Des-aspartate angiotensin I (DAA-I) reduces endothelial dysfunction in the aorta of the spontaneously hypertensive rat through inhibition of angiotensin II-induced oxidative stress

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Des-aspartate angiotensin I (DAA-I), an endogenous nonapeptide, counteracts several effects of angiotensin II on vascular tone. The aim of this study was to investigate the acute protective effect of DAA-I on endothelial function in the spontaneously hypertensive rat (SHR) as well as its effect on angiotensin II-induced contractions and oxidative stress. Aortic rings were incubated with DAA-I (0.1 μM) for 30 min prior to the assessment of angiotensin II-induced contractions (0.1 nM–10 μM) in WKY and SHR aortas. Total nitrate and nitrite levels were assessed using a colorimetric method and reactive oxygen species (ROS) were measured by dihydroethidium (DHE) fluorescence and lucigenin-enhanced chemiluminescence. The effect of DAA-I was also assessed against endothelium-dependent and -independent relaxations to acetylcholine and sodium nitroprusside, respectively. Angiotensin II-induced contractions were significantly reduced by DAA-I, losartan and tempol. Incubation with ODQ (soluble guanylyl cyclase inhibitor) and removal of the endothelium prevented the reduction of angiotensin II-induced contractions by DAA-I. Total nitrate and nitrite levels were increased in DAA-I, losartan and tempol treated-SHR tissues while ROS level was reduced by DAA-I and the latter inhibitors. In addition, DAA-I significantly improved the impaired acetylcholine-induced relaxation in SHR aortas whilst sodium nitroprusside-induced endothelium-independent relaxation remained unaffected. The present findings indicate that improvement of endothelial function by DAA-I in the SHR aorta is mediated through endothelium-dependent release of nitric oxide and inhibition of angiotensin II-induced oxidative stress.

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1. Introduction

Hypertension is a multifactorial disease that involves complex interactions between homeostatic control mechanisms and environmental factors [1]. The abnormal vascular tone and blood vessel wall remodeling characteristic of hypertension are major risk factors for vascular disease [1,2]. Chronic elevation of arterial blood pressure is associated with endothelial dysfunction [3]. The hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilatation due to attenuated production and release of nitric oxide (NO) and other endothelium-derived relaxing factors (EDRFs) and/or increased production of endothelium-derived contracting factors (EDCFs) [4,5]. The abnormal vascular reactivity in blood vessels of SHR is due in part to overproduction of reactive oxygen species (ROS), in particular superoxide anions which scavenge NO and reduce its bioavailability and thus contribute to the development of endothelial dysfunction [6–10].

The activity of the renin–angiotensin system (RAS) is enhanced in the SHR which leads to a decreased NO bioavailability [11]. RAS contributes to the regulation of arterial blood pressure in health and disease [12,13]. It does so primarily due to the vasoactive properties of angiotensin II and the sodium retaining properties of aldosterone. In particular, angiotensin II stimulates a number of cellular signaling pathways by binding to two distinct subtypes of specific G-protein coupled angiotensin receptors, AT1R and AT2R [14]. AT1R coupling to G-proteins activates phospholipase C to form second messengers including inositol triphosphate (IP3) and diacylglycerol (DAG) which increases the intracellular free Ca2⁺ concentration ([Ca2⁺]i) and eventually vascular tone [14]. AT1R activation also stimulates nicotinamide adenine dinucleotide production and release of nitric oxide (NO) and other endothelium-derived relaxing factors (EDRFs) and/or increased production of endothelium-derived contracting factors (EDCFs) [4,5]. The abnormal vascular reactivity in blood vessels of SHR is due to the overproduction of reactive oxygen species (ROS), which scavenge NO and reduce its bioavailability and thus contribute to the development of endothelial dysfunction [6–10].

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phosphate (NAD(P)H) oxidase and enhances the production of reactive oxygen species (ROS) which leads to vascular constriction, remodeling and inflammation resulting from the expression of adhesion molecules and the release of cytokines and chemokines [15,16]. AT1R activation has the opposite functional effects as it enhances bradykinin production which in turn stimulates NO release and promotes vasodilatation [17,18].

