretin hormones, such as gastric inhibitory polypeptide and glucagon-like peptide-1 (GLP-1).3 Consequently, DPPIV inhibitors raise circulating levels of incretin hormones and thereby exert antidiabetic actions by increasing insulin release.3 However, there are ≈35 known peptide substrates for DPPIV,4,5 and, therefore, inhibition of DPPIV increases levels of an array of biologically active peptides. Long-term increases in some of these peptide substrates of DPPIV may entail risks. For example, Brown et al6 provide evidence that DPPIV inhibitors increase angiogenesis risk in patients treated with angiotensin-converting enzyme inhibitors, most likely because of blockade of substance P metabolism.

With respect to other substrates for DPPIV that may entail risks, 2 peptides of particular importance are neuropeptide Y (NPY)1-36 and peptide YY (PYY)1-36. Both of these peptides are members of the pancreatic polypeptide-fold family,7 and the kidney (a major target organ for diabetes mellitus–induced damage) is likely exposed to high levels of these endogenous peptides. For example, renal sympathetic nerves release NPY1-36 in response to central nervous system–mediated activation of renal sympathetic tone,8 resulting in high local levels of NPY1-36 in sympathetically innervated renal microvessels. Also, NPY1-36 is made by renal epithelial cells and released into the renal interstitium, where it can affect vascular and glomerular cells.9 With regard to PYY1-36, fatty meals release this peptide into the systemic circulation from endocrine L-cells in the gastrointestinal tract producing physiologically active levels of PYY1-36 in plasma,10,11 and this circulating PYY1-36 would be delivered to the renal microcirculation and glomeruli via the blood stream. Type 2
diabetics, particularly those with the metabolic syndrome, have increased renal sympathetic tone\textsuperscript{12} and often ingest fatty meals.\textsuperscript{13} Thus, diabetics likely would have high renal levels of both NPY\textsubscript{1-36} and PYY\textsubscript{1-36} and inhibition of DPPIV would increase these elevated levels.

Both NPY\textsubscript{1-36} and PYY\textsubscript{1-36} are potent endogenous agonists of Y\textsubscript{1} receptors.\textsuperscript{7} However, DPPIV is anchored to the cell surface and efficiently converts PYY\textsubscript{1-36} to PYY\textsubscript{3-36} and NPY\textsubscript{1-36} to NPY\textsubscript{3-36} by cleaving 2 amino acids from the N-termini of PYY\textsubscript{1-36} and NPY\textsubscript{1-36}.\textsuperscript{3,5} DPPIV could just as logically be named “NPY-converting enzyme,” because the k\textsubscript{cat}/Michaelis constant of DPPIV for NPY\textsubscript{1-36} is 36-fold and 73-fold greater for NPY\textsubscript{1-36} compared with GLP-1 and gastric inhibitory polypeptide, respectively.\textsuperscript{5} Whereas PYY\textsubscript{1-36} and NPY\textsubscript{1-36} are potent Y\textsubscript{1} receptor agonists, PYY\textsubscript{3-36} and NPY\textsubscript{3-36} are inactive at Y\textsubscript{1} receptors but are selective Y\textsubscript{2} receptor agonists.\textsuperscript{14} Thus, likely DPPIV inhibitors chronically increase Y\textsubscript{1} receptor activation in preglomerular microvascular smooth muscle (PGVSMCs) and glomerular mesangial cells (GMCs). Because diabetic nephropathy entails hypercellularity of glomeruli and increased extracellular matrix production in glomeruli, a significant unanswered question is whether PYY\textsubscript{1-36} and NPY\textsubscript{1-36} stimulate proliferation of and collagen production by PGVSMCs and GMCs and whether DPPIV modulates these responses. Accordingly, the goals of the present study were to determine in PGVSMCs and GMCs: (1) the expression of DPPIV mRNA, the expression of DPPIV protein, and DPPIV activity; (2) whether there is an interaction between PYY\textsubscript{1-36} and DPPIV and NPY\textsubscript{1-36} and DPPIV such that the effects of PYY\textsubscript{1-36} and NPY\textsubscript{1-36} on proliferation and collagen production are enhanced by inhibition of DPPIV in PGVSMCs and GMCs; and (3) whether the expression and function of DPPIV and actions of PYY\textsubscript{1-36} and NPY\textsubscript{1-36} differ in PGVSMCs and GMCs from genetically hypertensive versus normotensive animals.
Methods
Animals, isolation and culture of PGVSMCs and GMCs, quantitative real-time PCR for DPPIV, Western blotting for DPPIV, assay for PYY3-36, extraction of sitagliptin, assessment of cell proliferation and collagen production, and statistical analysis are available in the online-only Data Supplement.

