Therapeutics

ORIGINAL RESEARCH COMMUNICATION

Uncoupling Protein-2 Mediates DPP-4 Inhibitor-Induced Restoration of Endothelial Function in Hypertension Through Reducing Oxidative Stress

Limei Liu,[1–3] Jian Liu,[2,3] Xiao Yu Tian,[2,3] Wing Tak Wong,[2,3] Chi Wai Lau,[2,3] Aimin Xu,[4,5] Gang Xu,[6] Chi Fai Ng,[7] Xiaoqiang Yao,[2,3] Yuansheng Gao,[1] and Yu Huang[2,3]

Abstract

Aims: Although uncoupling protein 2 (UCP2) negatively regulates intracellular reactive oxygen species (ROS) production and protects vascular function, its participation in vascular benefits of drugs used to treat cardiometabolic diseases is largely unknown. This study investigated whether UCP2 and associated oxidative stress reduction contribute to the improvement of endothelial function by a dipeptidyl peptidase-4 inhibitor, sitagliptin, in hypertension. *Results:* Pharmacological inhibition of cyclooxygenase-2 (COX-2) but not COX-1 prevented endothelial dysfunction, and ROS scavengers reduced COX-2 mRNA and protein expression in spontaneously hypertensive rats (SHR) renal arteries. Angiotensin II (Ang II) evoked endothelium-dependent contractions (EDCs) in C57BL/6 and UCP2 knockout (*UCP2KO*) mouse aortae. Chronic sitagliptin administration attenuated EDCs in SHR arteries and Ang II-infused C57BL/6 mouse aortae and eliminated ROS overproduction in SHR arteries, which were reversed by glucagon-like peptide 1 receptor (GLP-1R) antagonist exendin 9-39, AMP-activated protein kinase (AMPK)α inhibitor compound C, and UCP2 inhibitor genipin. By contrast, sitagliptin unaffected EDCs in Ang II-infused *UCP2KO* mice. Sitagliptin increased AMPKα phosphorylation, upregulated UCP2, and downregulated COX-2 expression in arteries from SHR and Ang II-infused C57BL/6 mice. Importantly, exendin 9-39, compound C, and genipin reversed the inhibitory effect of GLP-1R agonist exendin-4 on Ang II-stimulated mitochondrial ROS rises in SHR endothelial cells. Moreover, exendin-4 improved the endothelial function of renal arteries from SHR and hypertensive patients. *Innovation:* We elucidate for the first time that UCP2 serves as an important signal molecule in endothelial protection conferred by GLP-1-related agents. UCP2 could be a useful target in treating hypertension-related vascular events. *Conclusions:* UCP2 inhibits oxidative stress and downregulates COX-2 expression through GLP-1/GLP-1R/ AMPKα cascade. *Antioxid. Redox Signal.* 21, 1571–1581.

Introduction

ENDOTHELIUM-DEPENDENT CONTRACTIONS (EDCs) associated with endothelial dysfunction were first observed in the isolated aortae of spontaneously hypertensive rats (SHR) (21), and EDCs are correlated with the severity of hypertension (12). Impaired endothelial function can be initiated either as a consequence of reduced production/release of nitric oxide (NO) or by increased inactivation of NO due to the overproduction of reactive oxygen species (ROS) or the two factors combined (4). ROS overproduction or increased oxidative stress has been implicated in the onset and/or

[1]Department of Physiology and Pathophysiology, Peking University Health Science Center, Peking, China.
[2]Institute of Vascular Medicine, Chinese University of Hong Kong, Hong Kong, China.
[3]Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong, Hong Kong, China.
Departments of [4]Medicine and [5]Pharmacology and Pharmacy, University of Hong Kong, Hong Kong, China.
[6]School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China.
[7]Department of Surgery, Chinese University of Hong Kong, Hong Kong, China.

Innovation

The present study provides novel evidence showing that cyclooxygenase-2 (COX-2) upregulation mediates endothelium-dependent contractions (EDCs) in arteries from angiotensin II (Ang II)-infused mice and spontaneously hypertensive rats (SHR), whereas inhibition of reactive oxygen species (ROS) production downregulates COX-2 expression and attenuates EDCs in SHR arteries. Uncoupling protein 2 (UCP2) inhibits mitochondrial ROS production as an antioxidant protein. The present study demonstrates that a highly selective dipeptidyl peptidase-4 inhibitor, sitagliptin, protects endothelial function in hypertension through elevating the expression of UCP2 and subsequently scavenging mitochondrial ROS, which in turn downregulates COX-2 expression *via* the stimulation of the glucagon-like peptide (GLP)-1/GLP-1R/AMPKα cascade. We have therefore revealed that UCP2 is another effective target for drug intervention to combat against the hypertension-related vascular diseases.

progression of chronic diseases, including hypertension (2, 24). EDCs are induced by endothelium-derived contracting factors (EDCFs), including some endothelial cyclooxygenase (COX)-derived prostanoids or ROS. Constrictive prostanoids directly activate thromboxane/prostaglandin (TP) endoperoxide receptors of the vascular smooth muscle cells (VSMCs) to induce contraction, whereas ROS stimulate the activity of COX in endothelial cells or VSMCs to release prostanoids, such as $PGF_{2\alpha}$, which stimulate the TP receptors (27, 33, 34).

ROS participate in vascular tone regulation (32), and the mitochondrion is a major source of ROS (30). The mitochondrial uncoupling proteins (UCPs) have emerged as important natural antioxidants in maintaining cellular ROS homeostasis (3, 7). UCPs are the inner mitochondrial membrane-associated proteins, which dissipate the proton electrochemical gradient formed during mitochondrial respiration and generate heat production instead of ATP (13). Transcriptional upregulation of mitochondrial UCP2 abrogates angiotensin II (Ang II)-induced oxidative stress and alleviates oxidative stress-associated neurogenic hypertension (6). A significant association of hypertension with UCPs, especially UCP2, has been reported in hypertensive animals and patients (8, 17). UCP2 appears to be a negative regulator of ROS generation (23, 35). The promoter polymorphism of the UCP2 gene is associated with hypertension (17). Furthermore, overexpression of UCP2 inhibits ROS production in endothelial cells (19).

Our previous study showed that sitagliptin, a highly selective dipeptidyl peptidase-4 (DPP-4) inhibitor, improves endothelium-dependent relaxations and decreases systolic blood pressure *via* elevating NO production in patients and rats with hypertension (20). The present study aimed to investigate whether UCP2 contributes to the vascular benefit of sitagliptin in suppressing EDCs in hypertension.