Des-aspartate angiotensin I (DAA-I) is an endogenous nonapeptide formed by an alternative degradation pathway of angiotensin I to angiotensin III bypassing the formation of angiotensin II [19,20]. DAA-I attenuates the central pressor action of angiotensin II and III in the SHR [21]. It also exerts cardioprotective actions in pathologies involving angiotensin II. For example, it attenuates the hyperplastic effect of angiotensin II in cultured vascular smooth muscle cells [22], and reduces the age-related cardiac and vascular hypertrophy in the SHR [23]. In the renal and mesenteric vasculatures of hypertensive rats, DAA-I attenuates the vasoconstrictor effect of angiotensin II and III, an action mediated by AT1R [24,25].

The present study was originally initiated to compare the effect of DAA-I on contractions to angiotensin II in WKY and SHR aortas. However, exploratory experiments revealed that the nonapeptide reduced angiotensin II-induced contractions only in SHR but not in WKY preparations (Fig. 1). The present experiments were designed to define the mechanism[s] underlying this difference.

2. Materials and methods

2.1. Animals

Male Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs), 18 to 20 weeks old, were kept under controlled light (12 h:12 h light–dark cycle) and temperature (23 ± 1 °C) conditions. All rats used in the present study were born in the certified animal facility from BioLASCO (Yi-Lan Breeding Center, Taipei, Taiwan). The animals were fed with standard rat chow (Specialty Feeds Pty. Ltd., Glen Forrest, Australia) and had free access to tap water. All the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee.

2.2. Tissue preparation

The animals were sacrificed by cervical dislocation and the thoracic aorta was isolated and immediately placed in Krebs physiological salt solution (control solution) of the following composition (mM): NaCl, 118.9; NaHCO3, 25.0; MgSO4, 1.2; KCl, 4.7; KH2PO4, 1.0; glucose, 11.1 and CaCl2, 2.4. The perivascular tissues were removed, and the aorta was cut into rings (3 to 4 mm long) that were suspended in Jacketed organ chambers containing 5 ml of control solution, maintained at 37 °C and gassed with 95% O2 and 5% CO2. The rings were attached to isometric force transducers (Grass Instrument Co., Quincy, MA, USA).

The transducer outputs were amplified and recorded continuously using a PowerLab recording system (AD Instrument, Sydney, NSW, Australia) connected to a portable computer display monitor. The rings were stretched to a baseline tension of 1 g and allowed to equilibrate for 45 min. In some experiments, the endothelium was removed by gently rubbing the lumens of the rings with a small forceps (Furchgott and Zawadzki, 1980). After stabilization, the tissues were primed three times with KCl (80 mM) to obtain a consistent reference contraction. The presence or absence of functional endothelium was verified prior to the actual experiment by determining whether or not relaxation occurred upon exposure to acetylcholine (10 μM), in phenylephrine (0.1 μM)-contracted preparations. Rings with more than 70% relaxation in response to acetylcholine were considered to have sufficient functional endothelium while those which relaxed less than 5% were accepted as rings without endothelium.

2.3. Experimental protocols

To examine the effect of DAA-I (0.001 pM–10 μM) on angiotensin II, concentration–contraction curves to angiotensin II were obtained in rings from WKY and SHR. The rings were incubated with captopril (30 μM) for 20 min to prevent the conversion of DAA-I to angiotensin III [26] prior to incubation with the nonapeptide for 30 min. Each concentration of DAA-I was studied in separate experiments.

To evaluate the role of the endothelium, the response to angiotensin II (0.1 nM–10 μM) was obtained in rings with or without endothelium from SHR aortas, in the absence or presence of DAA-I (0.1 μM, the most effective concentration). To determine the mechanism underlying the effect of DAA-I, the response to angiotensin II was compared in the presence of losartan (10 μM, AT1R antagonist) [27], tempol (100 μM, a cell permeable superoxide dismutase (SOD) mimetic) [28], 1H-[1,2,4]- oxadiazolo[3,4-a]quinoxalin-1-one (ODQ, 3 μM, soluble guanylyl cyclase (sGC) inhibitor) [29] or apocynin (0.1 μM, an antioxidant and NAD(P)H oxidase inhibitor) [30]. Each inhibitor was added to the organ chamber at least 30 min prior to obtaining a concentration–response curve to angiotensin II.

