Dipeptidyl Peptidase-4 Inhibition Attenuates Arrhythmias via a Protein Kinase A-Dependent Pathway in Infarcted Hearts

Tsung-Ming Lee, MD; Wei-Ting Chen, MD; Nen-Chung Chang, MD, PhD

**Background:** The effect of dipeptidyl peptidase-4 (DPP-4) inhibitors on arrhythmias remains unknown. The aim of this study was to investigate whether sitagliptin attenuates arrhythmias through inhibiting nerve growth factor (NGF) expression, focusing on cyclic adenosine monophosphate (cAMP) downstream signaling such as protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac).

**Methods and Results:** Male Wistar rats were randomized to either vehicle or sitagliptin for 4 weeks starting 24 h after ligating the coronary artery. Post-infarction was associated with increased oxidative stress. Measurement of myocardial norepinephrine levels revealed a significant elevation in vehicle-treated rats compared with sham. Compared with the vehicle, infarcted rats treated with sitagliptin had significantly increased cAMP levels, decreased DPP-4 activity, oxidative stress, NGF levels and immunofluorescence-stained sympathetic hyperinnervation. Arrhythmic scores were significantly lower in the sitagliptin-treated infarcted rats than in vehicle. Ex vivo studies showed that sitagliptin increased the phosphorylated cAMP response element-binding protein (CREB), which can be reversed by H-89 (a PKA inhibitor), not brefeldin A (an Epac inhibitor). Heme oxygenase-1 (HO-1) expression was attenuated in KG-501 (a CREB inhibitor)-treated infarcted rats in the presence of a PKA agonist.

**Conclusions:** Sitagliptin protects ventricular arrhythmias by attenuating NGF-induced sympathetic innervation via upregulation of HO-1 expression in a cAMP/PKA/CREB-dependent antioxidant pathway in non-diabetic infarcted rats. (Circ J 2015; 79: 2461–2470)

**Key Words:** Arrhythmia; Cyclic adenosine monophosphate (cAMP); Myocardial infarction; Nerve growth factor; Reactive oxygen species
important trigger of NGF formation. A brief exposure to peroxynitrite induces NGF expression and secretion in astrocytes.

DPP-4 inhibitors may increase cyclic adenosine monophosphate (cAMP) levels by activating GLP-1-induced adenylyl cyclase. cAMP is a key intracellular signal that regulates neuronal differentiation, survival, neurite length, and neurite guidance in a variety of neurons. Downstream effectors of cAMP include protein kinase A (PKA) and the exchange protein activated by cAMP (Epac). While most studies of cAMP signaling have focused on PKA, cAMP has been shown to regulate gene transcription, cellular proliferation, and cytokine signaling through the Epac pathway. Epac belongs to a family of cAMP-regulated guanine nucleotide exchange factors that mediate PKA-independent signal transduction properties of the second messenger cAMP. Epac has been shown to either antagonize or synergize with PKA. Previous studies have shown that cAMP mediates axonal growth cone attraction or repulsion by distinctly activating PKA or Epac, depending on intracellular levels of cAMP. High levels mediate attraction in an Epac-dependent manner, whereas lower levels result in repulsion in a PKA-dependent manner. However, the specific involvement of cAMP effectors, PKA or Epac, on neurite outgrowth is still a subject of debate. Activation of these pathways in the cytoplasm ultimately affects nuclear transcription factors. One critical stimulus-induced transcription factor is the cAMP response element-binding protein (CREB). Transcriptional activity of CREB is positively regulated by phosphorylation of a critical serine residue, Ser133. Once phosphorylated, CREB recruits the CREB binding protein to the promoter regions of cAMP-responsive target genes such as heme oxygenase-1 (HO-1). HO-1, a stress-inducible protein, is the rate-limiting enzyme of heme degradation, and it is associated with protection against cellular injury and oxidative stress. HO-1 thus provides a relevant and sensitive index by which to assess alterations in a cellular redox state.