Results
Expression of DPPIV mRNA and Protein in PGVSMCs and GMCs
DPPIV mRNA was detected by quantitative real-time PCR in PGVSMCs and GMCs obtained from spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto rats (WKYs). DPPIV mRNA expression was similar in SHR PGVSMCs compared with WKY PGVSMCs and was similar in SHR GMCs compared with WKY GMCs; however, overall DPPIV mRNA levels were lower in GMCs compared with PGVSMCs (Figure 1A). Western blots for DPPIV revealed a single band at 55 kDa in both cell types for both strains using 2 different antibodies (Figure 1B). This 55-kDa band is consistent with the datasheet for the DPPIV antibody 10940-1-AP and with a report by Bauvois.15 Bands were digitized and normalized to β-actin. DPPIV protein levels were similar in SHR versus WKY PGVSMCs and tended to be lower in SHR compared with WKY GMCs, although this did not achieve statistical significance (Figure 1C). Overall, DPPIV protein levels were lower in GMCs compared with PGVSMCs.

Conversion of PYY1-36 to PYY3-36 in SHR PGVSMCs and GMCs
Confluent monolayers of cells were incubated in PBS for 15 minutes with 0, 3, or 10 nmol/L of PYY1-36 without or with sitagliptin, and the medium was assayed for PYY3-36. In both PGVSMCs (Figure 2A) and GMCs (Figure 2B), incubation with 3 and 10 nmol/L of exogenous PYY1-36 increased PYY3-36 medium levels to 6-fold and 13-fold of basal, respectively. Sitagliptin significantly attenuated the increase in PYY3-36 levels in PGVSMCs and GMCs; however, sitagliptin did not abolish the PYY1-36-induced increase in PYY3-36 levels in either cell type. The sitagliptin-inhibitable PYY3-36 production (PYY3-36 levels in the absence of sitagliptin minus the average levels of PYY3-36 in the presence of sitagliptin) was greater in PGVSMCs compared with GMCs (Figure 2C).

Effects of NPY1-36, PYY1-36, and Sitagliptin on [3H]-Thymidine Incorporation in SHR PGVSMCs and GMCs
In SHR PGVSMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 (Figure 3A) and sitagliptin and PYY1-36 (Figure 3C) on [3H]-thymidine incorporation. NPY1-36 and PYY1-36 stimulated [3H]-thymidine incorporation, and this response was enhanced by sitagliptin. In WKY PGVSMCs, NPY1-36 (Figure 3B) and PYY1-36 (Figure 3D) significantly but very mildly increased [3H]-thymidine incorporation, and these effects were not augmented by sitagliptin.
Effects of NPY1-36, PYY1-36, and Sitagliptin on Cell Number in SHR and WKY PGVSMCs

In SHR PGVSMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 (Figure 4A) and sitagliptin and PYY1-36 (Figure 4C) on cell number. NPY1-36 and PYY1-36 increased cell number, and sitagliptin enhanced the growth effects of NPY1-36 and PYY1-36. In WKY PGVSMCs, NPY1-36 (Figure 4B) and PYY1-36 (Figure 4D) significantly but very mildly increased cell number; however, these responses were not augmented by sitagliptin.

Effects of NPY1-36, PYY1-36, and Sitagliptin on [3H]-Proline Incorporation in SHR and WKY PGVSMCs

In SHR PGVSMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 on [3H]-proline incorporation (Figure 5A). NPY1-36 stimulated [3H]-proline incorporation, and this response was enhanced by sitagliptin. PYY1-36 also stimulated [3H]-proline incorporation in SHR PGVSMCs (Figure 5C); however, the interaction between sitagliptin and PYY1-36 was not statistically significant; there was a trend in this regard (P=0.1138). In WKY PGVSMCs, NPY1-36 (Figure 5B) and PYY1-36 (Figure 5D) significantly but very mildly increased [3H]-proline incorporation, and these effects were not augmented by sitagliptin.