2.4. Measurement of total nitrite/nitrate

Nitric oxide (NO) breaks down rapidly into nitrate (NO3-) and nitrite (NO2-) [31]. In order to measure NO products (total nitrate and nitrite, NOx), isolated aortas were pre-incubated with captopril in the presence or absence of DAA-I (0.1 μM) for 30 min. The isolated aortic rings were also incubated with or without inhibitors (losartan, 10 μM and tempol, 100 μM). In addition, angiotensin II (0.1 μM) was added to all groups from SHR aortas, in the presence or absence of DAA-I to mimic the experimental conditions of the organ chamber studies. Total NOx were determined with a Nitrate/Nitrite Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA), following the manufacturer’s instructions. Briefly, the tissues were homogenized in PBS (pH 7.4) using a glass tissue grinder (Wheaton™Tenbroeck, New
Jersey, USA) and centrifuged at 10,000 × g for 20 min. The absorbance of the sample was read using a microplate reader (CHAMELEON™ V; Hidec, Turku, Finland) at 540 nm. The measurement was based on the conversion of nitrate to nitrite, followed by the colorimetric determination of the total concentration of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. The amount of NO products is expressed in micromoles per milligram protein. Protein content of each sample was measured by the DC protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Vascular superoxide anion production

2.5.1. In situ detection of vascular superoxide anion production in cryostat section of rat aortas

To measure in situ vascular superoxide anion formation in rat aortas, dihydroethidium (DHE, Invitrogen, CA, USA) fluorescence staining was performed as described [32]. Briefly, aortic rings from respective treated-WKY and SHR groups were frozen in OCT compound (Sakura Finetek, Torrance CA, USA). Angiotensin II (0.1 μM) had been added to all groups of aortic rings to mimic the experimental conditions in the organ chambers. A cross section (10 μm thick) of frozen aortic segments was obtained and incubated in dark for 15 min in normal physiological saline solution [NPSS (mM): NaCl 140, KCl 5, CaCl2 1, MgCl2 1, glucose 10 and HEPES 5] containing 5 μM DHE. The fluorescence intensity was then measured with an Olympus FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) at excitation/emission wavelengths of 515/580 nm to visualize the signal. The images were analyzed using the Olympus Fluoview version 4 software.

2.5.2. Detection of vascular superoxide anion production in aortic rings using lucigenin-enhanced chemiluminescence

Vascular superoxide anion production was detected using lucigenin-enhanced chemiluminescence method as described [10,32]. Briefly, aortic rings from WKY and SHR were first pre-treated with or without DAA-I (0.1 μM), losartan (10 μM) and tempol (100 μM) for 30 min before addition of angiotensin II to mimic the experimental conditions of the organ chamber studies. After 30 min, the rings were then incubated for another 45 min at 37 °C in 2 ml of Krebs–HEPES buffer (in mM: NaCl 99.0, NaHCO3 25, KCl 4.7, KH2PO4 1.0, MgSO4·7 H2O 1.0 and HEPES 5) containing 5 μM DHE. The fluorescence intensity was obtained and incubated in dark for 15 min in normal physiological saline solution [NPSS (mM): NaCl 140, KCl 5, CaCl2·2H2O 2.5 and Na+–HEPES 20.0] in the presence of diethyliothiocarbamic acid (DETA, 1 mM, inactivator of superoxide dismutase) and β-nicotinamide adenine dinucleotide phosphate (NADPH, 0.1 mM, substrate for NADPH oxidase). Diphenyleneiodonium (DPI; 5 mM, NADPH oxidase inhibitor) was added for the positive controls. At the end of the incubation, the aortic rings were transferred to a 96-well Optiplate filled with 300 ml of Krebs–HEPES buffer containing lucigenin (5 mM) and NADPH. The luminescence signal was measured using microplate reader (CHAMELEON™ V; Hidec, Turku, Finland) in luminescent detection mode over 20 min. At the end of the measurement, the rings were dried for 48 h at 65 °C and weighed. The data are expressed as average counts per mg of vessel dry weight.

