

# Angiotensin-(1–7) Prevents Activation of NADPH Oxidase and Renal Vascular Dysfunction in Diabetic Hypertensive Rats

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## Key Words

Diabetes · Hypertension · Angiotensin · Kidney · Renal artery

## Abstract

**Background/Aim:** We examined the influence of chronic treatment with angiotensin-(1–7) [Ang-(1–7)] on renox (renal NADPH oxidase, NOX-4) and the development of renal dysfunction in streptozotocin-treated spontaneously hypertensive rats (diabetic SHR). **Methods:** Mean arterial pressure, urinary protein and vascular responsiveness of the isolated renal artery to vasoactive agonists were studied in vehicle- or Ang-(1–7)-treated SHR and diabetic SHR. **Results:** Ang-(1–7) decreased the elevated levels of renal NADPH oxidase (NOX) activity and attenuated the activation of NOX-4 gene expression in the diabetic SHR kidney. Ang-(1–7) treatment increased sodium excretion but did not affect mean arterial pressure in diabetic SHR. There was a significant increase in urinary protein ( $266 \pm 22$  mg/24 h) in the diabetic compared to control SHR ( $112 \pm 13$  mg/24 h) and treatment of diabetic SHR with Ang-(1–7) reduced the degree of proteinuria ( $185 \pm 23$  mg/24 h,  $p < 0.05$ ). Ang-(1–7) treatment also attenuated the diabetes-induced increase in renal vascular responsiveness to endothelin-1, norepinephrine, and angiotensin II in SHR, but significantly increased the vasodilation of the renal artery of SHR and diabetic SHR to the vasodilator ago-

nists. **Conclusion:** These results suggest that treatment with Ang-(1–7) constitutes a potential therapeutic strategy to alleviate NOX-mediated oxidative stress and to reduce renal dysfunction in diabetic hypertensive rats.

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## Introduction

The simultaneous occurrence of essential hypertension and diabetes mellitus is exceedingly common. Increased blood glucose and its oxidized metabolites cause vascular damage and abnormal endothelial function, resulting in increased vulnerability to higher blood pressure [1]. Overproduction of reactive oxygen species (ROS) is associated with diabetes mellitus and hypertension, and recent studies suggest that oxidative stress contributes to the enhanced renal damage and dysfunction in the diseased kidney [2]. NADPH oxidases (NOX) are a major source of superoxide anion production and may be a key player in the regulation of cellular redox [2–6]. Cardiovascular dysfunction in blood vessels is associated with altered vascular reactivity. An enhanced vascular responsiveness to vasoconstrictors and an attenuated response to vasodilators is often evident in diabetic and/or hypertensive vascular tissues. The streptozotocin (STZ)-treated spontaneously hypertensive rat (diabetic SHR)

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develops a hyperglycemic syndrome associated with other biochemical and morphological changes that to some extent approach insulin-dependent diabetes mellitus combined with hypertension [7]. Moreover, the diabetic SHR exhibits accelerated proteinuria and marked structural lesions, particularly podocyte damage, mesangial expansion and vascular dysfunction that make it a suitable model for investigation of diabetes-induced kidney dysfunction [7].

The activity of the renin-angiotensin system is elevated both in the circulation and in the renal tissue of diabetic nephropathies and increased activity of the renin-angiotensin system plays an important role in the hemodynamic and nonhemodynamic pathogenic mechanisms involved in kidney disease [8–14]. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin type-1 receptor (AT<sub>1</sub>) blockers (ARB) can prevent/delay the development of diabetic nephropathy independently of their beneficial blood pressure-lowering effect in patients with diabetes and microalbuminuria [15–20]. Importantly, ACEI and ARB shift the balance of the renin-angiotensin system from Ang II to increased formation and actions of angiotensin-(1–7) [Ang-(1–7)] [21–25]. Ang-(1–7) is a vasodilator peptide with antihypertensive properties [26–28]. Ang-(1–7) is formed from Ang I and Ang II by several endopeptidases and carboxypeptidases, including angiotensin-converting enzyme-2 (ACE2) [22, 23, 25]. Indeed, a recent study showed that ACE2 protein in renal cortical tubules is increased in diabetic mice, suggesting that increased formation of Ang-(1–7) may be a compensatory renoprotective response in early stages of diabetes [29]. Moreover, administration of an ACE2 inhibitor exacerbates the diabetic-induced renal damage [30]. In normotensive animals, we have shown that chronic treatment with Ang-(1–7) attenuates the extent of diabetic renal dysfunction [31]. Since diabetic injury in hypertensive models is associated with the activation of NOX and increased ROS, we determined whether chronic treatment with Ang-(1–7) can attenuate NOX activation and the development of abnormal renal function in diabetic SHR.

## Methods

### *Treatment Groups and Induction of Diabetes*

Seventeen-week-old male SHR and WKY rats were used in this study. Animals were divided into five groups (n = 15/group): group 1, WKY; group 2, SHR; group 3, SHR treated with daily intraperitoneal injections of Ang-(1–7) (576 µg/kg/day i.p.) [SHR-Ang-(1–7)]; group 4, STZ-treated SHR [diabetic SHR]; group 5,

Ang-(1–7)-treated diabetic SHR [diabetic SHR-Ang-(1–7)]. Animals were sacrificed at the end of the 4-week treatment period.