Results

Sitagliptin attenuated EDCs in SHR renal arteries

EDCs are associated with endothelial dysfunction in hypertension. First, acetylcholine (ACh)-triggered EDCs in renal arteries from SHR were exaggerated compared to those

from Wistar-Kyoto rats (WKY), and 2-week oral treatment with sitagliptin markedly attenuated EDCs in arteries from SHR without affecting those from WKY (Fig. 1A). The EDCs were likely mediated through COX-2-dependent mechanism since they were inhibited or abolished by selective COX-2 inhibitor NS398 (1 μM) and DuP697 (3 μM) or selective TP receptor antagonists S18886 (0.1 μM) but unaffected by COX-1 inhibitor valeryl salicylate (VAS) (10 μM) (Fig. 1B). Importantly, chronic sitagliptin treatment reduced the elevated COX-2 expression in SHR arteries (Fig. 1C). By contrast, COX-1 expression was similar in WKY and SHR with or without sitagliptin treatment (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). Furthermore, we have for the first time demonstrated that the UCP2 expression was significantly reduced in SHR renal arteries compared with WKY controls, and sitagliptin treatment increased the UCP2 expression in both SHR and WKY arteries (Fig. 1D), suggesting the possible involvement of UCP2 in contributing to the vascular benefits of sitagliptin.

Sitagliptin attenuated EDCs *via* GLP-1R/AMPKα/ UCP2 cascade in SHR renal arteries

To obtain insight into the molecular mechanism of sitagliptin to attenuate EDCs, we next explored the effects of some antagonist or inhibitors on the effects of sitagliptin. Glucagon-like peptide 1 receptor (GLP-1R) antagonist exendin 9-39 (100 nM), AMP-activated protein kinase
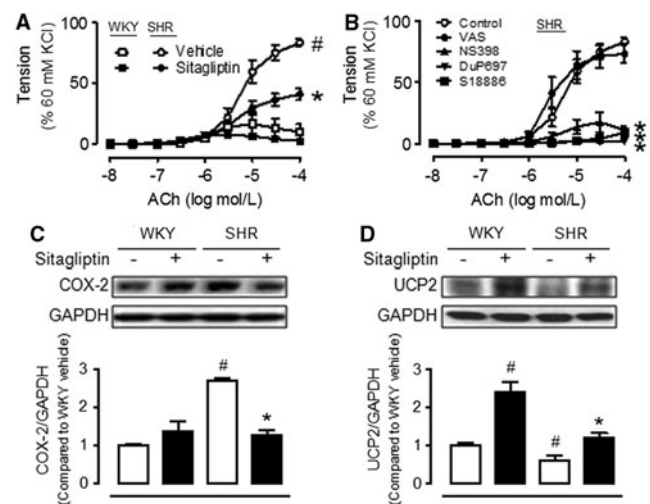

FIG. 1. Sitagliptin attenuates EDCs in SHR renal arteries. (A) In the presence of L-NAME, ACh elicited pronounced EDCs of renal arterial rings from SHR, which were inhibited by 2-week administration with sitagliptin. **(B)** EDCs in SHR arteries were attenuated or abolished by treatment with selective COX-2 inhibitor NS398 (1 μM) and DuP697 (3 μM) or selective TP receptor antagonist S18886 (0.1 μM) but unaffected by COX-1 inhibitor VAS (10 μM). COX-2 **(C)** and UCP2 **(D)** expressions in renal arteries from WKY and SHR. Results are means ± SEM of six to eight experiments. *$p < 0.05$ *versus* SHR vehicle, #$p < 0.05$ *versus* WKY vehicle. L-NAME, NG-nitro-L-arginine methyl ester; EDCs, endothelium-dependent contractions; VAS, valeryl salicylate; ACh, acetylcholine; COX-2, cyclooxygenase-2; TP, thromboxane/prostanoid; UCP2, uncoupling protein 2; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

(AMPK)α inhibitor compound C (10 μM), and UCP2 inhibitor genipin (1 μM) augmented the EDCs in renal arteries from sitagliptin-treated SHR (Fig. 2A) without affecting those from vehicle-treated SHR (Fig. 2B). Sitagliptin treatment increased AMPK phosphorylation (Fig. 2C) and UCP2 expression (Fig. 2D), whereas it reduced COX-2 expression (Fig. 2E) in SHR renal arteries. Moreover, the increased AMPK phosphorylation (Fig. 2C) and elevated UCP2 expression by sitagliptin were inhibited by exendin 9-39 (100 nM) and compound C (10 μM) but not by genipin (1 μM) (Fig. 2D). On the other hand, the inhibitory effect of sitagliptin on COX-2 upregulation was reversed not only by exendin 9-39 and compound C but also by genipin (Fig. 2E). These data suggest that UCP2 is likely to mediate the beneficial effect of GLP-1R and AMPK activation to suppress COX-2 expression in hypertension.

Sitagliptin reduced COX-2 expression via scavenging mitochondrial ROS in SHR renal arteries

Since the sitagliptin-induced reduction of the COX-2 expression was due to the upregulation of UCP2, we hypothesized that UCP2 activation decreased the ROS level and subsequently resulted in COX-2 downregulation. To test this hypothesis, we investigated the ROS level as well as the effect of ROS scavengers on EDCs and COX-2 expression in SHR arteries. First, ROS production increased in renal arteries from SHR compared to those from WKY (Supplementary Fig. S2). Sitagliptin (10 mg·kg^{-1}·day^{-1}) or *in vitro* treatment with tempol (100 μM) or tiron (1 mM) plus diethyldithiocarbamate (DETCA) (100 μM) reduced the ROS level in SHR arteries, whereas exendin 9-39, compound C, or genipin reversed the inhibitory effects of sitagliptin (Fig. 3A and Supplementary Fig. S3A, B). Likewise, the results from lucigenin-enhanced chemiluminescence assay (Fig. 3B) and electron paramagnetic resonance (EPR) spin trapping method (Fig. 3C and Supplementary Fig. S4) also showed that

sitagliptin-induced reduction of superoxide anion accumulation in SHR arteries was reversed by these inhibitors. Next, we explored the effects of ROS scavengers on EDCs in SHR renal arteries and showed that tempol (100 μM) or tiron (1 mM) plus DETCA (100 μM) reduced EDCs in these arteries (Fig. 3D). Similarly, three mitochondrial ROS scavengers, MitoQ (100 nM), coenzyme Q10 (10 μM), and idebenone (10 μM), attenuated EDCs (Fig. 3E). Furthermore, tempol, tiron plus DETCA, coenzyme Q10 (10 μM), or idebenone (10 μM) reduced the expression of COX-2 mRNA (Fig. 3F) and protein (Fig. 3G) in SHR arteries. Taken together, the present results suggest that sitagliptin reduces COX-2 expression most likely through scavenging mitochondrial ROS in SHR renal arteries.