Effects of NPY1-36, PYY1-36, and Sitagliptin on [3H]-Thymidine Incorporation in SHR and WKY GMCs

In SHR GMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 and sitagliptin and PYY1-36 on [3H]-thymidine incorporation (Figure S1A and S1C, respectively, available in the online-only Data Supplement). NPY1-36 and PYY1-36 stimulated [3H]-thymidine incorporation, and this response was enhanced by sitagliptin. In WKY GMCs, NPY1-36 and PYY1-36 significantly but very mildly increased [3H]-thymidine incorporation, and these effects were not augmented by sitagliptin (Figure S1B and S1D, respectively).

Effects of NPY1-36, PYY1-36, and Sitagliptin on Cell Number in SHR and WKY GMCs

In SHR GMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 and sitagliptin and PYY1-36 on cell number (Figure S2A and S2C, respectively). NPY1-36 and PYY1-36 increased cell number, and sitagliptin enhanced the growth effects of NPY1-36 and PYY1-36. In WKY GMCs, NPY1-36 and PYY1-36 significantly but very mildly increased cell number; however, these responses were not augmented by sitagliptin.

Effects of NPY1-36, PYY1-36, and Sitagliptin on [3H]-Proline Incorporation in SHR and WKY GMCs

In SHR GMCs, 2-factor ANOVA indicated a significant interaction between sitagliptin and NPY1-36 on [3H]-proline incorporation (Figure S3A). NPY1-36 stimulated [3H]-proline incorporation, and this response was enhanced by sitagliptin. PYY1-36 also stimulated [3H]-proline incorporation in SHR GMCs; although the interaction between sitagliptin and
PYY1-36 was not statistically significant, there was a trend in this regard (P = 0.0705; Figure S3C). In WKY GMCs, NPY1-36 and PYY1-36 significantly increased [3H]-proline incorporation, and the effect of NPY1-36 but not PYY1-36 was significantly augmented by sitagliptin (Figure S3B and S3D, respectively).

Effects of NPY3-36, PYY3-36, and Sitagliptin on [3H]-Thymidine Incorporation in SHR PGVSMCs and GMCs

In SHR PGVSMCs, neither NPY3-36 nor PYY3-36 affected [3H]-thymidine incorporation (Figure S4A and S4B, respectively) either in the absence or presence of sitagliptin. In SHR GMCs, NPY3-36 and PYY3-36 significantly but very mildly increased [3H]-thymidine incorporation, and these effects were not augmented by sitagliptin (Figure S3B and S3D, respectively).

Effects of BIBP3226 on [3H]-Thymidine Incorporation Induced by NPY1-36, PYY1-36, Sitagliptin Plus NPY1-36, and Sitagliptin Plus PYY1-36 in SHR PGVSMCs and GMCs

In SHR PGVSMCs and GMCs BIBP3226 abolished the ability of both NPY1-36 and PYY1-36 to stimulate [3H]-thymidine incorporation (Figure 6). This complete inhibition by BIBP3226 was also observed for the combination effects of sitagliptin plus NPY1-36 and sitagliptin plus PYY1-36.

Effects of GLP-1 on Cell Number in SHR and WKY PGVSMCs

Because GLP-1 is also an important substrate for DPPIV, GLP-1 induces vasodilation,16,17 and vasodilators often are associated with inhibition of vascular smooth muscle cell growth, we also examined the effects of GLP-1 (7-36) on proliferation of SHR and WKY PGVSMCs in the absence and presence of sitagliptin. At concentrations from 1 to 100 nmol/L, GLP-1 had little if any effect on platelet-derived growth factor (PDGF)-induced proliferation of either SHR or WKY PGVSMCs, and there was no interaction between sitagliptin and GLP-1 in either SHR or WKY PGVSMCs (Figure S5).

Discussion

The present results indicate that DPPIV message and protein are expressed in vascular and glomerular cellular elements and could, therefore, importantly influence the metabolism of NPY1-36 and PYY1-36. Because we do not have an assay that distinguishes NPY1-36 from NPY3-36 yet we do have an assay that detects PYY3-36 without cross-reacting with PYY1-36, in the present study we assessed the ability of DPPIV to metabolize pancreatic polypeptide-fold peptides by measuring the production of PYY3-36 from PYY1-36. Likely, both peptides are metabolized similarly by DPPIV, so the results for PYY3-36, which should reflect the ability of DPPIV to metabolize NPY1-36. Indeed, our results show that the expression of DPPIV message and protein by PGVSMCs and GMCs is associated with the ability of these cell types to metabolize PYY1-36 to PYY3-36. Importantly, sitagliptin, a potent and selective inhibitor of DPPIV,1 suppresses the conversion of PYY1-36 to PYY3-36 in both cell types. This finding confirms that at least a portion of the enzymatic activity that converts PYY1-36 to PYY3-36 in PGVSMCs and GMCs is attributed to DPPIV.