Diabetes was induced by a single intraperitoneal injection of 55 mg/kg body weight STZ dissolved in citrate buffer (pH 4.5) and verified by blood glucose concentrations above 200 mg/dl 48 h after STZ injection. Blood glucose levels were determined using an automated blood glucose analyzer (glucometer Elite XL). Blood glucose was reassessed and body weight determined after 4 weeks just before sacrificing the animals. All analyses were performed by investigators who were blinded to the treatment groups. The investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985) and was approved by Kuwait University Research Administration as the use of animals was in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

(±)-Norepinephrine-bitartrate, STZ, Ang-(1–7), Ang II, sodium nitroprusside, endothelin-1 (ET-1) and carbachol were obtained from Sigma Biochemical (USA).

### *Urine Analysis*

At the end of the 4-week treatment period, animals were placed in metabolic cages and food and water were provided ad libitum. Metabolic cages provided an effective separation of feces and urine into tubes outside the cage. The urine collection was carried out for 24 h, with the sample collection tube immersed in ice-cold water. Total protein was determined as reported [32]. Urinary electrolytes were measured by Ecolyte electrolyte analyzer (Eschweiler GmbH & Co. KG), which is a microprocessor-controlled analysis system for quantitative measurement of electrolyte parameters.

### *Blood Pressure Measurement*

Animals were anesthetized with sodium pentobarbital (60 mg/kg) and the left femoral artery was exposed surgically at the end of the 4-week period. A small incision was made in the femoral artery, and a catheter was inserted and connected to a pressure transducer for blood pressure measurement. Mean arterial pressure (MAP, expressed as mm Hg) was recorded on a polygraph.

### *NADPH Oxidase Studies*

At the end of the 4-week treatment with experimental agents, kidneys were removed from the euthanized animals. One part of the kidney was used to prepare tissue homogenates in 0.25 M sucrose buffer, pH 7.2, while the other half of the kidney was immediately frozen in liquid nitrogen. Kidney homogenates were used for assay of NADPH oxidase activity while the frozen tissue was used later to isolate RNA.

*NADPH Oxidase Assay.* NADPH oxidase activity was measured in tissue homogenates at room temperature in assay mixture that contained 50 mM phosphate buffer, pH 7.1, 0.01 mM EDTA and 250 µM lucigenin. Reaction was started by addition of 100 µM of NADPH and chemoluminescence was recorded over a period of 3 min. Specific enzyme activity was calculated as relative light units emitted per second per milligram of protein [6].

*RNA Isolation and Reverse Transcription.* In each experiment, total RNA was extracted from kidney tissues with RNA extraction kit based on the use of guanidinium thiocyanate lithium chloride and cesium trifluoroacetate. Isolated RNA was of high

**Table 1.** Effect of Ang-(1-7) on MAP, body weight, blood glucose and kidney function

Animal group	MAP mm Hg	Body weight, g	Blood glucose mg/dl	Urine volume ml/24 h	Protein excretion mg/24 h	Urine lysozyme µg/24 h	Na <sup>+</sup> excretion mmol/24 h
WKY	104 ± 4	319 ± 11	90 ± 3	12 ± 1	88 ± 6	43 ± 5	20 ± 1
SHR	205 ± 11 <sup>a</sup>	326 ± 8	95 ± 4	11 ± 2	112 ± 13 <sup>a</sup>	62 ± 15 <sup>a</sup>	16 ± 1
SHR-Ang-(1-7)	172 ± 5 <sup>a, b</sup>	364 ± 14	97 ± 6	12 ± 4	101 ± 12	44 ± 14 <sup>b</sup>	27 ± 2
Diabetic SHR	163 ± 3 <sup>a, b</sup>	262 ± 13 <sup>a, b</sup>	599 ± 13 <sup>a, b</sup>	106 ± 9 <sup>a, b</sup>	266 ± 22 <sup>a, b</sup>	463 ± 7 <sup>a, b</sup>	689 ± 12 <sup>a, b</sup>
Diabetic SHR-Ang-(1-7)	164 ± 8 <sup>a, b</sup>	317 ± 14 <sup>c</sup>	421 ± 11 <sup>a-c</sup>	122 ± 5 <sup>a-c</sup>	185 ± 23 <sup>a-c</sup>	452 ± 10 <sup>a</sup>	1,232 ± 22 <sup>a-c</sup>

Data presented as mean ± SEM (n = 5–8).

<sup>a</sup> Value significantly different compared to WKY, p < 0.05. <sup>b</sup> Value significantly different compared to SHR, p < 0.05. <sup>c</sup> Value significantly different compared to diabetic SHR, p < 0.05.

quality and was used immediately for synthesis of first strand cDNA according to protocols from Clontech's SMART PCR cDNA synthesis kit.