Exendin-4 ameliorated endothelial dysfunction in SHR

Our previous study revealed that sitagliptin improved endothelium-dependent relaxation in a GLP-1-dependent manner. The present study also confirmed that GLP-1 plays an important role in the sitagliptin-induced attenuation of EDCs. Treatment with GLP-1 analog exendin-4 (10 nM, 8 h) diminished EDCs (Fig. 4A) and the ROS level (Supplementary Fig. S5) in SHR renal arteries; both effects were reversed by cotreatment with exendin 9-39 (100 nM), compound C (10 μM), or genipin (1 μM) (Fig. 4B and Supplementary Fig. S5). Moreover, both the lucigenin-enhanced chemiluminescence assay (Fig. 3B) and the EPR method (Fig. 3C) showed that exendin-4 reduced superoxide anion level in SHR arteries, and this effect was abolished by genipin. To further demonstrate that the protective effect of GLP-1 analog is endothelium-dependent, we tested the action of exendin-4 in primary cultured SHR endothelial cells (SHRECs). Eight hours of incubation with exendin-4 (10 nM) reduced mitochondrial ROS production triggered acutely by Ang II (100 nM, 30 min) in SHRECs, which was also inhibited by cotreatment with exendin 9-39, compound C, or genipin (Fig. 4C, D).
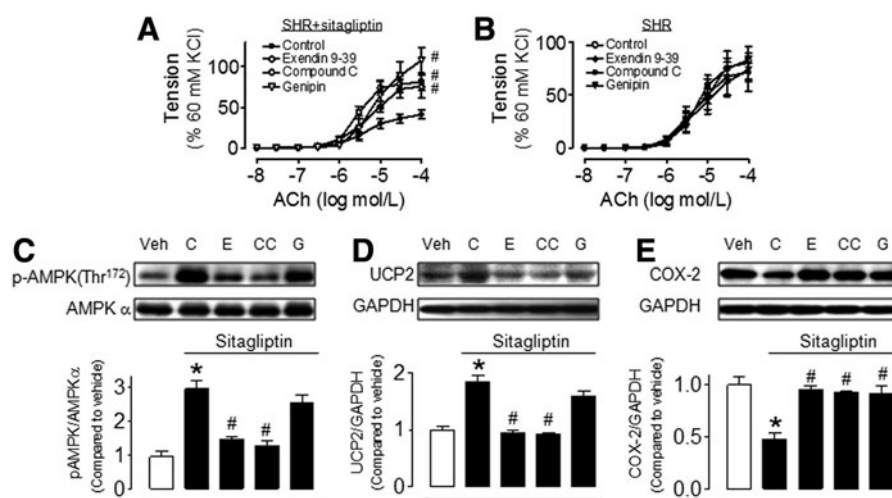

FIG. 2. Sitagliptin attenuates EDCs through the activation of GLP-1 receptor/AMPKα/UCP2 in SHR renal arteries. Effects of GLP-1 receptor antagonist exendin 9-39 (100 nM), AMPKα inhibitor compound C (10 μM), and UCP2 inhibitor genipin (1 μM) on EDCs in sitagliptin-treated SHR **(A)** and vehicle-SHR **(B)** groups. Level of AMPK phosphorylation **(C)** and expressions of UCP2 **(D)** and COX-2 **(E)** in SHR renal arteries. Results are means ± SEM of four to eight experiments. *$p < 0.05$ *versus* vehicle, #$p < 0.05$ *versus* sitagliptin. Veh, vehicle; C, control; E, exendin 9-39; CC, compound C; G, genipin; AMPK, AMP-activated protein kinase; GLP-1, glucagon-like peptide 1.
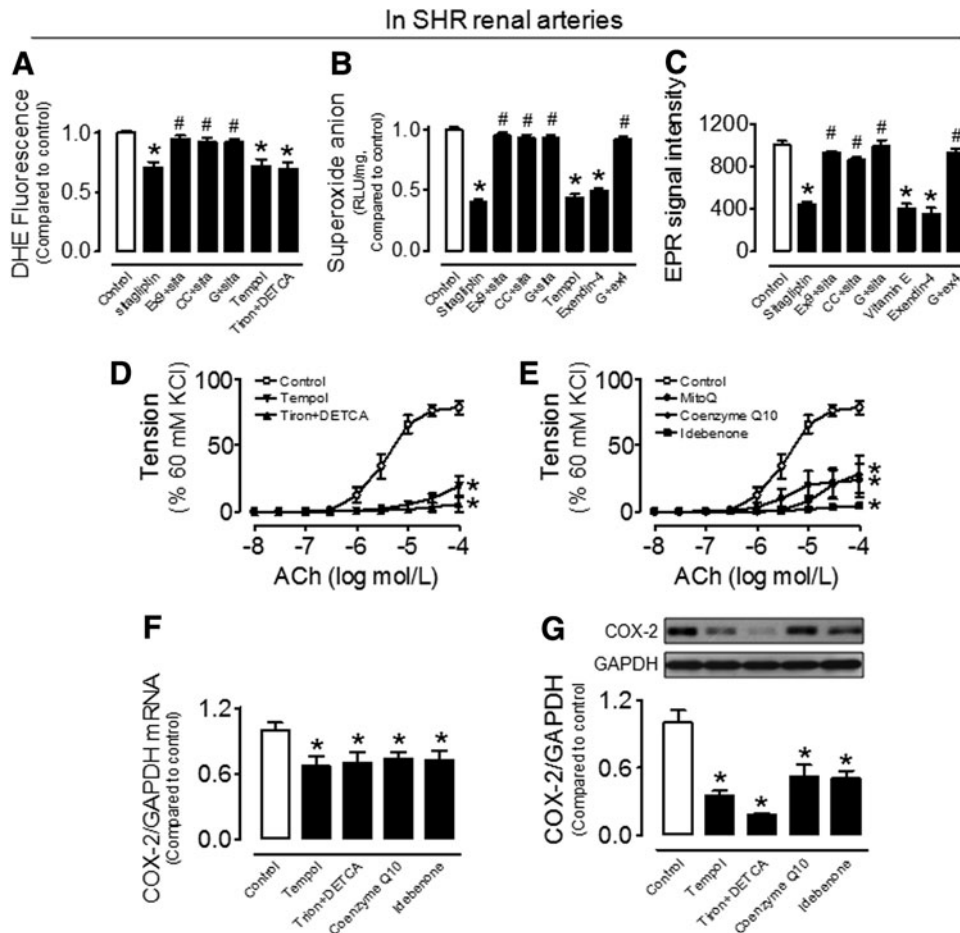
In SHR renal arteries

