Apelin modulates aortic vascular tone via endothelial nitric oxide synthase phosphorylation pathway in diabetic mice

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Abstract

Objective: The apelin receptor APJ is a putative receptor protein related to angiotensin (Ang) type 1 receptor. The apelin-APJ system has been implicated in diabetes, but its role in the diabetic vasculature and the mechanisms involved remain unclear. Our aim here was to explore the regulatory role of apelin in the aortic vascular tone in diabetic mice.

Methods: A Multi Myograph system was used to determine the isometric vessel tone in aortic rings from diabetic db/db and control db/+ mice. The mRNA, phosphorylation, and protein levels of APJ, Akt, and endothelial nitric oxide synthase (eNOS) were analyzed by reverse transcription–polymerase chain reaction and Western blotting, respectively.

Results: There is depressed expression of APJ, enhanced contractile response to Ang II, and reduced relaxation response to acetylcholine in aortas from db/db mice. Apelin treatment strikingly reversed the altered aortic vascular responsiveness to Ang II and acetylcholine in db/db mice, both of which were abolished by the eNOS inhibitor N⁶-nitro-L-arginine methyl ester. Finally, in db/db mice, considerable increases in phosphorylation of Akt on serine 473 and of eNOS on serine 1177 were found in aortas pretreated with apelin.

Conclusions: Apelin treatment modulates the abnormal aortic vascular tone in response to Ang II and acetylcholine by potentiating phosphorylation of Akt and eNOS in diabetic mice, suggesting that the apelin-APJ system might be an important regulator of vascular function in diabetes.

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1. Introduction

Impairment of vascular dilatation and constriction is a hallmark of type 2 diabetes mellitus (T2DM), which has been closely linked to the renin–angiotensin system (RAS) [1]. As a predominant effector of the RAS, angiotensin (Ang) II exerts various deleterious effects via Ang II type 1 (AT₁) receptor by promoting vascular contraction, impairing insulin signaling pathways, stimulating productions of growth factor, reactive oxygen species (ROS) and adhesions molecules, causing cardiovascular hypertrophy, endothelial dysfunction and insulin resistance [1–3]. Apelin, a newly identified Ang II homologue, has been shown to act as an endogenous ligand of the orphan receptor APJ and counterbalance the actions of Ang II [4,5]. APJ (a putative receptor protein related to AT₁) is a seven-transmembrane
domain G-protein-coupled receptor that was originally identified from a human genomic library using the polymerase chain reaction (PCR). In addition to the limited homology between apelin and Ang II, there is the sequence polymerase chain reaction (PCR). In addition to the limited homology between the APJ and AT₁ receptor (54% in the transmembrane regions), and the anatomical distribution of both receptors and peptides overlaps in the cardiovascular system [5,6]. However, the functional effects of apelin are independent of AT₁ receptor and APJ does not show specific binding of Ang II [5]. More intriguingly, apelin and Ang II have similar degradation pathways, and are both substrates of angiotensin-converting enzyme (ACE) 2 (ACE2), which is a critical regulator of the RAS and counteracts the function of ACE and Ang II [2,6–8]. The apparent involvement of apelin peptides in the enzymatic cascades of the RAS and the counter-regulatory activity of apelin against the action of Ang II support the possible importance of apelin in vascular reactivity maintenance in diabetes. The mechanisms and functional relevance of this remain, however, to be further studied.