2.6. Role of DAA-I in the endothelium-dependent and -independent relaxations

In order to test the effect of DAA-I (0.1 μM) on the endothelium-dependent and -independent relaxations, WKY and SHR aortas were pre-incubated with DAA-I for 30 min, followed by a sustained contraction with phenylephrine (1 μM) and exposure of the tissues to increasing concentrations of either acetylcholine (0.1 nM–10 μM) or sodium nitroprusside (0.01 nM–1 μM). For experiments designed to investigate the mechanism of action of DAA-I, only aortic rings from SHR were used. Following incubation of the preparations with DAA-I, concentration–response curves to acetylcholine were compared in the presence of Nω-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor [33]).

2.7. Chemicals

Acetylcholine chloride, captopril, Nω-nitro-L-arginine methyl ester (L-NAME), phenylephrine, sodium nitroprusside, angiotensin II, apocynin, lucigenin, diethylthiocarbamic acid (DETA), β-nicotinamide adenine dinucleotide phosphate (NADPH), and diphenyleneiodonium (DPI) were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Losartan potassium was from Fluka Analytical (St Louis, MO, USA). Des-aspartate angiotensin I (DAA-I) was purchased from GL Bachem (Shanghai, China). Tempol and 1H-[1,2,4]-oxadiazolo [3, 4-a]quinoxalin-1-one (ODQ) were purchased from Calbiochem® (EMD Chemicals, San Diego, CA, USA). All drugs were freshly prepared in distilled water, with the exception of ODQ and indomethacin, which was dissolved in dimethyl sulfoxide (DMSO) and 0.5% Na2CO3, respectively. The concentrations in the organ chamber solution are expressed in molar.

2.8. Data analysis

The responses to acetylcholine and sodium nitroprusside were calculated as percentage inhibition of phenylephrine (1 μM)-induced contractions, while the responses to angiotensin II were calculated as percentage of the reference contraction to KCl obtained at the beginning of the experiment. Data are reported as means ± standard error of the mean (S.E.M.) and ‘n’ indicates number of animals used in each set of data. All data are analyzed using GraphPad Prism version 5. For statistical analysis, either Student’s t-test for differences between two groups or one-way ANOVA followed by Dunnett’s multiple comparison tests for more than two groups were used. *p values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. DAA-I suppresses angiotensin II-induced contraction in SHR aortas

DAA-I had no effect on angiotensin II-induced contraction in WKY aortic rings, however, the three concentrations of DAA-I used in the present study reduced angiotensin II-induced contractions in SHR preparations (Fig. 1). Angiotensin II induced dose-dependent contractions in both WKY and SHR aortas. Angiotensin II-induced contractions were not significantly different in the two types of preparations (Fig. 2). Incubation with DAA-I did not significantly affect angiotensin II-induced contractions in WKY aortic rings (Fig. 2B). By contrast, it significantly attenuated the contractions to angiotensin II in SHR aortas. There were no significant differences in the angiotensin II-induced contraction in SHR aortic rings with endothelium compared to those without endothelium (Fig. 2C). The inhibitory effect of DAA-I to angiotensin II contraction was decreased significantly following removal of the endothelium (Fig. 2C).

The AT1R antagonist, losartan (10 μM) abolished and the superoxide dismutase (SOD) mimetic, tempol (100 μM) significantly reduced angiotensin II-induced contractions in SHR aortas (Fig. 3A). ODQ (3 μM), an inhibitor of soluble guanylyl cyclase (sGC), by itself had no effect on angiotensin II-induced contractions, but it reversed the inhibitory effect of DAA-I (Fig. 3B). Apocynin, an antioxidant and inhibitor of NAD(P)H oxidase reduced angiotensin II-induced contraction to a similar extent as DAA-I (Supplemental Fig. 1).