FIG. 3. Sitagliptin reduces COX-2 expression *via* scavenging ROS in SHR renal arteries. Exendin 9-39 (100 n*M*), compound C (10 μM), or genipin (1 μM) reversed the inhibitory effect of sitagliptin (10 mg·kg^{-1}·day^{-1}) on ROS production by DHE staining **(A)**, lucigenin-enhanced chemiluminescence assay **(B)**, and EPR **(C)**. Moreover, acute treatment with tempol (100 μM) or tiron (1 m*M*) plus DETCA (100 μM) **(A)** reduced ROS level, and genipin (1 μM) also reversed the ROS-lowering effect of exendin-4 (10 n*M*) **(B, C)**. ROS scavenger tempol (100 μM) or tiron (1 m*M*) plus DETCA (100 μM) **(D)** and mitochondrial ROS scavengers MitoQ (100 n*M*), coenzyme Q10 (10 μM), or idebenone (10 μM) **(E)** reduced or abolished EDCs in SHR arteries. Tempol (100 μM), tiron (1 m*M*) plus DETCA (100 μM), coenzyme Q10 (10 μM), or idebenone (10 μM) reduced COX-2 mRNA **(F)** and protein expression **(G)** in SHR arteries. Results are means ± SEM of four to eight experiments. *$p < 0.05$ *versus* control, #$p < 0.05$ *versus* sitagliptin or exendin-4. Ex9, exendin 9-39; CC, compound C; G, genipin; ex4, exendin-4; RLU, relative luminance unit; ROS, reactive oxygen species; EPR, electron paramagnetic resonance; DHE, dihydroethidium; DETCA, diethyldithiocarbamate.

The critical role of UCP2 in the improvement of endothelial function by sitagliptin

To explore the critical role of UCP2, we used UCP2 knockout (*UCP2KO*) mice and adenovirus overexpressing UCP2. First, we confirmed the beneficial effect of sitagliptin in Ang II-infused C57BL/6 mice. Ang II infusion unmasked EDCs in mouse aortae, which were inhibited by cotreatment with sitagliptin (3 mg·kg^{-1}·day^{-1}) (Fig. 5A). Moreover, acute treatment with exendin 9-39 (100 n*M*), compound C (10 μM), or genipin (1 μM) enhanced EDCs in aortae from Ang II-sitagliptin-cotreated mice (Supplementary Fig. S6A) without affecting those from mice treated with Ang II alone (Supplementary Fig. S6B). Next, we observed that EDCs evoked by Ang II were unaffected by sitagliptin (3 mg·kg^{-1}·day^{-1}) in *UCP2KO* mouse aortae (Fig. 5B). Furthermore, UCP2 over-expression by adenovirus (AdUCP2) diminished EDCs in SHR renal arteries (Fig. 5C), further supporting the importance of UCP2. Western blotting data showed that Ang II reduced

AMPK phosphorylation (Supplementary Fig. S6C), diminished UCP2 expression (Fig. 5D), and elevated COX-2 expression (Fig. 5E) in C57BL/6 mouse aortae, which were reversed by sitagliptin. In addition, exendin 9-39 (100 n*M*) and compound C (10 μM) but not genipin (1 μM) inhibited the increased AMPK phosphorylation (Supplementary Fig. S6C) and elevated UCP2 expression (Fig. 5D) by sitagliptin. Again, the inhibitory effect of sitagliptin on COX-2 upregulation was reversed by exendin 9–39, compound C, and genipin (Fig. 5E). We also revealed an elevated COX-2 expression (Fig. 5F) in *UCP2KO* mice, which was, however, unaffected by Ang II or sitagliptin.

Exendin-4 improved endothelial function in renal arteries from hypertensive patients

To further demonstrate the clinical relevance of the results from both SHR and Ang II-induced hypertensive mice, we tested the effect of exendin-4 in human renal arteries. We
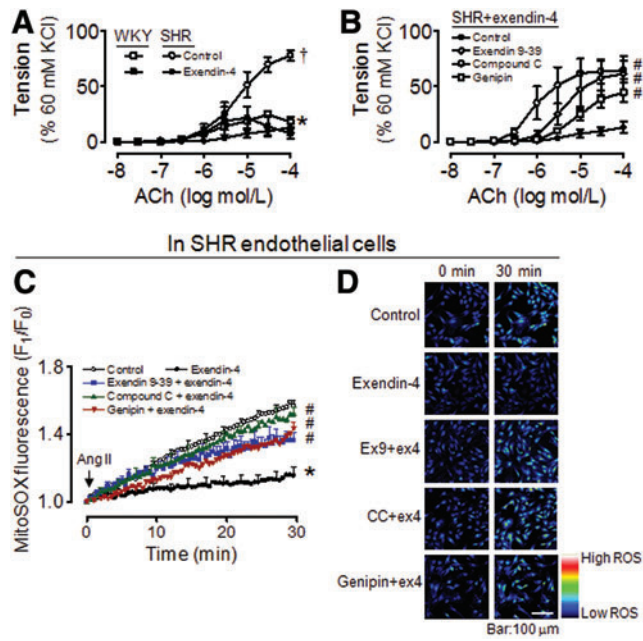
FIG. 4. Exendin-4 ameliorates endothelial dysfunction in SHR renal arteries. (A) Effects of GLP-1 analog exendin-4 (10 nM, 8 h) on EDCs in renal arteries from WKY and SHR. **(B)** Effects of exendin 9-39 (100 nM), compound C (10 μM), and genipin (1 μM) on exendin-4-induced reduction of EDCs. **(C)** Exendin-4 (ex4, 10 nM) reduced mitochondrial ROS production triggered by Ang II (100 nM) in primary cultured SHR endothelial cells, which was inhibited by cotreatment with exendin 9-39 (Ex9, 100 nM), compound C (CC, 10 μM), or genipin (1 μM). **(D)** Images showing mitochondrial ROS production under different treatments. Results are means ± SEM of four to six experiments. $^{\dagger}p < 0.05$ *versus* WKY control, $^{*}p < 0.05$ *versus* SHR control, $^{\#}p < 0.05$ *versus* exendin-4. Ang II, angiotensin II. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

observed that renal arteries from hypertensive patients exhibited a reduced UCP2 level (Fig. 6A) and an elevated COX-2 expression (Fig. 6B) compared to those from normotensive subjects, and such alterations were reversed by *ex vivo* treatment with exendin-4. Moreover, exendin-4 treatment normalized the ROS overproduction in renal arteries from hypertensive patients (Fig. 6C and Supplementary Fig. S7). These findings in human renal arteries further support the vascular benefit of GLP-1 and its analogues to restore the impaired endothelial function in hypertension.