Abnormalities in the apelin-APJ system contribute to decreased vasodilatation and increased vasoconstriction responses, which have been described in insulin resistance-related disorders such as diabetes and cardiovascular dysfunctions [1,3,9–11]. Apelin peptides cause endothelium-dependent vasorelaxation by triggering the release of nitric oxide (NO), this effect can be almost completely abolished in the presence of endothelial NO synthase (eNOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), indicating that apelin may exert vasorelaxation effect via activation of eNOS pathway [9,12]. In addition, apelin may phosphorylate serine/threonine kinase Akt and raise intracellular calcium, both of which will activate eNOS phosphorylation pathway and promote NO release [6,9,13,14]. Apelin may be a new member of the family of vasoactive agents that are known to activate eNOS in the endothelial cells. In contrast, Ang II can produce ROS and impair phosphatidylinositol 3-kinase (PI3K)-dependent activation of Akt/eNOS phosphorylation, which in turn diminishes NO generation, causing potent vasoconstriction [1,3]. We previously reported that increased levels of the apelin-APJ system revealed a counter-regulatory role against the Ang II-AT₁ signaling, leading to the fall of AT₁ expression and the subsequent reduction of NO inactivation in the spontaneously hypertensive rat, in which is often accompanied by insulin resistance [5,7]. Abnormalities in the Akt-eNOS phosphorylation pathway would result in reduction of NO production and impairment of vascular reactivity [1,14,15]. Therefore, we presume that apelin may play a crucial role in the vascular tone maintenance by counteracting the vasoconstrictor action of Ang II and potentiating the release of NO through activation of Akt–eNOS phosphorylation pathway. The aim of the present study was to determine the mRNA and protein levels of the apelin-APJ system and the Akt–eNOS phosphorylation pathway in the vasculature and further evaluate the regulatory roles of apelin in the vascular tone in diabetic db/db mouse, a rodent model for T2DM.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. The specific AT₁ receptor antagonist Losartan was from Merck (Darmstadt, Germany). PI₃K inhibitor LY294002 and 9, 11-dideoxy-11α, 9α-epoxy-methanoprostaglandin F (2α) (U-46619) were dissolved in dimethyl sulfoxide and others in distilled water. TRIzol was obtained from Invitrogen (Carlsbad, CA). Polyclonal antibodies against Akt, eNOS, phospho-Akt (Ser173), phospho-eNOS (Ser1177), APJ receptor and β-actin were obtained from Cell Signaling Technology (Beverly, Mass), Santa Cruz Biotechnology (Santa Cruz, CA) and Phoenix Pharmaceuticals (Belmont, CA), respectively.

2.2. Blood vessel preparation

All experiments described below were approved by the Animal Research Ethics Committee at the Chinese University of Hong Kong (CUHK). 15-week-old Male C57BL/KsJ diabetic (db/db) and age/gender-matched C57BL/KsJ control (db/m+) mice were supplied by the CUHK Laboratory Animal Service Center. The mice were previously kept in a temperature-controlled room (22–24 °C) with a 12-h light/dark cycle, and fed a standard diet and water ad libitum. The experiments were performed after the mice were acclimatized to our housing conditions for at least 1 week. The investigation 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). The mouse was killed by inhalation of carbon dioxide and the thoracic segment of aorta was rapidly removed. Half of the aorta was kept at −70 °C until mRNA and protein analyses, while the other half was placed on a dissecting plate containing ice-cold Krebs solution with the following composition in mmol/L (mM): 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 d-glucose. Each aorta was carefully cleaned of the surrounding connective tissue while preserving endothelial integrity and then cut into 2–4 mm slices (~ 1.5 mm in length) for parallel studies. Each arterial ring was mounted in a Multi Myograph System (Danish Myo Technology A/S, Denmark) and performed as previously described [16], and changes in arterial tone were recorded. Briefly, two tungsten wires were passed through the ring’s lumen and fixed to jaws of myograph. The chamber bath was filled with 5 ml Krebs solution and continually oxygenated with a gas mixture of 95% O₂ plus 5% CO₂. Krebs solution in the chamber was maintained at 37 °C using a built-in heat-exchanger device to give a pH value of between 7.35 and 7.45. The
ring was stretched until the optimal baseline tension of 1 mN and then allowed to equilibrate for about 60 min before the start of the experiment. High K+-containing solution was prepared by replacing NaCl with KCl on an equimolar basis in order to retain a constant ionic strength. Each experiment was performed on rings prepared from different mice.

2.3. Vascular reactivity

After equilibration in Krebs solution, high K⁺ (60 mM) was used to induce steady blood vessel tone. The contractions to cumulative concentration of K⁺ (20–80 mM) and thromboxane A2 receptor agonist U-46619 (1–300 nM) were subsequently studied. The second series of experiments were run to determine the contractile response to Ang II in the aortic ring with endothelium and the role of apelin in the effects of Ang II. Apelin (10 or 100 nM) and/or LY294002 (10 μM), L-NAME (100 μM), losartan (10 μM), AT₂ receptor antagonist PD123319 (1 μM) were added to aortic rings for 20 min in the chamber bath before exposure to Ang II (100 nM). Some aortic rings were prepared for Akt–eNOS phosphorylation analysis after 20 min of stimulation with apelin (100 nM) in the chamber bath; time-matched negative control (vehicle) protocol was also performed. In the last set of experiments, we investigated the dose-response vasoactive responses to phenylephrine (0.01–10 μM) and acetylcholine (0.01–10 μM, after addition of 3 μM phenylphrine) in rings with endothelia, which followed treatment with L-NAME (100 μM) for 30 min. The vasorelaxation effects of apelin (100 M) and sodium nitroprusside (SNP, 3 μM) were also examined in rings. Each constrictor or dilator was added cumulatively to Krebs solution in at least 1-min intervals without washout of the previous concentration. For comparison, the above-mentioned vascular reactivities were tested in different db/db and db/m+ mice.