**PCR Detection of NOX-4 and G3PDH mRNA.** Amplification of cDNA obtained from reverse transcription of RNAs was carried out using Advantage cDNA PCR kit (BD Biosciences Clontech) and the following primers: NOX-4, 5'-TGG GTC CAC AAC AGA AAA CA-3' and 5'-TTG GCT TTG GAT TTC TGG AC-3'. Primers for G3PDH were provided by Clontech. First strand of cDNA obtained from reverse transcription was denatured for 1 min at 95°C and subjected to PCR with the following parameters: 95°C for 30 s, 58°C or 62°C for 30 s, 68°C for 45 s, 25–30 cycles after denaturing at 95°C for 1 min. PCR products were then analyzed using agarose gel electrophoresis.

#### Vascular Reactivity Experiments

Vascular reactivity experiments were performed in a separate set of animals treated as above:

**Isolation of the Renal Artery.** The animals were euthanized at the end of the 4-week study by decapitation under light ether anesthesia. The renal artery was isolated carefully and transferred to a Petri dish containing oxygenated Krebs' solution. The vessel was cut into ring segments of about 5 mm. The ring segments were mounted in organ baths containing 50 ml Krebs-Henseleit solution at pH 7.4. The composition of Krebs-Henseleit solution is as follows (mM): NaCl (118.3), KCl (4.7), CaCl<sub>2</sub> (2.5), MgSO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (25), KH<sub>2</sub>PO<sub>4</sub> (1.2) and glucose (11.2). The tissue bath solution was maintained at 37°C and was aerated with 95% oxygen and 5% carbon dioxide mixture. Isometric contractions of the renal arteries were recorded through UFI dynamometers on a Lectromed two-channel recorder. A pretension of 0.5 g was applied and the preparations were allowed to stabilize (45 min) until a stable baseline tone was obtained.

**Vasoconstriction Studies.** Following the period of equilibration, the vasoconstrictor effects of ET-1 (10<sup>-10</sup>, 10<sup>-9</sup> and 10<sup>-8</sup> M), norepinephrine (NE, 10<sup>-7</sup> M) and Ang II (10<sup>-9</sup>, 10<sup>-8</sup> and 10<sup>-7</sup> M) were tested on the isolated renal artery ring segments. The different doses of the agonists were added successively to the organ baths to establish the vasoconstrictor responses. The maximal increase in tension was recorded as milligram per milligram tissue

weight. The effect of the vasoconstrictor agonists were tested on different tissue preparations.

**Vasodilation Studies.** The vasodilator responses of carbachol, Ang-(1-7) and sodium nitroprusside were investigated. Following the period of equilibration, the isolated renal artery segments were precontracted by a submaximal concentration of NE (3 × 10<sup>-7</sup> or 10<sup>-6</sup> M) added to the organ baths. After obtaining a steady level of precontraction, the vasodilator effects of carbachol (10<sup>-7</sup>, 10<sup>-6</sup> and 10<sup>-5</sup> M), Ang-(1-7) (10<sup>-8</sup>, 10<sup>-7</sup> and 10<sup>-6</sup> M) and sodium nitroprusside (10<sup>-8</sup>, 10<sup>-7</sup> and 3 × 10<sup>-7</sup> M) were examined. The vasodilator response was measured as the percent change in the maximal initial contraction induced by NE.

#### Statistical Analysis

Results were analyzed using Graphpad Prism software. Data are presented as mean ± SEM of the number of experiments. Mean values were compared using analysis of variance followed by post hoc test (Bonferroni). The difference was considered to be significant when the p value was less than 0.05.

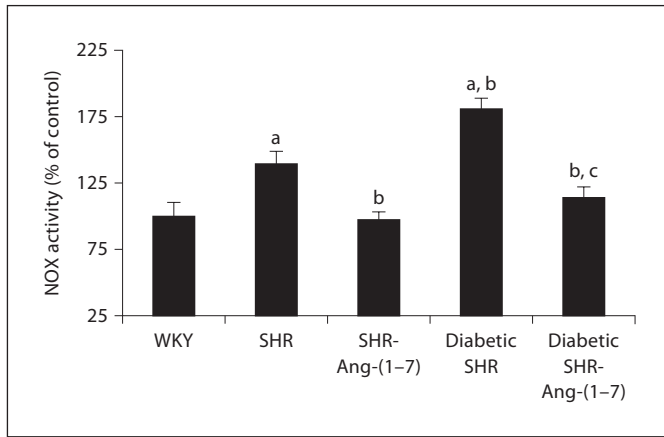
## Results

### Mean Arterial Pressure

As shown in table 1, the MAP was significantly reduced in SHR-Ang-(1-7) compared to SHR. MAP was not different in the diabetic SHR-Ang-(1-7) compared to diabetic-SHR (table 1).