Discussion

EDCs occur generally in arteries of aged or diseased animals, including high-fat diet–induced obese mice, SHR, and diabetic rats and also in young and healthy hamsters (28, 36, 38, 40), which are associated with endothelial dysfunction. The major findings of the present study include (i) EDCs were mediated by COX-2-dependent mechanisms in SHR renal arteries; (ii) *in vivo* sitagliptin and *in vitro* exendin-4 treatment normalized ROS overproduction and attenuated EDCs in SHR arteries; (iii) sitagliptin treatment upregulated UCP2 expression and downregulated the elevated expression of COX-2 mRNA and protein through scavenging mitochondrial ROS; (iv) sitagliptin treatment inhibited EDCs in aortae from Ang II-infused C57BL/6 mice, and this effect was absent in *UCP2KO* mice; (v) UCP2 inhibitor reversed the sitagliptin-induced beneficial effects in SHR and Ang II-infused mice, whereas UCP2 overexpression inhibited EDCs in SHR arteries; (vi) *ex vivo* exendin-4 treatment reversed endothelial dysfunction in renal arteries from hypertensive patients.

GLP-1, a 30 amino acid brain–gut insulinotropic peptide, which is rapidly degraded by DPP-4 in circulation, is now considered a leading hormone regulating glucose homeostasis in the absorptive state (10, 18). Apart from DPP-4 inhibitors (14), more stable GLP-1 analogs have been reported
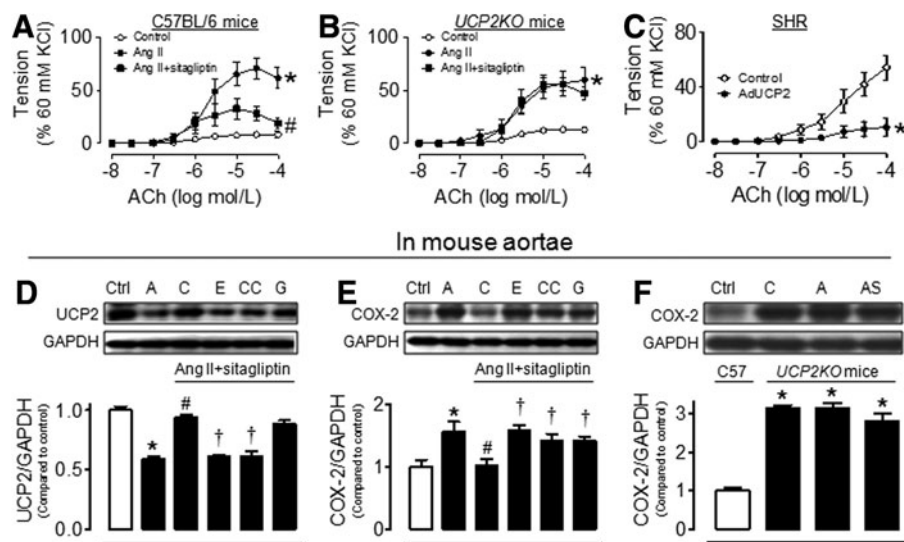

FIG. 5. The critical role of UCP2 in the improvement of endothelial function by sitagliptin. Ang II unmasked EDCs, which were inhibited by cotreatment with sitagliptin (3 mg·kg^{-1}·day^{-1}) in C57BL/6 mice **(A)** but not in *UCP2KO* mice **(B)**. Overexpression of UCP2 by adUCP2 diminished EDC in SHR renal arteries **(C)**. Expressions of UCP2 and COX-2 in C57BL/6 mice **(D, E)** and *UCP2KO* mice **(F)**. Results are means ± SEM of five to eight experiments. $^{*}p < 0.05$ *versus* respective control; $^{\#}p < 0.05$ *versus* Ang II or A, $^{\dagger}p < 0.05$ *versus* Ang II + sitagliptin control. C or Ctrl, control; E, exendin 9-39; CC, compound C; G, genipin; AS, Ang II + sitagliptin; *UCP2KO*, UCP2 knockout; adUCP2, adenovirus UCP2.
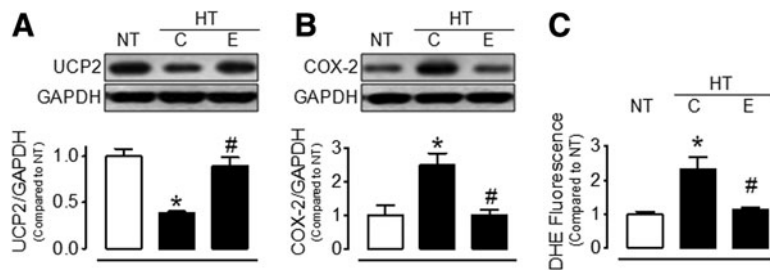
FIG. 6. Exendin-4 improves endothelial function in renal arteries from HT. UCP2 **(A)** and COX-2 **(B)** expressions in human renal arteries. **(C)** DHE fluorescence of ROS in human renal arteries. Results are means ± SEM of four experiments. *$p < 0.05$ *versus* NT; #$p < 0.05$ *versus* HT. C, control; E, exendin-4; NT, normotensive patients; HT, hypertensive patients.