Figure 5. Effects of neuropeptide Y (NPY)1-36 (10 nmol/L; A and B) and peptide YY (PYY)1-36 (10 nmol/L; C and D) in the absence or presence of sitagliptin (1 μmol/L) on [3H]-proline incorporation in spontaneously hypertensive rat (SHR; A and C) and Wistar-Kyoto rat (WKY; B and D) preglomerular microvascular smooth muscle cells (PGVSMCs). Letters above bars indicate significantly different (Fisher least significant difference [LSD] test) from control (a), peptide (b), sitagliptin (c), or sitagliptin plus peptide (d).
We hypothesize that inhibition of DPPIV may have adverse renal consequences because proliferation of PGVSMCs and GMCs mediates detrimental changes in renal structure and function; activation of Y₁ receptors may stimulate growth of aortic vascular smooth muscle cells, so it is conceivable that Y₁ receptors could stimulate growth of PGVSMCs and GMCs; NPY₁₋₃₆ and PYY₁₋₃₆ but not NPY₃₋₃₆ and PYY₃₋₃₆ activate Y₁ receptors; DPPIV is present in PGVSMCs and GMCs; and inhibition of DPPIV reduces the metabolism of pancreatic polypeptide-fold peptides in PGVSMCs and GMCs.

Importantly, the results of the present study demonstrate that NPY₁₋₃₆ and PYY₁₋₃₆ stimulate proliferation of PGVSMCs and GMCs. Although these effects of NPY₁₋₃₆ and PYY₁₋₃₆ are barely perceptible in PGVSMCs and GMCs obtained from normotensive rats, the ability of NPY₁₋₃₆ and PYY₁₋₃₆ to stimulate proliferation of PGVSMCs and GMCs is pronounced in cells from genetically hypertensive animals. These findings underscore the potential for NPY₁₋₃₆ and PYY₁₋₃₆ to regulate cell growth in renovascular and glomerular cellular elements in genetically susceptible animals.

Real-time PCR and Western blotting show similar DPPIV mRNA and protein levels in PGVSMCs and GMCs from SHRs versus WKYs, yet NPY₁₋₃₆ and PYY₁₋₃₆ have little effect on proliferation of WKY PGVSMCs and GMCs. Our previously published work demonstrates that Y₁ and Y₂ receptor expression is not different in freshly isolated preglomerular microvessels from SHRs versus WKYs. However, our previously published work does show that signaling via G_i-linked receptors is enhanced in the renal vasculature of SHRs versus WKYs. Inasmuch as Y₁ receptors are G_i-coupled receptors, the most likely reason that NPY₁₋₃₆ and PYY₁₋₃₆ stimulate growth more in SHR versus WKY PGVSMCs and GMCs is that postreceptor signaling via the G_i pathway is enhanced in SHR PGVSMCs and GMCs.

The present study also demonstrates that the progrowth effects of NPY₁₋₃₆ and PYY₁₋₃₆ in cells from genetically susceptible rats are enhanced by inhibition of DPPIV, whereas inhibition of DPPIV seems to have little effect on the progrowth effects of NPY₁₋₃₆ and PYY₁₋₃₆ in cells from genetically nonsusceptible rats. The enhancement of the progrowth effects of NPY₁₋₃₆ and PYY₁₋₃₆ in cells from genetically susceptible animals by inhibition of DPPIV is likely mediated by reducing the rate of metabolism of NPY₁₋₃₆ and PYY₁₋₃₆ to NPY₃₋₃₆ and PYY₃₋₃₆, respectively, leading to greater activation of Y₁ receptors. The evidence for this conclusion is that, unlike NPY₁₋₃₆ and PYY₁₋₃₆, NPY₃₋₃₆ and PYY₃₋₃₆ have little effect on cell growth even in cells from genetically susceptible animals, and the progrowth effects of both NPY₁₋₃₆ and PYY₁₋₃₆ are abolished by the selective Y₁ receptor antagonist BIBP3226.

