*Original Article*

# Vascular Angiotensin Type 1 Receptor Expression Is Associated with Vascular Dysfunction, Oxidative Stress and Inflammation in Fructose-Fed Rats

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**This study determined whether or not oxidative stress and vascular dysfunction in fructose-induced hyperinsulinemic rats are associated with activation of the vascular renin-angiotensin system (RAS). Four groups of rats were used. CONT rats were fed normal rat chow, CONT+CAP were fed normal rat chow and given 500 mg/L captopril in their drinking water, fructose-fed rats (FFR) were fed a high-fructose diet and FFR+CAP were fed the high-fructose diet plus captopril in water. After 8 weeks, the vascular reactivity of mesenteric artery segments was measured. Blood was analyzed for insulin, glucose, hydrogen peroxide and 8-isoprostane. Aortic and heart tissue were used for subjected to quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. Systolic blood pressure was significantly higher in FFR ($p<0.05$), and captopril treatment inhibited the blood pressure increase. Mesenteric artery dose-response curves to acetylcholine were shifted to the right in FFR ($p<0.05$) and were normal in FFR+CAP. Plasma insulin ($p<0.05$), hydrogen peroxide ($p<0.02$) and 8-isoprostane ($p<0.05$) were increased in FFR. Captopril treatment reducd hydrogen peroxide and 8-isoprostane concentrations. Aortic tissue mRNA expression levels were increased for angiotensin-converting enzyme (ACE, $p<0.05$), angiotensin type 1 receptor (AT1R, $p<0.02$), NOX4 ($p<0.02$) and VCAM-1 ($p<0.05$) in FFR aortic samples. Captopril treatment reduced AT1R, NOX4 and VCAM-1 expression in FFR to levels not different from CONT. Similar changes in heart tissue mRNA expression for angiotensinogen, AT1R and NOX4 were also observed. These results demonstrate that vascular RAS is upregulated in FFR and support the hypothesis that vascular RAS mediates vascular dysfunction and vascular oxidative stress in FFR. (*Hypertens Res* 2007; 30: 451–457)**

***Key Words*: angiotensin, hypertension, oxidative stress, vascular, insulin resistance**

## Introduction

Feeding normal rats a high-fructose diet induces insulin resistance, hypertension, dyslipidemia and vascular dysfunction (*1*, *2*). Although the fructose-fed rat (FFR) model exhibits many metabolic disorders, the cause of hypertension in these animals is not known. Other studies show that reactive oxygen species (ROS) are elevated in FFR (*3*–*5*) and can interfere with nitric oxide (NO) production, which maintains vascular relaxation in resistance arteries (*6*). Nitric oxide synthase (NOS) activity itself is also reduced in FFR aortas (*7*). Thus, a reduction of NOS activity and an increase of ROS production in FFR together could lead to decreased NO bioavailability, resulting in the increased vascular contraction and cardiovascular risk seen in this model of

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the metabolic syndrome.

Recent studies have shown that the renin-angiotensin system (RAS) may be involved in the vascular derangements that occur in patients with type 2 diabetes or the metabolic syndrome (*8*). Previous studies from our laboratory have shown that high insulin concentrations can stimulate the RAS and subsequent production of angiotensin II in cultured vascular smooth muscle cells (9, *10*). A fructose-fed rat study has shown that RAS inhibition with enalapril increases vascular endothelial nitric oxide synthase (eNOS) activity (*11*), and it can be argued that the angiotensin type 1 receptor (AT1R) is responsible for hypertension (*12*). However, it has yet to be demonstrated that gene expression of local vascular components of the RAS is increased in cardiovascular tissue from FFR, and this would be a necessary step in the implication of its role in hypertension. Elevated expression of the AT1R gene, but not that of any other RAS component, has been found in aortas of fructose-fed mice, but no heart tissue gene expression data are available (*4*). Further, it has yet to be shown that gene expression of components of oxidative stress and inflammation in FFR cardiovascular tissue can be inhibited by angiotensin-converting enzyme (ACE) inhibition. The present study was undertaken to determine whether or not hypertension, the production of vascular ROS and endothelial dysfunction in FFR are associated with increased expression of the cardiovascular RAS and whether or not any of these would form a mechanism for the induction of hypertension.