2.4. Measurement of apelin receptor mRNA levels in aortic tissues

APJ mRNA expression was measured by Superscript® First Strand Synthesis System for reverse transcription-PCR (RT-PCR, Invitrogen) according to the manufacturer’s instructions. Total RNA was extracted from aortic tissues using TRIzol reagent. RT-PCR was performed with mouse gene-specific primers based on sequences published in GenBank: APJ sense 5′-TCGGCTAAGGCTGCGAGTC-3′, anti-sense 5′-GTCTGTGGAACGGAACAC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5′-GGCCTCCTAGCTGACCCC-3′, anti-sense 5′-ACCTGGTCCTAGTGAAGCC-3′. After amplification, RT-PCR products were electrophoresed on a 2% agarose gel at 100 V for 40 min. Bands corresponding to RT-PCR products were measured by densitometry.

2.5. Western blotting

Lysates from aortic tissues were subjected to Western blotting analyses as previously reported [5,7]. Protein concentrations were measured using the BCA™ Protein Fig. 1. Quantification of apelin receptor mRNA in db/db mice by RT-PCR. Total RNA was extracted from aortic tissues. RT-PCR products were electrophoresed on a 2% agarose gel at 100 V for 40 min. APJ receptor mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expected RT-PCR product size was 102 bp (for APJ, A) and 149 bp (for GAPDH, B), respectively. M indicates DNA maker. Each bar represents mean±SEM (n=4). *, p<0.05 vs. db/m+ mice.

Fig. 2. Quantification of apelin receptor protein in db/db mice by Western blotting in aortas. APJ receptor protein (41 kD) was normalized to the mouse β-actin (45 kD). The bar graphs represent mean±SEM of 5 different mice. *, p<0.05 vs. db/m+ mice.
Array Kit (Pierce, USA). An equal amount of protein for each sample was separated by 8–12% precast sodium dodecyl sulfate–polyacrylamide denatured gels, transferred onto a nitrocellulose membrane (Hybond ECL, Amersham), and then blocked with 1% Blot-qualified bovine serum albumin (Promega) in Tris-buffered saline with Tween solution for 90 min. The membranes were incubated overnight at 4 °C with primary antibodies against Akt, Ser473–Akt, eNOS, Ser1177–eNOS, APJ and β-actin (APJ 1/200, others 1/1000 dilution) and then incubated with alkaline phosphatase (AP)-labeled secondary antibody (Santa Cruz; 1/5000) for 60 min at room temperature. The positive bands corresponding to aim proteins were developed with the Western Blue® Stabilized Substrate for AP (Promega), and quantified by an Automated Imaging System (Bio-Rad).

2.6. Data analysis

The relaxations of acetylcholine and SNP were expressed as percentage reduction in phenylephrine-induced contraction.
Response curves were constructed and analyzed with the use of Graphpad Prism software (version 4). Active tension in graph was expressed as mN/mm aorta ring length. Contractions or relaxations were determined as percentages of the maximal amount obtained for the first concentration-response curves. Data represent means±SEM. Statistical analysis was performed either by Student’s t test or by ANOVA followed by Bonferroni’s test, as appropriate. P<0.05 was considered statistically significant.

3. Results

3.1. Depressed mRNA and protein levels of apelin receptor in aortas from db/db mice

A diminishment of APJ receptor mRNA expression was shown in aortas from db/db mice compared with controls (n=4, p<0.05) (Fig. 1A). The mRNA levels of housekeeping gene GAPDH did not differ between db/db and db/m+ mice (n=4) (Fig. 1B). Consistent with the RT-PCR results, APJ receptor protein was obviously depressed in aortas from db/db mice compared with db/m+ mice (n=5, p<0.05) (Fig. 2), implying that abnormalities of the apelin-APJ system might be involved in the vascular pathophysiology of diabetes.