In addition to accelerating cell proliferation, our experiments support the conclusion that both NPY₁₋₃₆ and PYY₁₋₃₆ stimulate collagen production by both PGVSMCs and GMCs, with the effect being more pronounced in cells from genetically hypertensive animals. The ability of NPY₁₋₃₆ to stimu-

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**Figure 6.** Effects of BIBP3226 (10 μmol/L) in spontaneously hypertensive rat (SHR) preglomerular microvascular smooth muscle cells (PGVSMCs; A and B) and glomerular mesangial cells (GMCs; C and D) on [³H]-thymidine incorporation induced by neuropeptide Y (NPY)₁₋₃₆ (10 nmol/L; A and C) and peptide YY (PYY)₁₋₃₆ (10 nmol/L; B and D) in the absence or presence of sitagliptin (1 μmol/L). P values in panels compare corresponding treatment groups with and without BIBP3226 (unpaired Student t tests).
late extracellular matrix production was significantly enhanced by DPPIV inhibition in SHR PGVSMCs and SHR and WKY GMCs. Although the interaction between sitagliptin and PYY<sub>1-36</sub> did not reach statistical significance, there was a strong trend toward such an interaction in SHR PGVSMCs and GMCs.

Although the focus of the present study was NPY<sub>1-36</sub> and PYY<sub>1-36</sub>, these peptides are not the only substrates for DPPIV that could enhance proliferation of PGVSMCs and GMCs. An important question, therefore, is whether the effects of sitagliptin observed in the present study can be attributed entirely to an interaction with the metabolism of NPY<sub>1-36</sub> and PYY<sub>1-36</sub>. Importantly, in our experiments we used PDGF as our growth stimulant (ie, we did not add serum). So the only progrowth factors present in the medium were PDGF and NPY<sub>1-36</sub> or PYY<sub>1-36</sub>. Because PDGF is not a substrate for DPPIV, we are reasonably certain that the effects of sitagliptin were mediated in our experimental paradigm via inhibition of NPY<sub>1-36</sub> and PYY<sub>1-36</sub> metabolism. This hypothesis is further supported by the complete blockade of the effect of sitagliptin by antagonism of Y<sub>1</sub> receptors.

Nonetheless, in vivo sitagliptin certainly affects the metabolism of multiple peptides, and any adverse effects of sitagliptin on renal morphology in vivo could very well be because of enhancement of the levels of multiple progrowth peptides. To address this issue, it will be necessary to examine in vivo the interaction of sitagliptin with all of the possible growth-promoting peptides that are metabolized by DPPIV. The lack of an interaction between GLP-1 and sitagliptin on PDGF-induced proliferation of either SHR PGVSMCs or GMCs suggests that in vivo the progrowth effects of DPPIV inhibitors mediated by increases in NPY<sub>1-36</sub> and PYY<sub>1-36</sub> are unlikely to be offset by increases in GLP-1. However, in vivo DPPIV inhibitors decrease glucagon secretion somewhat, and glucagon may stimulate GMC proliferation. Thus, it is conceivable that in vivo decreases in glucagon-driven promotion of GMC growth could offset some of the progrowth effects attributed to enhanced Y<sub>1</sub> receptor activation.

Perspectives
In 2006, the US Food and Drug Administration approved sitagliptin, the first DPPIV inhibitor to attain US Food and Drug Administration approval for the treatment of type 2 diabetes mellitus. More recently, other DPPIV inhibitors have been approved by the US Food and Drug Administration, including saxagliptin and linagliptin, and many other DPPIV inhibitors are in development. In all likelihood, DPPIV inhibitors will become widely used to treat type 2 diabetics, many of whom will have hypertension, vascular disease, and renal diseases as comorbidities. A recent study by Kirino et al shows that, in DPPIV-deficient rats, streptozotocin-induced diabetes mellitus profoundly and chronically reduces creatinine clearance when compared with DPPIV-expressing rats. Moreover, case reports are now appearing linking DPPIV inhibitors to renal impairment in patients with type 2 diabetes mellitus. The present study demonstrates that cellular elements in the preglomerular renal microcirculation and glomeruli express DPPIV message, protein, and activity. Also, our results indicate that, in cells from genetically susceptible animals, proliferation of and extracellular matrix production by PGVSMCs and GMCs can be driven by NPY<sub>1-36</sub> and PYY<sub>1-36</sub> and that DPPIV inhibition enhances these effects of NPY<sub>1-36</sub> and PYY<sub>1-36</sub>. Because in vivo kidneys are normally exposed to high levels of NPY<sub>1-36</sub> and PYY<sub>1-36</sub>, long-term treatment with DPPIV inhibitors could enhance proliferation of and collagen production by PGVSMCs and GMCs in genetically susceptible hypertensive patients. Long-term treatment with DPPIV inhibitors might, therefore, entail a risk of renal dysfunction attributed to abnormal proliferation of cells in the preglomerular microcirculation and glomeruli. Therefore, the present findings suggest vigilance as DPPIV inhibitors become widely used in the treatment of type 2 diabetics with hypertension, vascular disease, or renal dysfunction. The recent finding that DPPIV inhibition may alter the antihypertensive effects of angiotensin-converting enzyme inhibitors further underscores this concern.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is New?**