3.2. DAA-I increases aortic NO production

The content of NO products were significantly lower in SHR compared to WKY aortas pre-treated with angiotensin II (Fig. 3C). In the presence of angiotensin II (0.1 μM), treatment with DAA-I (0.1 μM) for 30 min significantly increased NO production in SHR aortas. Similarly, both losartan (10 μM) and tempol (100 μM) significantly augmented NO production in SHR aortas exposed to angiotensin II.

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3.3. DAA-I reduces angiotensin II-induced oxidative stress level

In situ vascular superoxide anion formation, measured by DHE fluorescence, was significantly higher in SHR compared to WKY aortic rings in the presence of angiotensin II (0.1 μM). Treatment of SHR aortas with DAA-I (0.1 μM) for 30 min significantly decreased the accumulation of ROS. Additionally, the ROS levels in SHR aortas were also normalized by losartan (10 μM) and tempol (100 μM) (Fig. 4A and B).

NADPH-induced superoxide anion production was significantly higher in the aortic rings from the SHR compared to that of the WKY. Angiotensin II aggravated NADPH-stimulated superoxide anion in the SHR but not in the WKY. DPI, an inhibitor of NAD(P)H oxidase reduced ROS. Additionally, the ROS levels in SHR aortas were also normalized by losartan (10 μM) and tempol (100 μM) (Fig. 4A and B).

Fig. 2. Effect of DAA-I (0.1 μM) on angiotensin II (Ang II)-induced contraction in isolated aortic rings from the WKY and SHR. (A) Representative traces showing angiotensin II-induced contraction in WKY and SHR aortas, in the absence or presence of DAA-I. (B) Angiotensin II-induced contractions were similar in WKY and SHR aortic rings but pre-treatment with DAA-I for 30 min inhibited the response in SHR preparations. (C) The inhibitory effect of DAA-I was present in SHR aortas with but not in those without endothelium. Results are shown as means ± S.E.M. (n = 5–7). Significant difference from SHR is indicated by *p < 0.05, **p < 0.01, and ***p < 0.001.

Fig. 3. Concentration–contraction curves to angiotensin II (Ang II) in SHR aortas in the absence or presence of DAA-I (0.1 μM), losartan (10 μM, AT1R inhibitor), tempol (100 μM, SOD mimetic) or ODQ (3 μM, sGC inhibitor). (A) Treatment (30 min) with DAA-I, losartan and tempol reduced angiotensin II-induced contractions. (B) Treatment with ODQ for 30 min did not affect angiotensin II-induced contractions in SHR aortas but prevented the inhibition by DAA-I of the response to angiotensin II. (C) Measurement of total nitrite/nitrate content in angiotensin II (0.1 μM)-pretreated aortic rings. Results are shown as means ± S.E.M. (n = 6–7). Significant difference from the WKY is indicated by **p < 0.01; *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the SHR.
superoxide anion production in both the absence and presence of angiotensin II in preparations of both strains. DAA-I (0.1 μM) pre-incubation for 30 min had no effect on the basal release of NADPH-induced superoxide anion production in the rings from the WKY and SHR (Fig. 5).

However, treatment with DAA-I for 30 min significantly reduced the superoxide anion production in SHR in the presence of angiotensin II but had not such effect in WKY preparation. Both losartan and tempol significantly reduced the superoxide anion production in the SHR preparation exposed to angiotensin II.

3.4. DAA-I improves endothelial function in SHR aortas

Maximal relaxations to acetylcholine (10 μM) were significantly decreased in aortic rings of the SHR compared to the WKY (Fig. 6).

Incubation with DAA-I (0.1 μM) had no effect on the acetylcholine-induced relaxations in WKY rings but significantly improved the response in SHR preparations compared to their respective controls (Fig. 6B). The maximal acetylcholine-induced relaxation in SHR preparations was normalized by DAA-I compared to the normotensive WKY rings (Fig. 6B). Incubation with DAA-I had no significant effect on relaxations to sodium nitroprusside. In the SHR aorta, the acetylcholine-induced relaxation was abolished by the ENOS inhibitor, l-NAME (100 μM); DAA-I did not significantly affect the acetylcholine-induced relaxation in the presence of l-NAME (Fig. 7).