### Hyperglycemia and Body Weight

Induction of diabetes by STZ resulted in a significant increase in blood glucose concentration (table 1). Hyperglycemia persisted in the diabetic SHR and was 599 ± 13 mg/dl at 4 weeks as compared with WKY and SHR controls. Blood glucose concentration was significantly reduced in comparison with diabetic SHR after 4 weeks of



**Fig. 1.** NADPH oxidase enzyme activity in kidneys of hypertensive and diabetic hypertensive rats with or without Ang-(1-7) treatment. Enzyme activity is shown as percent of control. (Mean  $\pm$  SD, n = 6 per group; significantly different compared to WKY, <sup>a</sup> p < 0.05; significantly different compared to SHR, <sup>b</sup> p < 0.05; significantly different compared to diabetic SHR, <sup>c</sup> p < 0.05.)

treatment with Ang-(1-7), but these values in diabetic SHR-Ang-(1-7) were still greatly elevated over control rats. There was a significant reduction in the body weight of diabetic SHR compared to SHR. Treatment of diabetic SHR with Ang-(1-7) resulted in a significant increase in body weight compared to diabetic SHR (table 1).

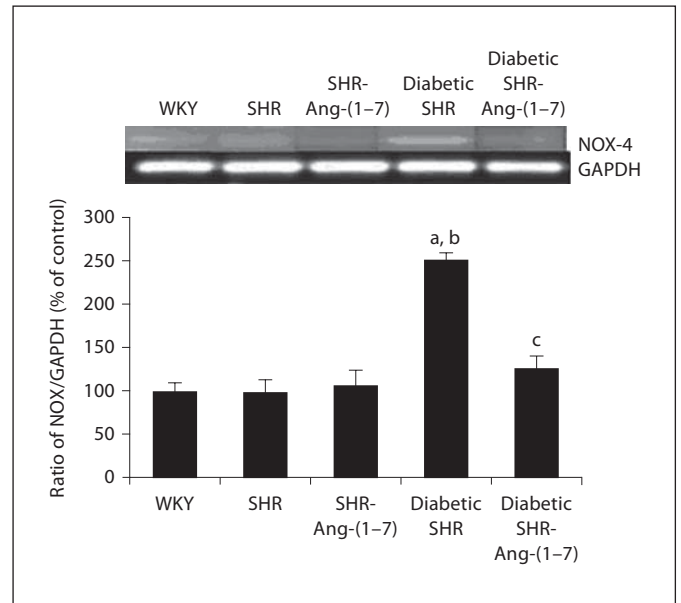
#### NOX Activity and Gene Expression of NOX-4

Figure 1 shows that kidneys of SHR have significantly higher enzymatic activity of NOX compared to WKY. Renal NOX activity in SHR was further increased by STZ-induced diabetes. Treatment with Ang-(1-7) significantly prevented the diabetes-induced activation of NOX enzymatic activity in kidneys of SHR.

Figure 2 shows NOX-4 gene expression. NOX-4 mRNA was significantly increased (2- to 3-fold) in diabetic SHR as compared to nondiabetic WKY rats or SHR, suggesting that transcriptional regulation of NOX-4 may account for the increase in activity. Ang-(1-7) treatment prevented the diabetes-induced activation of NOX-4 gene expression in SHR (fig. 2).

#### Urine Analysis

There was a significant increase in urinary protein in diabetic SHR as compared to SHR. Treatment of diabetic SHR animals with Ang-(1-7) resulted in a 30% reduction in urinary protein compared to diabetic SHR (table 1). Lysozyme excretion was also increased in both SHR as



**Fig. 2.** RT-PCR analysis of NOX-4 and GAPDH mRNA levels in kidneys of Ang-(1-7)-treated hypertensive and diabetic hypertensive rats. Bars show the ratio (as percent of control) of NOX-4 and GAPDH. (Mean  $\pm$  SD, n = 3 per group; significantly different compared to WKY, <sup>a</sup> p < 0.05; significantly different compared to SHR, <sup>b</sup> p < 0.05; significantly different compared to diabetic SHR, <sup>c</sup> p < 0.05.)

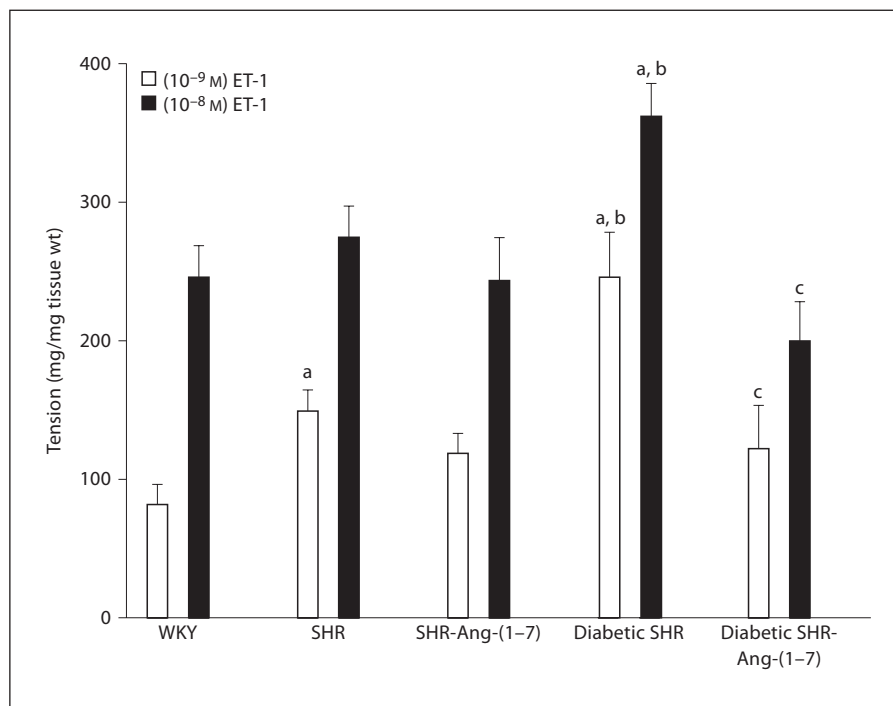
compared to WKY and in diabetic SHR. Although Ang-(1-7) treatment corrected the increase in lysozyme excretion in hypertensive animals, there was no significant reduction by the peptide in the diabetic SHR-Ang-(1-7) group. Diabetes induction resulted in a significant increase in Na<sup>+</sup> excretion in diabetic SHR compared to SHR or WKY. Na<sup>+</sup> excretion was increased further in diabetic SHR-Ang-(1-7) compared to diabetic SHR (table 1).