3.2. Effects of apelin on aortic vascular reactivity in response to Ang II

As presented in Fig. 3, both high K+ and U-46619 induced concentration-dependent contractions in aortic rings with endothelia (n=5–8). High-K+-, Ang II-induced contractile responses were obviously enhanced in rings from db/db mice compared with controls (n=5–8, p<0.05, respectively) (Fig. 3, A and C). Treatment with apelin (10 and 100 nM) or losartan (10 μM) significantly attenuated Ang II-induced vasoconstriction in aortic rings from db/db mice (n=4–6, p<0.01, respectively) (Fig. 3C). The inhibitory effect of apelin (100 nM) on vasoconstriction response to Ang II was abolished by administration with L-NAME (100 μM) and LY294002 (10 μM) in db/db mice (n=4, p<0.01, respectively), whereas no effect was observed with PD123319 (1 μM) supplement (Fig. 3C). The contractile response to U-46619 did not differ between db/db and db/m+ mice (n=5–8, p>0.05) (Fig. 3B).

3.3. Effects of apelin on Akt and eNOS phosphorylations in aortic rings in vitro

Fig. 4 describes that phosphorylations of Ser473–Akt and Ser1177–eNOS were greatly decreased in vehicle-treated...
aortic rings from \(\text{db/db}\) mice compared with controls, but significantly elevated in apelin-treated rings from \(\text{db/db}\) mice \((n=4–5, \ p<0.01, \ \text{respectively})\). In contrast, phosphorylations of Ser\(^{473}-\text{Akt}\) and Ser\(^{1177}-\text{eNOS}\) in aortic rings from \(\text{db/m}^+\) mice were unchanged after pretreatment with apelin \((n=4–5)\), suggesting that apelin accelerated the serine phosphorylations of Akt and eNOS in \(\text{db/db}\) mice but had no effect in \(\text{db/m}^+\) mice.

### 3.4. Effects of apelin on aortic vascular reactivity in response to acetylcholine

In the current study, apelin and L-NAME were initially used as stimulators in aortic rings before stimulation with acetylcholine or phenylephrine, aiming to examine specifically the regulatory role of endothelium-derived NO in aortic vascular tone. The diabetic \(\text{db/db}\) mice exhibited a marked increase in concentration-dependent contractile response to phenylephrine \((0.01–10 \mu \text{M})\) when compared with controls \((n=5–8, \ p<0.05)\) (Fig. 5, A, B and E). L-NAME \((100 \mu \text{M})\) augmented phenylephrine-induced contractions in aortic rings from \(\text{db/db}\) and \(\text{db/m}^+\) mice \((n=4–5, \ p<0.01)\) (Fig. 5, C, D and E), suggesting that diminished endothelial NO would potentiate phenylephrine-induced constrictor effect on aortic vascular tone. In addition, the aortic rings from \(\text{db/db}\) mice exhibited a decreased dose-dependent relaxation response to acetylcholine with a maximal relaxation of 84.1±9.8% of phenylephrine-induced tone compared with 44.3±9.3% of the control \((n=6–8, \ p<0.05)\) (Fig. 6, A, B, G and H). Pretreatment with apelin \((100 \text{ nM})\) improved the dose-dependent vasorelaxation response to acetylcholine, which was virtually abolished by L-NAME \((100 \mu \text{M})\) treatment in aortic rings from diabetic \(\text{db/db}\) and nondiabetic \(\text{db/m}^+\) mice \((n=4–5, \ p<0.01, \ \text{respectively})\) (Fig. 6, E, F and H).