4. Discussion

The present study demonstrates that in the SHR aorta: (i) DAA-I improves the impaired endothelium-dependent relaxations and decreases the angiotensin II-induced contractions; (ii) the action of DAA-I is endothelium-dependent; and (iii) DAA-I inhibits angiotensin II-induced superoxide anion production and thus increases NO bioavailability. The concentration of DAA-I used in the present study was based on the exploratory experiments which showed that 0.1 μM DAA-I was the most effective concentration in attenuating angiotensin II-induced contraction.

Angiotensin II induced a comparable concentration-dependent increase in tension in both WKY and SHR aortas. Similar observations have been made in aortic preparations from young hypertensive rats [34–36]. The effect of DAA-I on angiotensin II-induced contractions was similar to that on acetylcholine-induced relaxations in that it affected the responses to agonists in SHR but not in WKY aortas. This result is in line with previous findings that DAA-I counteracts the central pressor action of angiotensin II [22,37] and angiotensin II-induced vasoconstrictions in the renal and mesenteric vasculature of the SHR [38]. The present data also show that the action of DAA-I is endothelium-dependent, since its attenuating effect on angiotensin II-induced contractions was abolished by the removal of the endothelium.

Binding of angiotensin II to AT1R stimulates the mobilization of intracellular calcium ([Ca2+]) and activation of protein kinase C (PKC)
causing vascular contraction. Losartan, an AT1 antagonist [27], reduced the angiotensin II-induced contractions. Perfusion of the kidney with DAA-I reduces AT1R density in SHR [25] and AT1R can mediate effects of DAA-I both in vitro and in vivo [39,40]. In the present study, the inhibitory actions of DAA-I parallel those of losartan suggesting that DAA-I probably interacts with AT1R preventing the activation of its downstream signaling pathway. Tempol significantly reduced the contractile response to angiotensin II, supporting the role of reactive oxygen species (i.e. superoxide anion) [28,41]. Attenuation of the inhibitory effects of DAA-I by both the ENOS inhibitor, l-NAME and the inhibitor of soluble guanylyl cyclase, suggests that the nonapeptide protects and improves endothelial function by activating the NO/cyclic GMP signaling cascade.

Angiotensin II mediates the production of superoxide anions via the activation of AT1R and this contributes to the pathological effects of angiotensin II such as vascular remodeling, vascular contraction, inflammation and increased systolic arterial blood pressure [41,42]. The major source of angiotensin II-dependent ROS in the vascular system is NAD[P]H oxidase which importantly contributes to the angiotensin II-induced contraction [41,43,44]. Consistently, the present study demonstrates that apocynin, an antioxidant and inhibitor of NAD[P]H oxidase blunted the contraction evoked by angiotensin II to a similar extent as DAA-I suggesting the role of NADPH-mediated, ROS-induced contraction in SHR preparation. In addition, DAA-I attenuated the production of superoxide anion in angiotensin II-stimulated (NAD(P)H oxidase activation) aortic rings from the SHR, indicating that it interferes with oxidative stress generating action of angiotensin II. As shown in previous studies [28,43,45], tempol and losartan reduced superoxide anion production in SHR aortic rings in the presence of angiotensin II. DAA-I reduced the elevated pulmonary ROS levels in mice with 2-chloroethyl ethyl sulfide (CEES)-induced lung intoxication [46], and the skeletal muscle content of gp91 protein (a major constituent of NADPH oxidase, an enzyme that generates superoxide anions [47,48]) in mice with diet-induced hyperglycaemia [49]. The present study shows that the total nitrite/nitrate level of SHR aortas is significantly lower compared to WKY in the presence of angiotensin II and that DAA-I augmented the level of NO products. Blocking AT1R and reducing the generation of superoxide anion (with losartan and tempol, respectively) also increased the level of NO products. Taken in conjunction, the present data suggests that DAA-I protects endothelial function by antagonizing the effects of angiotensin II and attenuating superoxide anion production.