#### Vascular Reactivity

**Vasoconstriction Studies.** The vasoconstrictor responses to ET-1, NE and Ang II were significantly augmented in the isolated renal artery of SHR as compared to WKY, as well as the diabetic SHR versus the SHR (fig. 3-5). Chronic treatment of SHR and diabetic SHR with Ang-(1-7) resulted in a significant attenuation in the vasoconstrictor responses to ET-1 (10<sup>-9</sup> and 10<sup>-8</sup> M), NE (10<sup>-7</sup> M) and Ang II (10<sup>-9</sup>, 10<sup>-8</sup> and 10<sup>-7</sup> M) in the renal arteries compared to WKY and SHR, respectively (fig. 3-5).

**Vasodilation Studies.** The vasodilator response to sodium nitroprusside was similar in all the groups studied

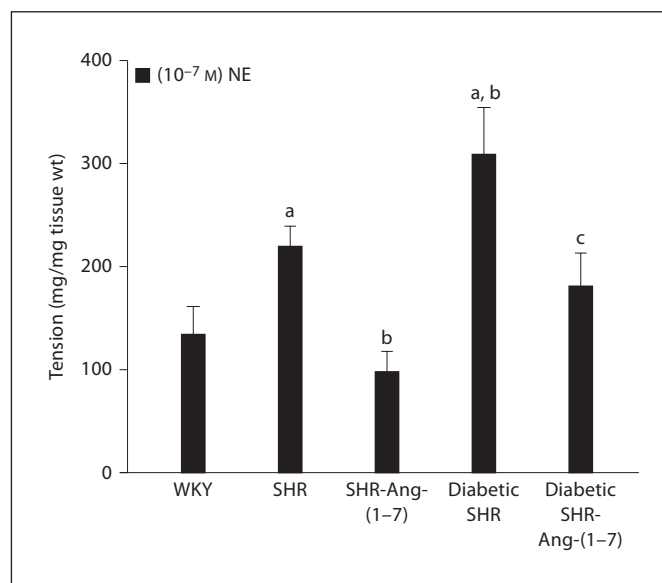
**Fig. 3.** ET-1-induced vasoconstriction ( $10^{-9}$  and  $10^{-8}$  M) in the renal artery ring segments of WKY, SHR, SHR-Ang-(1-7), diabetic SHR and diabetic SHR-Ang-(1-7). (Mean  $\pm$  SE, n = 6 per group; significantly different compared to WKY, <sup>a</sup>  $p < 0.05$ ; significantly different compared to SHR, <sup>b</sup>  $p < 0.05$ ; significantly different compared to diabetic SHR, <sup>c</sup>  $p < 0.05$ .)



(data not shown). As shown in figures 6 and 7, both the vasodilator responses to carbachol ( $10^{-7}$  and  $10^{-6}$  M) and Ang-(1-7) ( $10^{-8}$ ,  $10^{-7}$  and  $10^{-6}$  M) were reduced in the renal artery of SHR versus control WKY rats. There was no further impairment of the vasodilator responses in the diabetic animals. Ang-(1-7) treatment significantly increased the vasodilation of the renal artery of the SHR and diabetic SHR to both vasodilator agonists, but did not fully restore the impaired responses.