to exert protective effects in the cardiovascular system (26). Our previous study also demonstrated that sitagliptin improves endothelium-dependent vasodilatation *via* restoring the diminished NO bioavailability in hypertension (20). So far, the main cause of hypertension-related endothelial dysfunction in humans has been identified with an increased NO breakdown. In particular, hypertension-related endothelial dysfunction has been demonstrated to be the consequence of increased oxidative stress (31). Increased ROS level has a major impact on vascular tone regulation (9), which is highly reactive and inactivates NO, thus reducing its bioavailability and producing peroxynitrites (37). In the present study, we found that *in vivo* sitagliptin and *in vitro* exendin-4 treatment reduced ROS production based on the following observations. First, 2-week sitagliptin treatment normalized the elevated ROS accumulation in SHR renal arteries. Second, 8 h-incubation with exendin-4 reduced the ROS level in SHR arteries as reflected by the dihydroethidium (DHE) staining, lucigenin-enhanced chemiluminescence assay, and EPR method. ROS can serve as the trigger for the release of EDCFs, which facilitates a greater amplitude of EDCs in hypertensive rats (33), and this is consistent with the present results that EDCs in SHR arteries were attenuated by ROS scavengers, such as tempol, or mitochondrial ROS scavengers, such as coenzyme Q10 and idebenone. Previous studies showed that both GLP-1 (16) and sitagliptin (15) suppress ROS production induced by advanced glycation end products, whereas exendin-4 decreases endogenous ROS production and increases ATP production in diabetic rat islets (25).

UCP2, a negative regulator of ROS generation (5, 19), has been detected in a large spectrum of tissues, including endothelial cells (11), and plays an important role in the prevention of the development of salt sensitivity-related hypertension (22). The UCP2-dependent action of ghrelin on neurons is driven by AMPK-related signaling (1), and ghrelin inhibits insulin secretion in β cells through the AMPK-UCP2 pathway (39). In addition, AMPK can suppress ROS synthesis *via* mitochondrial uncoupling mediated through UCP2 (42). Our previous study showed that GLP-1 signaling leads to AMPK activation (20). Thus, here we show that the inhibitory effect of GLP-1 signaling on EDCs also contributes to endothelial cell protection in hypertensive animals, which is mediated by AMPK and UCP2. Furthermore, we confirmed the sitagliptin-induced elevation of UCP2 expression was mediated through AMPK activation by using dominant-negative AMPK (DN-AMPK) adenovirus in SHR renal arteries (Supplementary Fig. S8). We then verified a critical role of UCP2 in sitagliptin-induced vascular benefits using both *UCP2KO* mice and adUCP2-carried UCP2 overexpression in SHR renal arteries.

Finally, levels of COX-2 expression were assessed in hypertensive animals. The formation of EDCF, which partici-

pates in moment-to-moment regulation in vascular tone, depends on the activity of endothelial COX (33, 41). Both COX isoforms, namely COX-1 and COX-2, contribute to the induction of EDCs (27, 29). The present study shows that COX-2 expressions increased in renal arteries from SHR and aortae from Ang II-infused mice compared with respective control. But COX-1 expression was similar in arteries from both WKY and SHR and was unaffected by the treatment with sitagliptin or exendin-4 (Supplementary Fig. S1). Chronic sitagliptin treatment downregulated COX-2 expression in arteries from SHR and Ang II-infused mice; this benefit was reversed by exendin 9-39, compound C, and genipin. A previous study showed that ROS mediated COX-2-dependent contraction in renovascular hypertension (34) and bone morphogenic protein-4 upregulates COX-2 through ROS (41). Here, we showed that scavengers for ROS and mitochondrial ROS reduced the raised level of COX-2 mRNA and protein in SHR arteries. In addition, we observed that H_2O_2 elevated ROS level and COX-2 expression in mouse aortic endothelial cells (MAECs), which were unaffected by sitagliptin, but only inhibited by catalase (Supplementary Fig. S3C–E). We further evaluated the COX-2 levels in the vascular wall of mouse aortae. In consistence with western blotting results on intact aortae, the immunohistochemical staining also showed a significantly higher basal COX-2 expression in VSMCs of aortae from *UCP2KO* mice compared to C57BL/6 mice (Supplementary Fig. S9A), suggesting that UCP2 might be a physiological inhibitor of the COX-2 expression in aortic vascular wall of healthy mice. Two-week Ang II infusion significantly elevated the COX-2 expression in C57BL/6 mouse aortic VSMCs with little effect in *UCP2KO* mouse aortic VSMCs. Chronic sitagliptin treatment downregulated the COX-2 expression in aortic VSMCs from C57BL/6 mice but only induced a small decrease in the COX-2 expression of aortic VSMCs from *UCP2KO* mice. Although the *en face* immunofluorescence staining showed that the basal COX-2 expression was comparable, but it was elevated by Ang II treatment to a similar degree in aortic endothelium from both C57BL/6 mice and *UCP2KO* mice (Supplementary Fig. S9B). These results suggest that endothelial cell COX-2 upregulation is likely the primary mediator of EDCs in Ang II-treated aortae from both C57BL/6 and *UCP2KO* mice, and this also explains the observation that sitagliptin treatment reduces the COX-2 expression in the endothelium and inhibits EDCs in aortae from Ang II-infused C57BL/6 mice without affecting both alterations in Ang II-infused *UCP2KO* mouse aortae. Taken together, the present results indicate that sitagliptin and exendin-4 attenuate EDCs by reducing COX-2 expression *via* UCP2 upregulation and subsequent inhibition of mitochondrial ROS production. Of importance, the present study also

shows the reduced UCP2 level, elevated COX-2 expression, and increased ROS level in renal arteries from patients with hypertension, and such changes were normalized by *ex vivo* treatment with exendin-4.

In conclusion, EDCs visualized in arteries from Ang II-infused mice and SHR are COX-2-dependent. Sitagliptin treatment attenuates EDCs through normalizing COX-2 overexpression *via* the activation of GLP-1R/AMPKα/UCP2 signaling cascade (Fig. 7). Based on the therapeutic profile of sitagliptin to prevent hypertension, the orally active and stable GLP-1 analogs might be useful for the treatment of vascular dysfunction in hypertension-related vascular events.