- PGVSMCs and GMCs from SHR express DPPIV mRNA, protein, and activity.
- NPY<sub>1-36</sub> and PYY<sub>1-36</sub>, which are high-affinity endogenous substrates for DPPIV, stimulate proliferation of and collagen synthesis by SHR PGVSMCs and GMCs.
- In SHR PGVSMCs and GMCs, inhibition of DPPIV enhances the induction of proliferation and collagen production by NPY<sub>1-36</sub> and PYY<sub>1-36</sub>.

**What Is Relevant?**

- Because in vivo kidneys are normally exposed to high levels of NPY<sub>1-36</sub> and PYY<sub>1-36</sub>, long-term treatment with DPPIV inhibitors could enhance proliferation of and collagen production by PGVSMCs and GMCs in genetically susceptible hypertensive patients.

**Summary**

Long-term treatment with DPPIV inhibitors might entail a risk of renal dysfunction attributed to abnormal proliferation of cells in the preglomerular microcirculation and glomeruli, and recent case reports support this concern.
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DIPEPTIDYL PEPTIDASE IV REGULATES PROLIFERATION OF PREGLOMERULAR VASCULAR SMOOTH MUSCLE CELLS AND MESANGIAL CELLS

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METHODS

Animals. Studies utilized male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) obtained from Taconic Farms (Germantown, NY). The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolation and Culture of PGVSMCs. A complete description of our method for culturing and confirming the identity of rat PGVSMCs can be found in Mokkapatti et al.¹

Isolation and Culture of GMCs. A full description of our method for culturing and confirming the identity of rat GMCs can be found in Inoue et al.²

Quantitative Real Time PCR for DPPIV. Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) as per the manufacturer’s instructions. Quantitative real time PCR for DPPIV was performed with appropriate primers (forward primer, 5'-acaagagaaagcggaacaga-3'; reverse primer, 5'-gtcccttgactctggagga-3'; amplicon, 223 base pairs) by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the AB 7300 Real-time PCR System. The threshold cycle (Ct) for target was subtracted from the Ct for β-actin to calculate ΔCt, and relative mRNA levels were expressed as 2^ΔΔCt.

Western Blotting for DPPIV. To ensure that the signal detected by Western blotting was indeed DPPIV, we performed Western blotting for DPPIV protein as described in Jackson et al.³ using two different primary antibodies: 1) rabbit polyclonal anti-DPPIV antibody (Proteintech Group, Inc.; Chicago, IL; catalog No. 10940-1-AP; 1:600); and 2) rabbit polyclonal anti-DPPIV antibody (Thermo Scientific, Rockford, IL; catalogue number PA1-8455; 1:2500). The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Life Technologies, Grand Island, NY; catalogue number G-21234; 1:15,000).

Assay for PYY3-36. PYY3-36 levels in the medium were measured using a radioimmunoassay kit (Millipore, Billerica, MA, Catalogue number PYY-67HK) that recognizes only PYY3-36, and does not cross-react with PYY1-36.

Extraction of Sitagliptin. Sitagliptin is not available from chemical supply companies, so we purchased Januvia® tablets containing sitagliptin phosphate monohydrate from the University of Pittsburgh Medical Center hospital pharmacy, pulverized the tablets using a mortar and pestle, extracted the pulverized tablets with water and filtered the extract with a 2 micron filter. Sitagliptin phosphate monohydrate is highly water soluble. The solution was then tested by ion-trap mass spectrometry. The mass spectrum of the extract was characterized by a single peak at 408 m/z corresponding to protonated sitagliptin (407 + 1 = 408).