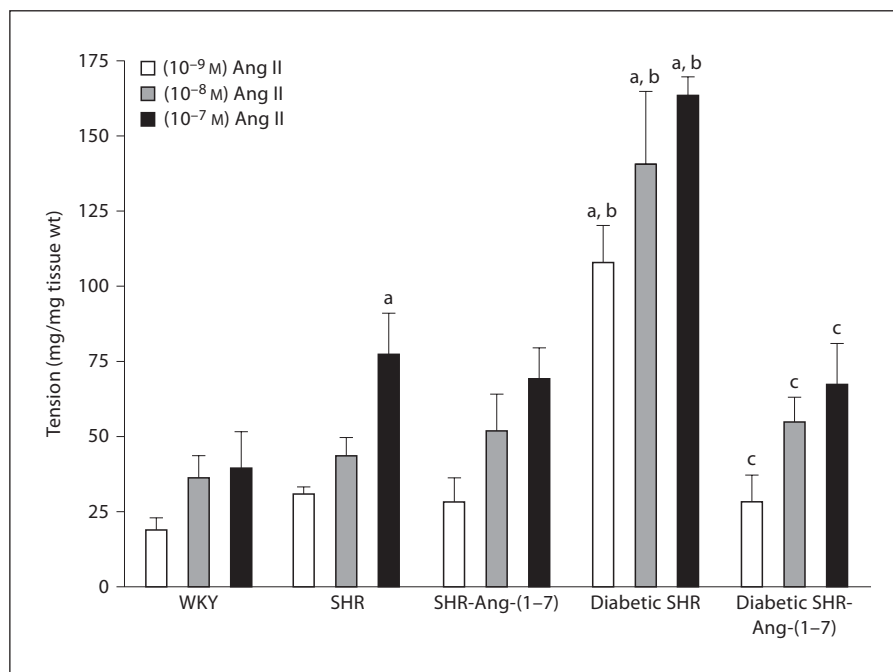
## Discussion

The induction of hyperglycemia in the SHR model resulted in decreases in MAP and body weight, and increases in blood glucose, proteinuria, lysozyme excretion, sodium excretion and renal NOX activity and NOX-4 gene expression. The vascular reactivity to the vasoconstrictors ET-1, NE and Ang II were enhanced whereas carbachol- or Ang-(1-7)-mediated vasorelaxation were reduced in the diabetic SHR. Chronic treatment with Ang-(1-7) prevented the increases in both renal NOX activity and NOX-4 gene expression. Hyperglycemia was modestly attenuated in the diabetic SHR by Ang-(1-7), but was not corrected. Treatment of diabetic SHR with Ang-(1-7) also resulted in increased natriuresis

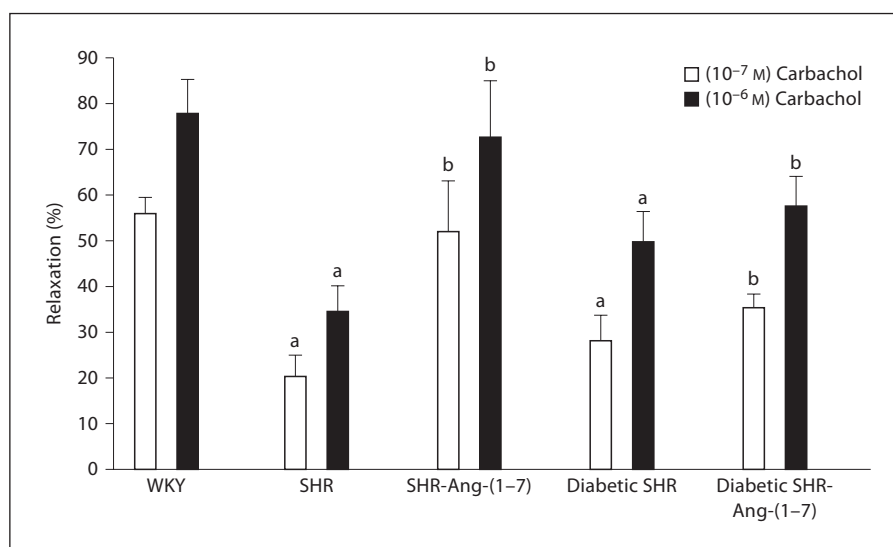


**Fig. 4.** NE-induced vasoconstriction ( $10^{-7}$  M) in the renal artery ring segments of WKY, SHR, SHR-Ang-(1-7), diabetic SHR and diabetic SHR-Ang-(1-7). (Mean  $\pm$  SE, n = 6 per group; significantly different compared to WKY, <sup>a</sup>  $p < 0.05$ ; significantly different compared to SHR, <sup>b</sup>  $p < 0.05$ ; significantly different compared to diabetic SHR, <sup>c</sup>  $p < 0.05$ .)

**Fig. 5.** Ang II-induced vasoconstriction ( $10^{-9}$ ,  $10^{-8}$  and  $10^{-7}$  M) in the renal artery ring segments of WKY, SHR, SHR-Ang-(1-7), diabetic SHR and diabetic SHR-Ang-(1-7). (Mean  $\pm$  SE, n = 8 per group; significantly different compared to WKY, <sup>c</sup>  $p < 0.05$ ; significantly different compared to SHR, <sup>b</sup>  $p < 0.05$ ; significantly different compared to diabetic SHR, <sup>c</sup>  $p < 0.05$ .)