Administration of DPP-4 inhibitors has been shown to attenuate increased ROS in diabetic db/db mouse islets. Very recently, the GLP-1 receptor agonist, exendin-4, has been shown to attenuate ROS production. The GLP-1 receptor couplings with Gs and activates adenylyl cyclase to upregulate cAMP. There were contradictory observations regarding the GLP-1-induced cAMP downstream molecules such as PKA and Epac. GLP-1 prevents ROS-induced endothelial cell senescence only through the activation of PKA. In contrast, the GLP-1 receptor agonist, exendin-4, attenuated ROS formation in an Epac-dependent manner in diabetic Goto-Kakizaki rat islets. Thus, it appears that in different cell types and different targets, different pathways are involved in GLP-1-induced antioxidant mechanisms. However, it remains unknown whether PKA and Epac collaborate in modulating cAMP-induced NGF expression upon DPP-4 inhibition in the infarcted heart. Thus, we assessed: (1) whether sitagliptin, a DPP-4 inhibitor, after infarction modulates NGF expression; (2) whether sitagliptin-induced superoxide changes are PKA or Epac dependent in a rat MI model by the use of inhibitors of PKA and Epac as well as the measurement of cyclic nucleotides; and (3) whether PKA activation increases the phosphorylation of CREB (Ser-133).

Methods

Ethics

The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals at the China Medical University and conformed with 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).

Animals

**Experiment 1 (In Vivo)** Healthy non-diabetic Male Wistar rats (300–350g) were subjected to ligation of the left anterior descending artery, resulting in infarction of the left ventricular (LV) free wall. Twenty-four hours after inducing MI, survived rats were randomly assigned into either the vehicle (saline) or sitagliptin (5 mg/kg per day; Merck, NJ, USA) groups that were administered orally by gastric gavage once a day. The study duration was designed to be 4 weeks. For further details on the methods, please refer to Supplementary Methods.

**Experiment 2 (Ex Vivo)** To test the relative importance of PKA and Epac in sitagliptin-related NGF levels, we used inhibitors of PKA and Epac in an ex vivo model. Four weeks after induction of MI by coronary ligation, infarcted rat hearts were isolated and subjected to no treatment (vehicle), sitagliptin (5 μmol/L), sitagliptin+H-89 (0.1 μmol/L, a highly specific inhibitor of PKA), and sitagliptin+brefeldin A (100 μmol/L, an Epac-signaling inhibitor). The doses of sitagliptin, H-89, and brefeldin A have been shown to be effective in modulating biological activities. The H-89 and brefeldin A were all from Sigma (St Louis, MO, USA). The heart was perfused with a non-circulating modified Tyrode’s solution containing (in mmol/L): glucose 5.5, NaCl 117.0, NaHCO3 23.0, KCl 4.6, NaH2PO4 0.8, MgCl2 1.0, and CaCl2 2.0, equilibrated at 37°C and oxygenated with a 95% O2 to 5% CO2 gas mixture. The drugs were infused for 60 min. At the end of the study, all hearts (n=5 each group) were used for performing Western analysis for p-CREB (Ser-133) and NGF protein at the remote zone (>2 mm outside the infarct).

**Experiment 3 (Ex Vivo)** To confirm the relative importance of PKA and Epac in HO-1 expression, we used activators of PKA and Epac in HO-1 expression, we used activators of PKA and Epac in HO-1 expression, we used activators of PKA and Epac in HO-1 expression, we used activators of PKA and Epac in HO-1 expression. We used activators of PKA and Epac in HO-1 expression. We used activators of PKA and Epac in HO-1 expression. We used activators of PKA and Epac in HO-1 expression. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used. Experiments were performed in triplicate. Non-circulating modified Tyrode’s solution was used.
of SIN-1 was used. At the end of the study, hearts (n=5 per group) were used for Western blot of NGF at the remote zone.

**Hemodynamics and Infarct Size Measurements**

Hemodynamic parameters and infarct size were measured in anesthetized rats at the end of the study, as described in detail in **Supplementary Methods**.

**In Vivo Electrophysiological Studies**

Following arterial pressure measurement, the rats were intubated. Electrophysiological studies were performed with electrodes sewn to the epicardial surface of the right ventricular outflow tract. To determine the ventricular effective refractory period (VERP), single extrastimuli were introduced at progressively shorter intervals. The VERP was the longest S1S2 interval that did not evoke a premature ventricular depolarization. For detailed methods, please refer to **Supplementary Methods**.