Assessment of Cell Proliferation and Collagen Production. PGVSMCs and GMCs were plated at a density of 5000 cells per well in 24-well tissue culture dishes. For cell number experiments,
cells were allowed to attach overnight, growth arrested for 48 hours, and then treated every 24 hours for 4 days with platelet-derived growth factor–BB (PDGF, 25 ng/mL) and various treatments, and on day 5 cells were dislodged and counted on a Coulter counter. For $^{[\text{3}]\text{H}}$-thymidine incorporation studies (index of DNA synthesis, and therefore proliferation), cells were allowed to grow to subconfluence and then were growth arrested by feeding DMEM containing 0.4% albumin for 48 hours. $^{[\text{3}]\text{H}}$-thymidine incorporation was initiated by treating growth-arrested cells for 20 hours with DMEM supplemented with PDGF (25 ng/mL) in the presence or absence of various treatments. After 20 hours of incubation, the treatments were repeated with freshly prepared solutions but supplemented with $^{[\text{3}]\text{H}}$-thymidine (1 µCi/mL). Four hours later, the experiments were terminated and the radioactivity in cells counted in a liquid scintillation counter. For assessment of collagen production, PGVSMCs and GMCs were cultured in 24-well tissue culture dishes and allowed to grow to subconfluence in DMEM/F12 containing 10% FCS under standard tissue culture conditions. Cells were rendered quiescent with DMEM containing 0.4% BSA for 48 hours. Collagen synthesis was initiated by treating growth-arrested cells for 48 hours with culture medium supplemented with PDGF (25 ng/mL) and $^{[\text{3}]\text{H}}$-L-proline (2 microCi/mL) and containing or lacking the various treatments. Experiments were terminated and the radioactivity in cells counted in a liquid scintillation counter.

**Statistical Analysis.** 2-Factor analysis of variance (2-factor ANOVA) is a statistical method that provides p-values not only for the separate effects of two independent variables per se, but also yields a p-value for the interactions between the two independent variables. It is important to note that when the interaction p-value is significant, the other two “main-effect” p-values have limited meaning since the effects of one variable depend on the level of the second variable. In this context, the nature of the interaction is then justifiably explored using post-hoc analyses to elucidate the nature of the interaction. If the interaction term in a 2-factor ANOVA is not significant, then one is generally not justified in doing post-hoc analyses and the main-effect p-values can be directly interpreted. Because the focus of the present study was the interaction between concentration of a particular PP-fold peptide and sitagliptin, in the present study we chose to use 2-factor ANOVA followed by post-hoc comparisons (using Fisher’s LSD tests) if and only if the interaction term was significant. The criterion of significance was $p<0.05$. Data are presented as means and SEMs.

**Figure S1**

**SHR GMCs**

2-Factor ANOVA
- NPY1-36: p=0.0354
- Sitagliptin: p=0.1099
- NPY1-36 x Sitagliptin: p=0.0001

**WKY GMCs**

2-Factor ANOVA
- NPY1-36: p<0.0001
- Sitagliptin: p=0.0857
- NPY1-36 x Sitagliptin: p=0.5378

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**SHR GMCs**

2-Factor ANOVA
- PYY1-36: p=0.0060
- Sitagliptin: p=0.0085
- PYY1-36 x Sitagliptin: p=0.0012

**WKY GMCs**

2-Factor ANOVA
- PYY1-36: p<0.0001
- Sitagliptin: p=0.6297
- PYY1-36 x Sitagliptin: p=0.4917
**Figure S1:** Bar graphs illustrate the effects of neuropeptide Y<sub>1-36</sub> (NPY<sub>1-36</sub>; 10 nmol/L; panels A and B) and peptide YY<sub>1-36</sub> (PYY<sub>1-36</sub>; 10 nmol/L; panels C and D) in the absence or presence of sitagliptin (1 μmol/L) on [³H]-thymidine incorporation in glomerular mesangial cells (GMCs) from spontaneously hypertensive rats (SHR; panels A and C) and Wistar-Kyoto normotensive rats (WKY; panels B and D). P-values in panels are from 2-factor analysis of variance, and letters above bars indicate significantly different (Fisher’s LSD test) from control (a), peptide (b), sitagliptin (c) or sitagliptin plus peptide (d).
SHR GMCs

2-Factor ANOVA
NPY\textsubscript{1-36}: p<0.0001
Sitagliptin: p<0.0001
NPY\textsubscript{1-36} x Sitagliptin: p=0.007