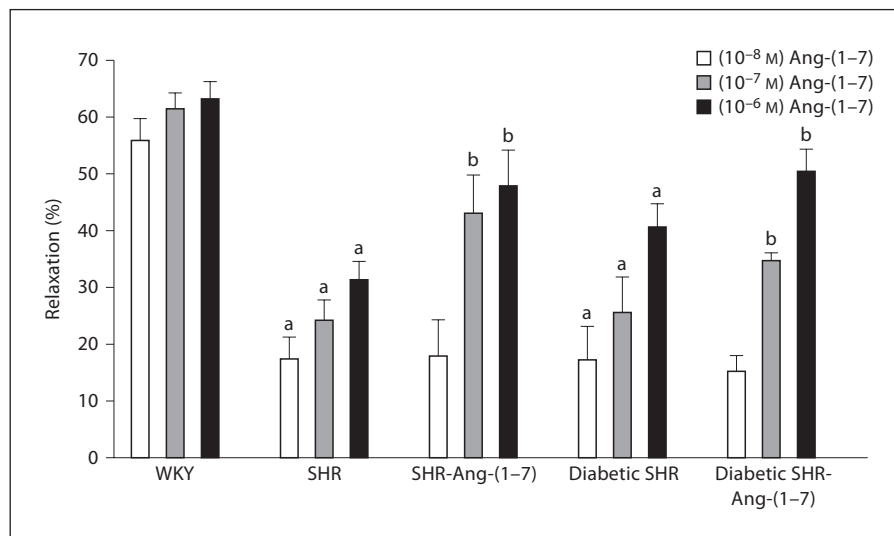
**Fig. 6.** Carbachol-induced vasodilation ( $10^{-7}$  and  $10^{-6}$  M) in the renal artery ring segments of WKY, SHR, SHR-Ang-(1-7), diabetic SHR and diabetic SHR-Ang-(1-7). (Mean  $\pm$  SE, n = 8 per group; significantly different compared to WKY, <sup>a</sup>  $p < 0.05$ ; significantly different compared to SHR, <sup>b</sup>  $p < 0.05$ .)



without an overall effect on MAP. Ang-(1-7) treatment significantly attenuated the proteinuria and reduced the development of abnormal vascular reactivity to constrictor and dilator stimuli in the diabetic animals. Further, a significant prevention of weight loss was observed following Ang-(1-7) treatment implying that Ang-(1-7) is either inhibiting pathways that lead to diabetes-induced weight loss in SHR or alternatively influencing weight gain subsequent to the correction of renal dysfunction and protein wasting.

The rat with STZ-induced diabetes is a widely used animal model of human diabetic nephropathy. In this model, diabetic nephropathy progresses without significant elevation in blood pressure [7-11]. Studies in the rat with STZ-induced diabetes demonstrate cardiovascular abnormalities such as depressed MAP and heart rate, endothelial dysfunction, and altered responses to vasoactive agents [7]. The current results are in agreement with other studies which report that STZ-induced diabetes reduces arterial pressure in SHR [7]. The factors that cause

**Fig. 7.** Ang-(1-7)-induced vasodilation ( $10^{-8}$ ,  $10^{-7}$  and  $10^{-6}$  M) in the renal artery ring segments of WKY, SHR, SHR-Ang-(1-7), diabetic SHR and diabetic SHR-Ang-(1-7). (Mean  $\pm$  SE, n = 6 per group; significantly different compared to WKY, <sup>a</sup> p < 0.05; significantly different compared to SHR, <sup>b</sup> p < 0.05.)



lowering of MAP in diabetic SHR are not known but we find an increase in urine volume and sodium excretion in the diabetic state. This condition may lead to volume depletion that was further exacerbated by Ang-(1-7). Lower blood pressure and the loss of salt and water may culminate to minimize end-organ damage. In the present study, induction of diabetes in SHR resulted in a significant increase in proteinuria, a marker of renal glomerular injury. It has been well documented that inhibiting the actions of Ang II improves proteinuria, and that this benefit is independent of blood pressure lowering [12–21]. These studies suggested that Ang II has deleterious effects directly on the kidney, independent of its hemodynamic actions. Indeed, Ang II directly causes podocyte apoptosis, an effect that is mediated through the AT<sub>1</sub> receptor. When cultured podocytes are placed under stress-tension (induced by mechanical stretch), they undergo apoptosis. However, blocking the AT<sub>1</sub> receptor pharmacologically significantly reduces stress-tension-induced podocyte apoptosis [33]. Taken together, in addition to reducing systemic and intraglomerular pressures, inhibitors of Ang II likely reduce podocyte apoptosis, thereby minimizing podocyte loss, providing an additional mechanism for reductions in proteinuria and glomerulosclerosis [33]. Ang II blockade with ACEI or ARB increases renal ACE2 activity and Ang-(1-7) formation [22–24]. Treatment with Ang-(1-7), which antagonizes the actions of Ang II and participates in the effects of ACEI and ARB, significantly attenuated diabetes-induced proteinuria in SHR but not in vehicle-treated SHR. This could be due to the fact that proteinuria is much higher in diabetic SHR