**Real-Time RT-PCR of HO-1 and NGF**

mRNAs were quantified by using real-time RT-PCR, with cyclophilin as a loading control. For detailed methods, please refer to **Supplementary Methods**.

**Western Blot Analysis of p-CREB (Ser-133) and NGF**

Samples were obtained from the remote zone at week 4 after infarction. Rabbit polyclonal antibodies to NGF (Chemicon, Hamburg, Germany), p-CREB (Ser-133, Cell Signaling) and CREB (Cell Signaling) were used. Western blotting procedures were described previously. Experiments were replicated three times and results were expressed as the mean value.

**Immunofluorescent Studies of Tyrosine Hydroxylase, Growth Associated Factor 43 and Neurofilament**

In order to investigate the spatial distribution and quantification of sympathetic nerve fibers, analysis of immunofluorescent staining was performed on the LV muscle from the remote zone. The analysis of the immunofluorescent staining is described in detail in **Supplementary Methods**.

**In Situ Detection of Superoxide**

Myocardial intracellular superoxide production was determined by using in situ dihydroethidium (DHE; Invitrogen Molecular Probes, Eugene, OR, USA) fluorescence, as previously described. For a detailed method, please refer to **Supplementary Methods**.

**Laboratory Measurements**

We measured the DPP-4 activity and active GLP-1 levels in plasma at the end of the study to confirm that continuous administration of sitagliptin was indeed associated with suppression of plasma DPP-4 activity, and an increase in the active GLP-1 levels. EDTA plasma was used to measure active GLP-1 (Millipore Corporation, Billerica, MA, USA) and DPP-4 activity (Quantizume AssaySystem, BIOMOL International, Plymouth Meeting, PA, USA). Insulin was measured by an ultrasensitive rat enzyme immunoassay (Merckodia, Uppsala, Sweden).

Although cardiac innervation was detected by immunofluorescent staining of tyrosine hydroxylase, growth-associated factor 43, and neurofilament, it did not imply that the nerves are functional. Thus, to examine the sympathetic nerve function after administering sitagliptin, we measured LV norepinephrine levels from the remote zone using a commercial ELISA kit (Noradrenal ELISA, IBL Immuno-Biological Laboratoraries Co, Hamburg, Germany).

Myocardial cAMP was measured by using an enzyme-linked immunoassay kit (R&D Systems, Abingdon, UK), and the protein content was determined by the BCA protein assay kit and CAMP levels were expressed as pmol/g LV tissue.

Superoxide production by myocardium from the remote zone was measured using lucigenin (5μmol/L bis-N-methyl-acridinium nitrate; Sigma, St. Louis, MO, USA) enhanced chemiluminescence, as previously described. The specific chemiluminescence signal was calculated after subtraction of background activity, and expressed as counts per minute per milligram weight (cpm/mg).

To estimate myocardial peroxynitrite formation, we measured free nitrotyrosine (as a marker for peroxynitrite forma-

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**Table. Cardiac Morphology, Hemodynamics and Glucose, Insulin, and Tissue NE Levels at the End of Study**

<table>
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<tr>
<th>Parameters</th>
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</tr>
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<tbody>
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<td>No. of rats</td>
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<td>10</td>
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<tr>
<td>Body weight, g</td>
<td>405±10</td>
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<td>Heart rate, bpm</td>
<td>397±9</td>
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<tr>
<td>Infarct size, %</td>
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<tr>
<td>LVW/BW, mg/g</td>
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<td>RVW/BW, mg/g</td>
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<td>LungW/BW, mg/g</td>
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<td>Glucose, mg/dl</td>
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<td>Insulin, μg/ml</td>
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<td>52±11*</td>
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<tr>
<td>NE, μg/g protein</td>
<td>1.38±0.38</td>
<td>1.27±0.32</td>
<td>2.78±0.22*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. BW, body weight; LungW, lung weight; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; LVW, left ventricular weight; NE, norepinephrine levels from remote myocardium; RVW, right ventricular weight. *P<0.025 compared with respective sham; **P<0.025 compared with the saline-treated infarcted group.
thin, and was totally replaced by fully differentiated scar tissue. The weight of the LV inclusive of the septum remained essentially constant for 4 weeks between the infarcted groups (Table). Compared with vehicle-treated infarcted rats in sitagliptin-treated infarcted rats, the maximal rate of LV $+\frac{dP}{dt}$ and $-\frac{dP}{dt}$ was significantly increased, and the lung weight/body weight ratio was significantly lower, which is consistent with favorable LV remodeling. LV end-systolic pressure and infarct size did not differ between the infarcted groups. No changes in plasma glucose and insulin levels were observed between the 2 infarcted groups.