## Methods

### Animals

The animal protocols were approved by the Institutional Animal Care and Use Committee of the VA Greater Los Angeles Healthcare System, and the animals were housed in an AAALAC-approved animal research facility. Male CD:IGS rats (Charles River Laboratories, Wilmington, USA) weighing 250–300 g were divided into four groups. The control (CONT) and control with captopril (CONT+CAP) groups were fed normal rat chow (Purina, Richmond, USA). The FFR and fructose-fed with captopril (FFR+CAP) groups were fed a high-fructose diet (60% fructose by weight, Harlan-Teklad, Madison, USA). All rats were given regular drinking water for the first 4 weeks. The CONT+CAP and FFR+CAP were thereafter given drinking water containing 500 mg/L captopril (ICN Chemicals, Costa Mesa, USA). Systolic blood pressure was measured weekly using a tail-cuff method with an optical sensor (IITC, Woodland Hills, USA) as previously described (*2*, *13*). Measures were taken to reduce stress in the rats while their blood pressures were determined. To accustom the rats to the restraints used for blood pressure measurement, they were kept in regular shoebox cages with a 14 cm length of 10 cm diameter plastic pipe. The IITC system we use to measure blood pressure requires minimal heating of the animals (29°C). After 8 weeks on the diets, the animals were euthanized, and blood, heart, aorta and mesenteric artery samples were removed for study.

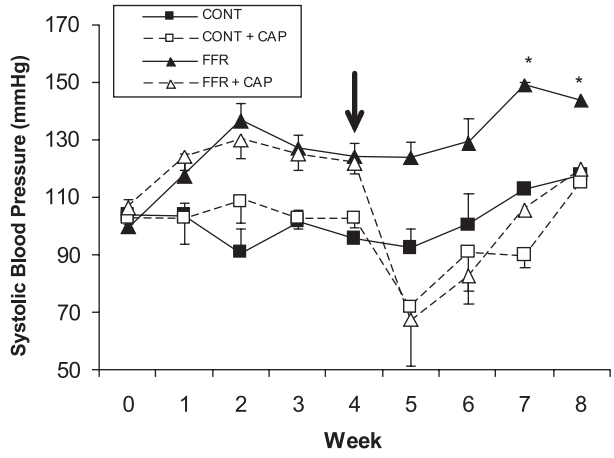


**Fig. 1.** *Systolic blood pressure changes during the 60% fructose vs. control dietary study in male CD:IGS rats (n = 6 in each group). Blood pressure increased in both FFR and FFR+CAP groups until week 4, when captopril treatment was initiated (indicated by arrow). At weeks 7 and 8, blood pressure was significantly lower in FFR+CAP than in FFR (*p < 0.05).*

### Vascular Reactivity Studies

Proximal sections of the duodenum and intact mesenteric vessel arcade were excised from euthanized rats and used as previously described (*13*). Briefly, segments of mesenteric resistance artery were transferred to a perfusion chamber (Living Systems, Burlington, USA). The perfusion chamber was mounted on an inverted TMS microscope (Nikon, Tokyo, Japan) and the video image of the superfused vessel was analyzed using a video imaging system (Living Systems). Lumen diameter measurements were recorded in response to serotonin or acetylcholine while the intraluminal pressure was maintained at 40 mmHg. The vessels were contracted with $1 \times 10^{-6}$ mol/L serotonin (Sigma Chemical, St. Louis, USA). After 5 min, acetylcholine (Sigma Chemical) was added to the reservoir in cumulative amounts at 2-min intervals.

### Plasma Analyses

Plasma glucose concentration was determined using a Glucose Analyzer 2 (Beckman Instruments, Fullerton, USA), and insulin concentration was determined by ELISA (Alpco Diagnostics, Windham, USA). 8-Isoprostane and aldosterone were determined in plasma using ELISA methods (Cayman Chemical, Ann Arbor, USA). Hydrogen peroxide in plasma samples was determined using an amplex red assay (Molecular Probes, Eugene, USA).

**Table 1. Plasma Concentrations of Metabolic and Oxidative Stress Parameters**

| | Glucose (mg/dL) | Insulin (ng/mL) | $H_2O_2$ (μmol/L) | 8-Isoprostane (pmol/L) |
|---|---|---|---|---|
| CONT | 395±38 | 1.56±0.32 | 7.78±0.65 | 78.47±11.73 |
| CONT+CAP | 340±19 | 2.24±0.23 | 9.13±0.68 | 71.66±16.27 |
| FFR | 458±19* | 2.87±0.32* | 23.34±4.37** | 112.6±7.29* |
| FFR+CAP | 445±44 | 2.72±0.67* | 12.96±1.26$^{\#}$ | 82.51±8.25$^{\#}$ |

CONT, control rats; CONT+CAP, control rats given captopril; FFR, fructose-fed rats; FFR+CAP, fructose-fed rats given captopril; $H_2O_2$, hydrogen peroxide. Values are means±SEM, $n=6$ per group. *$p<0.05$ *vs*. CONT; **$p<0.01$ *vs*. CONT; $^{\#}p<0.05$ *vs*. FFR.