### 4. Discussion

To our knowledge, this is the first report of depressed expression of apelin receptor and the regulatory role of apelin in the aortic vascular tone in diabetic \(\text{db/db}\) mice.
Both mRNA and protein levels of apelin receptor were diminished in the aortas from adult spontaneously diabetic db/db mice. It is not yet known whether this reduction in APJ receptor is of pathophysiological significance, but it has been demonstrated that APJ receptor-deficient mice exhibited an enhanced vasoconstrictor response to Ang II [10]. We have shown that an obvious increase in contractile response to Ang II was exhibited in aortic rings with endothelia from diabetic db/db mice. It is now clear that the vascular endothelium is an important target for the RAS and the apelin–APJ system, in which abnormalities have been implicated in diabetes. The APJ receptor mediates the biological effects of the various apelin peptides such as long isoform apelin-36 and short isoforms apelin-17, -13 and -12 [13,17,18]. Recently, roles have been established for the apelin system in producing vasodilatation and lowering blood pressure [12,17], as a potent cardiac inotrope [4,19,20], in modulating pituitary hormone release and food and water intake [21], in maintaining glucose homeostasis and regulating insulin secretion [11,22]. The reciprocal relationship between the apelin–APJ system and the Ang II–AT1 signaling has been described in our previously [5] and present work. As shown in our study, treatment with apelin strikingly attenuated Ang II-induced enhancement of aortic vascular tone in db/db mice. This inhibitory effect of apelin was abolished by PI3K inhibitor LY294002 and eNOS inhibitor L-NAME, indicating that apelin revealed a counter-regulatory role against the actions of Ang II involving the PI3K/Akt eNOS signaling pathway. The uncovering of the interactions between the RAS and the apelin–APJ system in the vasculature would shed new light on our understanding of the vascular pathophysiology in diabetes and open new possible venue for drug explore aimed at T2DM.

Endothelial dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilatation properties, which is responsible for diabetic vascular pathophysiology [3]. Mechanisms that participate in the reduced vasodilatory responses in diabetes include increased production of Ang II, decreased NO generation, oxidative excess and diminished expression of the apelin–APJ system [1,3,9,11]. The db/db mouse is a well-characterized and extensively used T2DM rodent model, in which couples insulin resistance with endothelial dysfunction [1]. The current study described that a marked decrease in dose-dependent vasorelaxation response to acetylcholine was shown in aortic rings from diabetic db/db mice, implying reduced bioavailability of NO under basal conditions in diabetes. More intriguingly, pretreatment with apelin strikingly improved the vasorelaxation responses to acetylcholine, which was abolished by L-NAME in aortic arteries from db/db mice, indicating that apelin may potentially improve endothelial function to some extent via eNOS phosphorylation pathway in diabetes. Taken together, our findings have demonstrated that apelin supplement strikingly reversed the altered vasoactive responses to Ang II and acetylcholine in db/db mice, both of which were abolished by eNOS inhibitor L-NAME, suggesting that endothelial eNOS signaling pathway mediated the regulatory roles of apelin in aortic vascular tone in diabetic mice.

Another striking observation in the present work is that diminished phosphorylations of Ser473–Akt and Ser1177–eNOS were found in aortic tissues from diabetic db/db mice. Our results implied that abnormal Akt/eNOS phosphorylation pathway might serve to dampen endothelial NO signaling in aortic rings, thereby leading to abnormal vasoactive responses to Ang II and acetylcholine. In aortic rings from db/db mice, apelin treatment significantly enhanced phosphorylations of Ser473–Akt and Ser1177–eNOS, which is consistent with previously published findings [10,14]. These findings have given strong support to the concept that the beneficial effects of apelin on aortic vascular tone are likely due to activation of phosphorylations of Ser473–Akt and Ser1177–eNOS in diabetic db/db mice. In contrast, in endothelial cells from APJ receptor-deficient mice, apelin-induced activation of eNOS phosphorylation was abolished, supporting the role of APJ receptor in apelin-mediated aortic vascular tone regulation [10]. Thus, diminished expression of APJ receptor observed in the current study may be, at least in part, responsible for reduced phosphorylations of Ser473–Akt and Ser1177–eNOS in the aortas from db/db mice. Generalising from the results, apelin may act as a “good peptide” in the vasculature, serving to modulate the abnormal aortic vascular tone in response to Ang II and acetylcholine by enhancing the phosphorylations of Ser473–Akt and Ser1177–eNOS, suggesting that apelin may be an important regulator of vascular function and exert a protective role in diabetes.

In summary, the present study defines for the first time that apelin treatment strikingly modulates the abnormal aortic vascular reactivity in responses to Ang II and acetylcholine by augmenting phosphorylations of Akt and eNOS, likely contributing to the improvement of the vascular function in diabetic mice. A shift in balance between vasodilator and vasoconstrictor actions of the apelin–APJ system and the Ang II–AT1 signaling may play an important role in the vascular pathophysiology of diabetes and cardiovascular diseases associated with insulin resistance.

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