Plasma GLP-1 and DPP-4 Activity and Myocardial cAMP, Superoxide, Nitrotyrosine, and Norepinephrine Levels

Plasma DPP-4 activity and GLP-1 levels were determined to confirm the successful oral delivery of sitagliptin. This was associated with a significant increase of the active GLP-1 level in the sitagliptin-treated group (Figure 1A). Plasma DPP-4 activity was significantly reduced by 63% in the sitagliptin-treated group compared to the vehicle group (Figure 1B). Compared with sham, ventricular remodeling after MI was associated with a significant increase in cAMP content (239±52

**Statistical Analysis**

Results are presented as mean±SD. Statistical analysis was performed using the SPSS statistical package (SPSS, version 12.0, Chicago, IL, USA). Differences among the groups of rats were tested by an ANOVA. In case of a significant effect, the measurements between the groups were compared with Bonferroni’s correction. Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. The significant level was assumed at a value of P<0.05.

**Results**

**Part 1. In Vivo Study (Experiment 1)**

Differences in mortality between the 2 infarcted groups were not found throughout the study. Sitagliptin had little effect on cardiac gross morphology in the sham-operated rats. Four weeks after infarction, the infarcted area of the LV was very

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**Figure 1.** Plasma (A) glucagon-like peptide-1 (GLP-1) and (B) dipeptidylpeptidase-4 (DPP-4) activity and myocardial (C) cyclic adenosine monophosphate (cAMP), (D) superoxide and (E) nitrotyrosine levels from the remote zone. *P<0.01 compared with sham- and vehicle-treated infarcted rats; †P<0.01, compared with sham and sitagliptin.
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in sham vs. 378±58 pmol/g LV, P<0.001, Figure 1C). Treatment with sitagliptin had a significant increase of cAMP content compared with vehicle.

Myocardial superoxide production, as assessed by lucigenin-enhanced chemiluminescence, was markedly increased in remote LV myocardium after MI as compared with sham (P<0.001, Figure 1D). Superoxide was significantly decreased in sitagliptin-treated rats to the level of sham.

Figure 2. (A) Detection of superoxide in the myocardium by dihydroethidium (DHE) staining (magnification 400x). Compared with sham, the DHE fluorescence intensity in the myocardium of the vehicle-treated infarcted group was significantly increased. DHE staining (%) at the remote zone. (B) Immunofluorescent staining for tyrosine hydroxylase from the remote regions. Tyrosine hydroxylase-positive nerve fibers are located between myofibrils and are oriented in a longitudinal direction as that of the myofibrils. (C) Immunofluorescent staining for growth-associated protein 43 from the remote regions. (A) Sham group; (B) infarction treated with vehicle group; (C) infarction treated with sitagliptin group. Bar=50 μm. Nerve density area fraction (%) at the remote zone. Each column and bar represents mean±SD. The number of animals in each group is indicated in parentheses. *P<0.05, compared with sham and sitagliptin.
Figure 2B. Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated infarcted rats than that in sham group. Sitagliptin-treated rats showed lower nerve density at the remote regions than vehicle-treated rats (0.23 ± 0.11% vs. 0.09 ± 0.03% in sitagliptin group, both P<0.001). Similar to the tyrosine hydroxylase results, densities of growth-associated protein 43-(Figure 2C) and neurofilament-positive nerves were significantly attenuated in the sitagliptin-treated infarcted rats compared with those in the vehicle-treated infarcted group. These morphometric results mirrored those of norepinephrine contents.