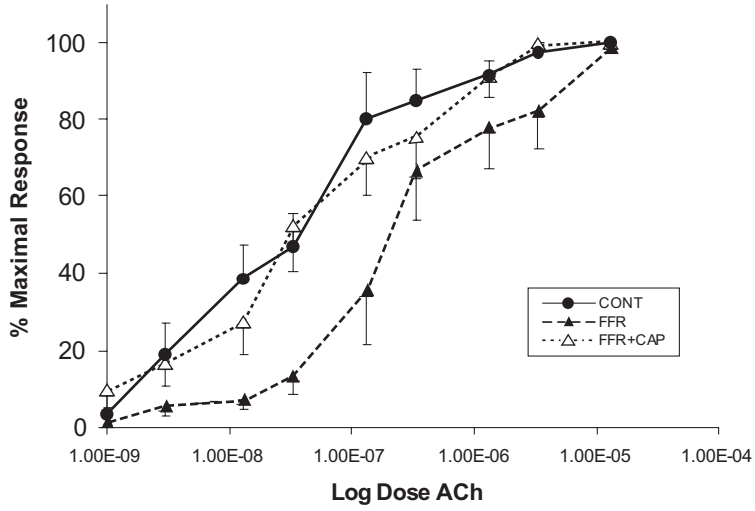


**Fig. 2.** *Cumulative dose-response curves to acetylcholine (ACh) in mesenteric artery segments taken from the different study groups. Segments were pre-contracted with serotonin and then exposed to increasing cumulative concentrations of ACh. The curve from FFR is shifted to the right ($p<0.05$ vs. CONT by two-way ANOVA). Treatment of FFR with captopril (FFR+CAP) prevented this shift to the right.*

### Quantitative Reverse Transcription–Polymerase Chain Reaction

Aorta and heart tissue samples for quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis were stored in RNA*later* (Ambion, Austin, USA) at −20°C. RNA was extracted from tissue samples using TRI Reagent (Sigma Chemical) and reverse-transcribed using an Omniscript RT kit (Qiagen, Valencia, USA). TaqMan Gene expression assay kits (catalog numbers are in parentheses) for angiotensinogen (AGT, Rn00593114), ACE (Rn00561094), AT1R (Rn00578456), angiotensin type 2 receptor (AT2R, Rn00560677), NOX4 (Rn00585380), VCAM-1 (Rn00563627) and GAPDH (Rn99999916) were purchased from Applied Biosystems (Foster City, USA). The gene expression assay kits were used in conjunction with Universal Master Mix (Applied Biosystems) in a 7300 Real-Time PCR System (Applied Biosystems). Standard curves were constructed for GAPDH as an internal standard and for each gene of interest. Gene expression was normalized to the GAPDH expression measured in each sample and expressed as fold increases or decreases from control values.

### Statistics

Values shown are means±SEM. Student's *t*-test was used to determine the significance of differences between groups. Two-way ANOVA was used to determine the significance of differences in the dose-response curves. $p<0.05$ indicated a significant difference.

## Results

During the 8-week study, animal weight did not differ among the four treatment groups. The high-fructose diet caused a significant increase in systolic blood pressure within the first 2 weeks in both the FFR and FFR+CAP groups. Captopril treatment lowered the SBP in both the CONT and FFR (Fig. 1). The maximum reduction in blood pressure with captopril occurred at week 5. At the end of 8 weeks, the blood pressure returned to normal in both CONT+CAP and FFR+CAP (Fig. 1).