Materials and Methods

Chemicals

Anti-phospho-AMPKα (Thr172) and anti-AMPKα were purchased from Cell Signaling Technology (Beverly, MA). Anti-COX-2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-UCP2 antibody was purchased from R&D Systems (Minneapolis, MN). Antibody against GAPDH was obtained from Ambion (Austin, TX). HRP-conjugated swine anti-goat, anti-rabbit, or anti-mouse IgG were from DakoCytomation (Carpinteria, CA). Immobilon-P polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA), and chemiluminescence
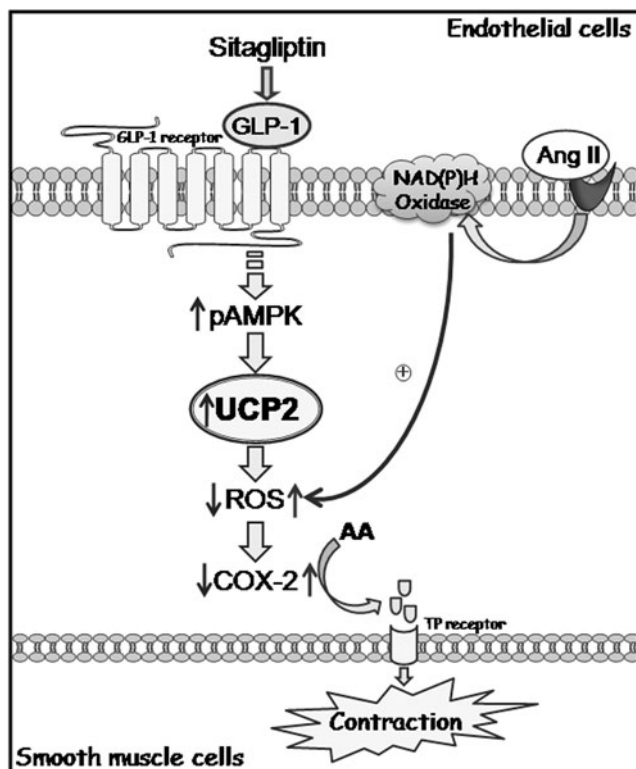

FIG. 7. **The proposed cellular mechanism for the protective effect of sitagliptin against endothelial dysfunction in hypertension.** Ang II evoked EDCs by increasing ROS level/COX-2 expression. Sitagliptin elevates UCP2 expression *via* the activation of GLP-1 receptor/AMPKα through GLP-1-dependent pathway, leading to reduced ROS production and subsequent downregulation of COX-2 expression, thus attenuates EDCs in hypertension.

(ECL reagents) was obtained from Amersham Pharmacia (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). ACh, NG-nitro-L-arginine methyl ester (L-NAME), compound C, exendin-4, exendin 9-39, DuP697, tempol, tiron, DETCA, *bis-N*-methylacridinium nitrate (lucigenin), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich Chemical (St Louis, MO). VAS and genipin were from Cayman Chemical (Ann Arbor, MI). Ang II, coenzyme Q10, idebenone, and NS398 were from Tocris Bioscience (Bristol, United Kingdom). MitoQ was from Antipodean Pharmaceuticals. S18886 was a gift from the Institut de Recherches Servier (Suresnes, France). 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) was from Alexis Biochemical Corp. (San Diego, CA). The cell culture media, DHE, and MitoSOX™ were from Invitrogen (Carlsbad, CA). Sitagliptin was a kind gift from Merck Research Laboratories (Rahway, NJ). DHE, MitoSOX, DuP697, NS398, idebenone, VAS, S18886, genipin, and compound C were dissolved in DMSO. Coenzyme Q10 and MitoQ were dissolved in ethanol. Other drugs were dissolved in distilled water. DMSO and ethanol (0.1% v/v) did not modify ACh-induced contraction.

Animals

Male SHR, WKY, and C57BL/6 mice were supplied by the Chinese University of Hong Kong (CUHK) Laboratory Animal Service Center. Male *UCP2KO* mice of C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Hong Kong. This investigation was approved by the CUHK Animal Experimentation Ethics Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). SHR (32–40 weeks old) and WKY (32–40 weeks old) received sitagliptin ($10\,mg\cdot kg^{-1}\cdot day^{-1}$ by oral gavage) or vehicle for 2 weeks. Ang II- or PBS-loaded osmotic pumps were implanted under ketamine/xylazine anesthesia (75 and $6\,mg\cdot kg^{-1}$ body weight) in C57BL/6 and *UCP2KO* mice (8–10 weeks) and received sitagliptin administration ($3\,mg\cdot kg^{-1}\cdot day^{-1}$) or vehicle for 2 weeks.

Human artery specimen

The present study was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Human renal arteries were obtained after informed consent from normotensive and hypertensive patients undergoing nephrectomy at ages between 50 and 80 years. The indications for surgery included tumor (three in normotensive patients and two in hypertensive patients) and poorly functioning kidney (one in normotensive patients and two in hypertensive patients). History of hypertension was defined as having persistent elevated blood pressure, systolic blood pressure of >140 mm Hg, or diastolic blood pressure of >90 mm Hg and requiring medical therapy.

Isometric force measurement

Rats and mice were sacrificed by CO_2 suffocation. Renal arteries from rats and aortae from mice were removed and placed in ice-cold Krebs solution (m*M*): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Arteries were cleaned of adhering tissue and cut into

ring segments of 2 mm in length. Rings were suspended in myograph (Danish Myo Technology, Aarhus, Denmark) for recording of changes in isometric tension (41). Briefly, to visualize the EDCs, rings with endothelium were exposed to 100 μM L-NAME for 30 min to eliminate the relaxant effect of endothelium-derived NO before the application of ACh (0.01 to 100 μM). The effects of various inhibitors and antagonists (*e.g.*, UCP2 inhibitor, COX-2 inhibitors, or TP receptor antagonist) were tested on ACh-induced contractions following 30-min incubation with each drug. In some experiments, SHR renal arteries were incubated with ROS scavengers or exendin 9-39 (100 nM, GLP-1R antagonist), compound C (10 μM, AMPKα inhibitor), and genipin (1 μM, UCP2 inhibitor) along with exendin-4 (10 nM, GLP-1 analog) for 8 h.

Primary culture of rat aortic endothelial cells

Aortae of SHR were dissected in sterilized phosphate-buffered saline (PBS) under a stereoscopic microscope. After digestion by 0.2% collagenase for 15 min at 37°C, RPMI-1640 was added, and endothelial cells were then collected by centrifugation at 1000 rpm for 5 min. Thereafter, the pellet was gently re-suspended in RPMI-1640 supplemented with 10% FBS and cultured in a 75-mm culture flask. To remove other cell types, the medium was changed after 1-h incubation and then maintained until 70% confluence before use.

Primary culture of MAECs

Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine. Heparin (100 U/ml in PBS) was infused into the circulation from the left ventricle. The aortae were dissected in DMEM and incubated with collagenase type II for 8 min at 37°C. Detached endothelial cells were collected by centrifugation, resuspended in 20% FBS-DMEM and then cultured in endothelial cell growth medium supplemented with bovine brain extract (Lonza, Walkersville, MD) till confluency before use (35). The identity of endothelial cells was confirmed by immunocytochemical staining for an endothelial cell-specific maker, PECAM-1.