NGF Protein and mRNA Expression

Western blot shows that NGF levels were significantly upregulated 2.1-fold at the remote zone in the vehicle-treated infarcted rats than in sham-operated rats (P<0.001, Figure 3A). When compared with vehicle-treated infarcted rats, sitagliptin-treated infarcted rats had significantly lower NGF levels.

PCR amplification of the cDNA revealed that the NGF mRNA levels showed a 2.7-fold upregulation at the remote zone in the vehicle-treated infarcted rats compared with sham (2.78±0.22 vs. 1.38±0.38µg/g protein, P<0.001, Table). When compared with vehicle-treated infarcted rats, sitagliptin-treated infarcted rats had a significantly lower LV norepinephrine level.

DHE Staining in Myocardium

DHE reacts with superoxide radicals to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide radical generation. As shown in Figure 2A, postinfarction remodeling markedly enhanced the intensity of the DHE staining in the remote zone in the vehicle-treated rats compared with sham. However, the intensity of the fluorescent signal in the sitagliptin group was significantly reduced relative to the vehicle group.

Immunofluorescent Analyses

The tyrosine hydroxylase-immunostained nerve fibers appeared to be oriented in the longitudinal axis of adjacent myofibers (Figure 2B). Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated infarcted rats than that in sham group. Sitagliptin-treated rats showed lower nerve density at the remote regions than vehicle-treated rats (0.23±0.11% vs. 0.09±0.03% in sitagliptin group, both P<0.001). Similar to the tyrosine hydroxylase results, densities of growth-associated protein 43-(Figure 2C) and neurofilament-positive (data not shown) nerves were significantly attenuated in the sitagliptin-treated infarcted rats compared with those in the vehicle-treated infarcted group. These morphometric results mirrored those of norepinephrine contents.

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Electrophysiological Stimulation

To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was per-
increased to 1.52-fold over the vehicle. Moreover, this effect of sitagliptin on CREB phosphorylation was inhibited by coadministration of H-89 (a PKA inhibitor), not brefeldin A (an Epac inhibitor). Compared with sitagliptin alone, the PKA inhibitor significantly increased NGF levels to 2.05-fold. Intriguingly, the Epac antagonist did not change NGF levels induced by sitagliptin.

Effect of PKA on HO-1 Expression (Experiment 3)
Treatment with a PKA inhibitor confirmed that CREB phosphorylation by sitagliptin occurs through the PKA pathway. We further determined the effect of PKA on the CREB target gene, HO-1.

As shown in Figure 5, N6Bz, not 8-CPT, increased HO-1 mRNA expression, which can be reversed by administering KG-501, a specific antagonist that disrupts the CREB:CBP complex and attenuates target gene induction. This result further confirms that PKA can activate CREB, which in turn upregulates the downstream target gene, HO-1.

Effect of Superoxide on NGF Levels (Experiment 4)
To elucidate the role of superoxide in modulating NGF, SIN-1 was assessed in an ex vivo model. Figure 6 shows that SIN-1 significantly increased NGF levels compared with sitagliptin alone, thus confirming the role of superoxide in mediating NGF levels.

Discussion
Our present study shows for the first time that chronic treatment for 4 weeks with sitagliptin leads to attenuated NGF expression, probably through a cAMP/PKA-dependent HO-1 pathway, independently from its glucose-lowering action. The induction of HO-1 along with an increase of CREB phosphorylation by PKA is responsible for attenuated sympathetic innervation and arrhythmias against oxidant stress. These results were concordant for beneficial effects of sitagliptin, as documented structurally by reduction in cardiac nerve sprouting, molecularly by myocardial NGF protein and mRNA levels, biochemically by tissue cAMP, superoxide and norepinephrine levels, pharmacologically by inhibition of PKA and Epac, and electrophysiologically by reduced extent of infarc-
tion restores GLP-1 signaling within the physiological, not pharmacological, range. Myocardial benefits of GLP-1 were carried out using either native GLP-1 or recombinant GLP-1 analogues at high concentrations or in a way that induced supraphysiological GLP-1 signaling. The supraphysiological GLP-1 levels by exendin-4 may explain, at least in part, the different mechanisms responsible for attenuated ROS in diabetic Goto-Kakizaki rat islets.