WKY GMCs

2-Factor ANOVA
NPY\textsubscript{1-36}: p<0.0001
Sitagliptin: p=0.0084
NPY\textsubscript{1-36} x Sitagliptin: p=0.4435

Figure S2
**Figure S2:** Bar graphs illustrate the effects of neuropeptide Y$_{1-36}$ (NPY$_{1-36}$; 10 nmol/L; panels A and B) and peptide YY$_{1-36}$ (PYY$_{1-36}$; 10 nmol/L; panels C and D) in the absence or presence of sitagliptin (1 µmol/L) on cell number in glomerular mesangial cells (GMCs) from spontaneously hypertensive rats (SHR; panels A and C) and Wistar-Kyoto normotensive rats (WKY; panels B and D). P-values in panels are from 2-factor analysis of variance, and letters above bars indicate significantly different (Fisher’s LSD test) from control (a), peptide (b), sitagliptin (c) or sitagliptin plus peptide (d).
**Figure S3**

SHR GMCs

![Graph A](image1)

- **2-Factor ANOVA**
  - NPY1-36: p<0.0001
  - Sitagliptin: p=0.0108
  - NPY1-36 x Sitagliptin: p=0.0190

WKY GMCs

![Graph B](image2)

- **2-Factor ANOVA**
  - NPY1-36: p<0.0001
  - Sitagliptin: p=0.0024
  - NPY1-36 x Sitagliptin: p=0.0335

SHR GMCs

![Graph C](image3)

- **2-Factor ANOVA**
  - PYY1-36: p<0.0001
  - Sitagliptin: p=0.0211
  - PYY1-36 x Sitagliptin: p=0.0705

WKY GMCs

![Graph D](image4)

- **2-Factor ANOVA**
  - PYY1-36: p<0.0001
  - Sitagliptin: p=0.0497
  - PYY1-36 x Sitagliptin: p=0.1532
Figure S3: Bar graphs illustrate the effects of neuropeptide Y1-36 (NPY1-36; 10 nmol/L; panels A and B) and peptide YY1-36 (PYY1-36; 10 nmol/L; panels C and D) in the absence or presence of sitagliptin (1 µmol/L) on [3H]-proline incorporation in glomerular mesangial cells (GMCs) from spontaneously hypertensive rats (SHR; panels A and C) and Wistar-Kyoto normotensive rats (WKY; panels B and D). P-values in panels are from 2-factor analysis of variance, and letters above bars indicate significantly different (Fisher’s LSD test) from control (a), peptide (b), sitagliptin (c) or sitagliptin plus peptide (d).
**Figure S4**

**A**

**SHR PGVSMCs**

2-Factor ANOVA

NPY<sub>3-36</sub>: p=0.1082
Sitagliptin: p=0.8623
NPY<sub>3-36</sub> x Sitagliptin: p=0.2705

**B**

2-Factor ANOVA

PYY<sub>3-36</sub>: p=0.2108
Sitagliptin: p=0.7944
PYY<sub>3-36</sub> x Sitagliptin: p=0.4490

**C**

**SHR GMCs**

2-Factor ANOVA

NPY<sub>3-36</sub>: p=0.0003
Sitagliptin: p=0.5572
NPY<sub>3-36</sub> x Sitagliptin: p=0.2279

**D**

2-Factor ANOVA

PYY<sub>3-36</sub>: p=0.0012
Sitagliptin: p=0.2560
PYY<sub>3-36</sub> x Sitagliptin: p=0.5913

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[Graphs showing the effects of NPY and Sitagliptin on SHR PGVSMCs and SHR GMCs with respective ANOVA results and [3H]-Thymidine Incorporation data.]
Figure S4: Bar graphs illustrate the effects of neuropeptide Y$_{3-36}$ (NPY$_{3-36}$; 10 nmol/L; panels A and C) and peptide YY$_{3-36}$ (PYY$_{3-36}$; 10 nmol/L; panels B and D) in the absence or presence of sitagliptin (1 µmol/L) on $[^3]$H-thymidine incorporation in preglomerular vascular smooth muscle cells (PGVSMCs; panels A and B) or glomerular mesangial cells (GMCs; panels C and D) from spontaneously hypertensive rats (SHR). P-values in panels are from 2-factor analysis of variance.
Figure S5

A  SHR PGVSMCs

Sitagliptin x GLP-1: p=0.5660

B  WKY PGVSMCs

Sitagliptin x GLP-1: p=0.3461