compared to SHR. Previous studies have shown that Ang-(1-7) produces blood pressure-lowering effects only when MAP is much higher than normal [22, 23, 27]. Interestingly, Ang-(1-7) treatment corrected the lysozyme excretion, a marker of tubular injury, in the hypertensive animals relative to WKY, but did not correct the diabetic-induced increase in lysozyme excretion. These findings may reveal a greater effect of Ang-(1-7) in maintaining vascular/glomerular function over tubular function in diabetes. Renoprotection and improvements in vascular health, without an alteration in lysozyme excretion, were also observed with Ang-(1-7) treatment in diabetic Wistar rats [31]. The cellular signaling responsible for the actions of Ang-(1-7) in the kidney most likely involves prostaglandins and nitric oxide. Ang-(1-7) infusion into SHR causes diuresis and natriuresis concomitant with increases in urinary prostaglandins [27]. The natriuretic and diuretic actions of Ang-(1-7) in the perfused kidney are associated with increased prostacyclin levels [22]. Ang-(1-7) also induces vasodilation of precontracted afferent arterioles by local release of nitric oxide [22]. These observations suggest that Ang-(1-7) treatment produces renal protection by antagonizing the hyperglycemia-induced activation of factors that promote glomerular capillary hypertension, renal fibrosis and inflammation.

Functional and anatomical abnormalities of the vascular endothelium are commonly associated with diabetes [1]. The pathogenesis of diabetes-induced vascular injury is affected by both a nitric oxide deficit and an Ang II increase [1, 12–14, 34, 35]. Hyperglycemia results in the impairment of endothelial cell nitric oxide production

possibly by protein kinase C activation [34], which has also been shown in human subjects [36], that leads to leukocyte adhesion to the endothelium through up-regulation of cell surface-specific adhesion molecules [35]. Increased oxidative stress plays an important role in the pathogenesis of several inflammatory diseases such as atherosclerosis, diabetes, hypertension and ischemia-reperfusion [2–5, 14]. Renal dysfunction associated with diabetes and or hypertension has also been suggested to occur due to increased production of ROS [2–5]. Although several sources of ROS may be involved, NOX appear to be important for regulation of cellular redox [2]. The localization of phagocytic NOX as well as NOX-1 and NOX-4 in the kidney has been well demonstrated and NOX-4, also called renox, is the predominant form in the kidney [2]. Ang II up-regulates renal NOX activity and increases oxidative stress [2, 3]. Activation of AT<sub>1</sub> receptors by Ang II results in activation of NADH/NADPH oxidases in vascular smooth muscle cells that lead to the production of O<sub>2</sub> [2]. Our findings that Ang-(1–7) prevents activation of NOX activity in diabetic SHR underscores its beneficial effects in blocking pathogenic events of hypertension. Our study further illustrates that the observed effects of Ang-(1–7) on NOX activity are occurring at the transcriptional level, suggesting a role for Ang-(1–7) in postreceptor regulation of signaling events during hypertension. Extracellularly O<sub>2</sub> inactivates nitric oxide, whereas intracellularly it activates mitogen-activated protein kinases and leads to vascular smooth muscle cell hypertrophy [37]. Treatment with Ang-(1–7) led to correction of the altered vasoconstrictor responses to ET-1, NE or Ang II in the renal artery of SHR and diabetic SHR. The normalization of vascular reactivity by Ang-(1–7) may therefore be explained by increased nitric oxide synthesis by Ang-(1–7) that counteracts the downstream signaling of protein kinase C. Ang-(1–7) treatment resulted in a decrease in Ang II-induced vasoconstriction in diabetic SHR but not in SHR. This could be due to the fact that the degree of impairment is more pronounced in diabetic SHR. Interestingly, diabetes did not further reduce the already impaired vasodilator responses to carbachol and Ang-(1–7) in the SHR, in contrast to the impairment seen with diabetes in WKY. The long-term Ang-(1–7) treatment improved the dilator capacity to these two agents in both SHR and diabetic SHR. These observations are in agreement with previous studies where Ang-(1–7) was shown to dilate precontracted renal afferent arterioles and increase renal blood flow in rabbits, and to attenuate Ang II-induced pressor responses in the rat isolated kidney [38–41]. Vasodilator responses

to sodium nitroprusside were the same in all groups studied, indicating that the responsiveness of vascular smooth muscle cells to nitric oxide was not altered in diabetes. Further studies will be needed to see if the present observations in the renal artery are also true in resistance arteries.

## Conclusion

Our results suggest a renoprotective role of Ang-(1–7) during the simultaneous occurrence of hypertension and diabetes. As previously observed with ACEI and ARB [13–16], Ang-(1–7)-mediated improvements were primarily observed in the vascular and glomerular functions of the kidney. Endogenous Ang-(1–7) may contribute to the renoprotective actions of blockade of Ang II since ACEI and ARB increase renal ACE2 activity and Ang-(1–7) formation. The regulation of NADPH oxidases by Ang-(1–7) in conditions of diabetes and hypertension may provide a therapeutic target for controlling oxidative stress-mediated pathogenesis.

## Acknowledgments

This work was supported by Kuwait University Research Grant RM02/03 (I.F.B., M.H.M.Y.), and HL51952 (D.I.D., M.C.C.).

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