Furthermore, the rise in cAMP treated with sitagliptin is within the physiological range and limited to those subcellular regions where adenylate cyclase is localized (e.g., cell membrane), by the maximal activity level of adenylate cyclase, and by cAMP degradation by cyclic nucleotide phosphodiesterases. Depending on their relative abundance, distribution, partners, and localization, PKA and Epac may act independently, cooperate, or oppose each other in regulating a specific cellular function.

Because our results using specific inhibitors reveal that PKA mediates inhibitory effects on NGF, it appears likely that the PKA pathway is of physiological relevance for NGF levels.

PKA signaling may regulate HO-1 expression through CREB activation. mRNA expression of the HO-1 gene is induced by the PKA-stimulating agent, N6Bz. Addition of a CREB:CBP antagonist, KG-501, suppressed N6Bz-induced HO-1 expression. The Epac agonist did not affect the expression of HO-1. These results suggest that PKA and CREB activation were involved in cAMP-induced HO-1 expression. The HO-1 gene is a CREB-dependent gene.

Increasing evidence has demonstrated...
that HO-1 catalysis provides the potent antioxidant activity. HO-1 activates the expression of mitochondrial superoxide dismutase in neonatal rat astroglia.\textsuperscript{24} In addition, the HO-1-deficient mice exhibit serious damage to iron metabolism, resulting in organ oxidative injury.\textsuperscript{30} The specific role of PKA was further supported by the ex vivo observation that sitagliptin treatment induced CREB phosphorylation, which can be reversed by administering H-89 in infarcted rats. Thus, our findings indicate the close correlation between PKA activation and HO-1 expression in sitagliptin-treated hearts. Indeed, our results were consistent with the findings of Immenschuh et al.,\textsuperscript{36} showing HO-1 gene induction by PKA activation. This extends a previous observation in which in vivo vildagliptin treatment resulted in HO-1 induction of cardiac tissue.\textsuperscript{24}

In this study, we demonstrated attenuated sympathetic reinnervation in sitagliptin-treated hearts. The detailed mechanisms by which sitagliptin affected sympathetic reinnervation remain undefined; however, several factors can be excluded. First, infarct sizes. The degree of sympathetic reinnervation has been related to the infarct sizes.\textsuperscript{37} Successful fiber reinnervation appears dependent on repopulating sheaths with Schwann cells, which would be injured according to the size of infarction. This possibility was excluded in this study due to similar infarct sizes between the infarcted groups. Second, insulin concentrations. Our results showed a robust phenomenon despite any potentially confounding effect of insulin-mediated sympathetic reinnervation. Initially, it was surmised that DPP-4 inhibitors, acting as a potent incretin, could increase levels of insulin, thereby suggesting a possible mechanism to explain our findings in the in vivo study. However, although a rise in insulin and a decrease in glucose is possible, our results showed that sitagliptin-mediated stimulation of insulin release is not significant because insulinotropic effects of sitagliptin are glucose dependent. Importantly, the attenuated NGF levels observed in our study are reproduced in both the in vivo and ex vivo models; the latter being specifically relevant because in this setting, there is an absence of circulating insulin. Any residual insulin in these hearts at the time of harvesting from the rats would be lost from the tissues during the stabilization period on the Langendorff apparatus, implying that the protective effects that we observe may not be a direct consequence of insulin itself.

Conclusions

These data provide new evidence that sitagliptin protects fatal arrhythmias by attenuating NGF-induced sympathetic reinnervation via the cAMP/PKA/CREB signaling pathway. The results of the present study support the concept of pleiotropic anti-oxidant properties of sitagliptin, suggesting that DPP-4 inhibitors might have antiarrhythmic benefits in diabetic patients.

Acknowledgments

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Disclosure

The authors declare that there are no conflicts of interest associated with this manuscript.

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Supplementary Files

Supplementary File 1

Methods

Please find supplementary file(s):