Western blot analysis

Isolated renal arteries, mouse aortae, or MAEC were homogenized in RIPA lysis buffer that contained 1 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml PMSF, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, and 2 μg/ml β-glycerolphosphate, and centrifuged at 20,000 g for 20 min at 4°C. Protein lysates (25 μg for arteries, 10 μg for cells) were separated by electrophoresis and transferred onto immobilon-P PVDF membrane. Blots were blocked with 1% bovine serum albumin or 5% nonfat milk for 1 h and incubated overnight at 4°C with antibodies against phosphorylated AMPKα, total AMPKα, UCP2, COX-2, and GAPDH. After washing, blots were incubated with HRP-conjugated swine anti-rabbit, anti-mouse, or anti-goat IgG (DakoCytomation, Carpinteria, CA). Immunoreactive bands were visualized by chemiluminescence (ECL reagents, Amersham Pharmacia) and exposed to Kodak Image Station 440 for densitometric analysis.

Real-time polymerase chain reaction

The effect of ROS scavengers on COX-2 transcription was examined by real-time polymerase chain reaction (PCR).

Total RNA from rat renal arteries was extracted using the Aurum total RNA Mini kit (BioRad, Hercules, CA) according to the manufacturers' instructions and then reverse transcribed using the iScriptTMcDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed with SYBR®Premix Ex TaqTM (Invitrogen) with thermal cycles at 95°C for 5 min, 40 cycles at 95°C for 1 min, 1 min Tm (melting temperature), 72°C for 1 min, finally followed at 72°C for 5 min with the specific primer sets. The forward primer for COX-2: 5′-GCAAATCCTTGCTGTTCCAATC-3′, the reverse primer for COX-2: 5′-GGAGAAGGCTTCCC AGCTTTTG-3′. The forward primer for GAPDH: 5′-TGCAC CACCAACTGCTTAGC-3′, the reverse primer for GAPDH: 5′-CAGTCTTCTGAGTGGCAGTGATG-3′. PCR conditions were as follows: 1 cycle at 95°C for 3 min, followed by 45 cycles at 95°C for 20 s, 63°C for 20 s, and 72°C for 20 s. COX-2 mRNA levels were normalized by GAPDH.

DHE staining

Frozen sections or MAEC seeded on glass coverslips were loaded with 5 μM DHE at 37°C for 10 min. ROS fluorescence was measured by a confocal scanning unit (Olympus, Tokyo, Japan) at excitation 515 nm and emission 585 nm. Data were analyzed by the Fluoview software (Olympus).

Detection of superoxide anions

The formation of superoxide anions in arteries was measured using the lucigenin-enhanced chemiluminescence method (43). Briefly, isolated SHR renal arteries was preincubated for 45 min at 37°C in 2 ml of Krebs–HEPES buffer (in mM: NaCl 99.0, NaHCO$_3$ 25.0, KCl 4.7, KH$_2$PO$_4$ 1.0, MgSO$_4$·7H$_2$O 1.2, glucose 11.0, CaCl$_2$·2H$_2$O 2.5, and Na-HEPES 20.0) in the presence of DETCA (1 mM) to inactivate superoxide dismutase and β-NADPH (NADPH oxidase substrate, 0.1 mM). The arteries were then transferred to vials containing 300 μl Krebs–HEPES buffer with 10 μM lucigenin, and the repeated measurement were taken over 10 min in 1-min intervals using the luminometer (GloMax® 20/20 Luminometer, Madison, WI). The data were expressed as average counts per mg of tissue dry weight.

ROS detection by EPR spin trapping

ROS levels in SHR renal arteries were also determined by EPR using 1-hydroxy-1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H; Alexis Biochemical Corp., San Diego, CA) as the spin trapping agent (40).

Mitochondrial ROS measurement

SHR aortic endothelial cells seeded on glass coverslips were incubated with exendin-4 (10 nM, 8 h) in the presence or absence of various inhibitors and then placed in a chamber designed for fluorescence imaging. Mitochondrial ROS production triggered by Ang II (100 nM) was monitored using a fluorescent ROS indicator MitoSOX (5 μM). ROS fluorescence was measured by a confocal scanning unit (FV1000; Olympus) at excitation of 515 nm and emission of 585 nm. Data were expressed as percentage change before (F0) and after (F1) the addition of Ang II.

Statistical analysis

EDCs were expressed as the percentage of 60 mM KCl-induced tension. Results are means ± SEM of n rings from

different animals. For statistical analysis, the Student's *t*-test or two-way ANOVA, followed by Bonferroni *post hoc* tests were used when more than two treatments were compared (GraphPad Software, San Diego, CA). $p < 0.05$ was considered significantly different.

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Author Disclosure Statement

There are no competing financial interests.

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Address correspondence to:
Dr. Yu Huang
School of Biomedical Sciences
Chinese University of Hong Kong
Shatin, NT
Hong Kong 00852
China

E-mail: yu-huang@cuhk.edu.hk

Abbreviations Used

ACh = acetylcholine
adUCP2 = adenovirus UCP2
AMPK = AMP-activated protein kinase
Ang II = angiotensin II
COX-2 = cyclooxygenase-2
DETCA = diethyldithiocarbamate
DHE = dihydroethidium
DN-AMPK = dominant-negative AMPK
DPP-4 = dipeptidyl peptidase-4
EDC = endothelium-dependent contraction
EDCFs = endothelium-derived contracting factors
EPR = electron paramagnetic resonance
GLP-1R = glucagon-like peptide 1 receptor
HT = hypertensive patients
L-NAME = NG-nitro-L-arginine methyl ester

Abbreviations Used (Cont.)

MAECs = mouse aortic endothelial cells
NADPH = nicotinamide adenine dinucleotide
phosphate
NO = nitric oxide
NT = normotensive patients
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
PVDF = polyvinylidene difluoride
RLU = relative luminance unit

ROS = reactive oxygen species
TP = thromboxane/prostanoid
UCP2 = uncoupling protein 2
UCP2KO = UCP2 knockout
SHR = spontaneously hypertensive rat
SHREC = SHR endothelial cell
VAS = valeryl salicylate
VE-cadherin = vascular endothelial cadherin
VSMC = vascular smooth muscle cell
WKY = Wistar-Kyoto rats