In the present study, judging from the blunted response to acetylcholine, a significant impairment of endothelial function was observed in the aorta of the SHR compared to the WKY. This is consistent with previous studies on the SHR [8,50,51]. DAA-I did not alter the relaxation to acetylcholine in WKY aortas but enhanced it in SHR preparations with endothelium. This suggests that DAA-I potentiates acetylcholine-induced relaxations by increasing NO release. This interpretation is supported by the observation that indeed the nonapeptide enhances the content of NO products in the SHR aorta. l-NAME, an eNOS inhibitor (a major constituent of NADPH oxidase, an enzyme that generates superoxide anions [47,48]) in mice with diet-induced hyperglycaemia [49]. The present study shows that the total nitrite/nitrate level of SHR aortas is significantly lower compared to WKY in the presence of angiotensin II and that DAA-I augmented the level of NO products. Blocking AT1R and reducing the generation of superoxide anion (with losartan and tempol, respectively) also increased the level of NO products. Taken in conjunction, the present data suggests that DAA-I protects endothelial function by antagonizing the effects of angiotensin II and attenuating superoxide anion production.

![Fig. 6](image_url) Effect of DAA-I (0.1 μM) pre-treatment on acetylcholine (ACh)-induced endothelium-dependent and sodium nitroprusside (SNP)-induced endothelium-independent relaxations in isolated aortic rings from the WKY and SHR. (A) Representative traces showing acetylcholine-induced relaxations from WKY and SHR aortas, in the absence or presence of DAA-I. (B) Pre-treatment of DAA-I improved acetylcholine-induced endothelium-dependent relaxations; (C) whilst sodium nitroprusside-induced endothelium-independent relaxations were unaltered. Results are means ± S.E.M. (n = 5–7). Significant difference from the WKY is indicated by *p < 0.05, **p < 0.01; ***p < 0.001. Significant difference compared to the SHR; and #p < 0.05, ##p < 0.01, ###p < 0.001 compared to DAA-I.

![Fig. 7](image_url) Concentration–relaxation curves to acetylcholine (ACh) in isolated SHR aortas with or without DAA-I (0.1 μM) or l-NAME (100 μM) or both. Treatment with l-NAME or l-NAME + DAA-I inhibited endothelium-dependent relaxation induced by acetylcholine compared to control-treated and DAA-I treated SHR aortas. Results are shown as means ± S.E.M. (n = 5–7). Significant difference is indicated by *p < 0.05, **p < 0.01 compared to the WKY; *p < 0.05, **p < 0.01, ***p < 0.001 compared to the SHR; and *p < 0.05, **p < 0.01, ###p < 0.001 compared to DAA-I.
modulation of the release of endothelium-derived NO rather than to changes in the sensitivity of the effectors cells to NO.

In summary, DAA-I improves acetylcholine-induced relaxations and decreases angiotensin II–induced contractions in the SHR but not WKY aorta. The endothelial protective actions of DAA-I is due to higher NO bioavailability through increasing ENSO activity and reducing ROS production in the SHR, achieved at least in part by interfering with Ang II–mediated, NADPH derived oxidative stress. The activity of aminopeptidase X, the enzyme which degrades angiotensin I to DAA-I, is higher in both the endothelium and the plasma of the SHR than in the WKY [52]. However, the plasma DAA-I level is lower in the hypertensive strain as in essential hypertensive patients [53]. This may result from increased activity of angiotensin converting enzyme (ACE) which not only converts angiotensin I into angiotensin II but also DAA-I into angiotensin III [54]. Indeed, inhibiting these two ACE pathways with ACE inhibitors while maintaining the activity of aminopeptidase X, increased plasma DAA-I level in the SHR [53]. The chronic lower level of DAA-I may cause a relative supersensitivity of its binding site on the AT1R which may explain why reintroducing the nonapeptide ex vivo (in the presence of captopril to prevent its conversion into angiotensin III), counteracted the effect of angiotensin II only in SHR preparations.

Supplementary data to this paper can be found online at dx.doi.org/10.1016/j.vph.2015.03.011.

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