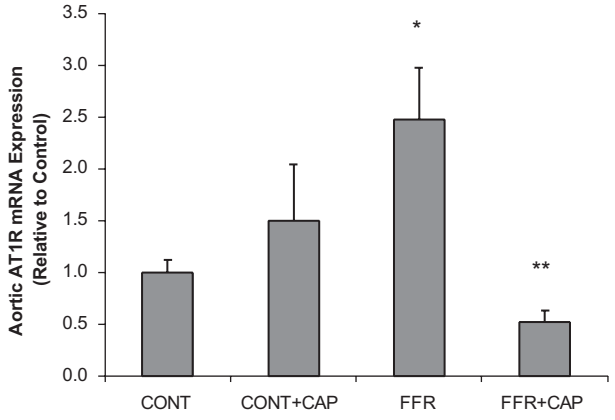


**Fig. 3.** *Angiotensin type 1 receptor (AT1R) mRNA expression in aortic tissue samples measured by qRT-PCR. Captopril did not significantly change AT1R expression in control rats (CONT+CAP). However, AT1R expression was increased significantly in FFR. Treatment of FFR with captopril (FFR+CAP) prevented the increase in AT1R mRNA expression. *$p<0.05$ vs. CONT, **$p<0.05$ vs. FFR. Each bar represents mean±SEM of 6 samples.*

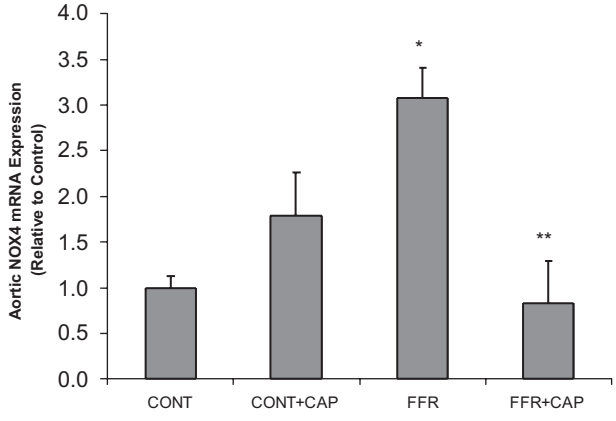


**Fig. 4.** *Aortic NOX4 mRNA expression measured by qRT-PCR. Aortic NOX4 mRNA expression was increased significantly in FFR. This increase was prevented by captopril treatment (FFR+CAP). *$p<0.05$ vs. CONT; **$p<0.05$ vs. FFR. Each bar represents mean±SEM of 6 samples.*

Fructose feeding significantly elevated plasma insulin concentrations, suggesting the induction of insulin resistance (Table 1). RAS blockade with captopril did not alter this increase in insulin. However, plasma indicators of oxidative stress, both hydrogen peroxide and 8-isoprostane, were increased in FFR, and were significantly returned to near normal by RAS blockade with captopril (Table 1). There was no difference in plasma aldosterone among the groups.

Vascular reactivity of mesenteric artery segments to acetylcholine was decreased in FFR vessels. However, the defective vascular reactivity returned to almost control levels by the addition of captopril. Thus, the acetylcholine dose-response curve was significantly shifted to the right in FFR (Fig. 2, $p<0.05$ *vs*. CONT). Captopril treatment (FFR+CAP) returned the dose-response curve toward the left ($p<0.05$ *vs*. FFR), and was not significantly different from CONT. Captopril had no effect on acetylcholine dose response in the CONT+CAP (data not shown).

Quantitative RT-PCR from aortic RNA extracts revealed that mRNA expression for ACE was increased 1.45±0.13-fold over CONT ($p<0.05$). Figure 3 shows that AT1R mRNA expression was also significantly increased in FFR aortas. Captopril treatment prevented the increase in aortic AT1R mRNA expression (Fig. 3) but did not change ACE mRNA expression (data not shown). Gene expression for NOX4 (Fig. 4) and VCAM-1 (Fig. 5) was also increased in FFR aortas. These increases were prevented in the captopril-treated FFR. In heart tissue, AGT mRNA expression was increased (2.6±0.79-fold over control, $p=0.069$) and AT1R mRNA expression and NOX4 mRNA expression were also increased in the FFR (Figs. 6 and 7, respectively). The increased expression of AT1R and NOX4 were attenuated by RAS blockade (Figs. 6 and 7, respectively). In FFR, captopril treatment did not affect AT2R mRNA expression. When administered to control diet animals, captopril caused insignificant but consistent increases in both aorta and heart AT1R and NOX4 (Figs. 3, 4, 6, 7).

## Discussion

The current study shows that the vascular RAS is upregulated *in vivo* in a rat model of the metabolic syndrome, the FFR, which is characterized by hypertension, decreased glucose tolerance, hypertriglyceridemia and hyperinsulinemia. Stimulation of the vascular tissue RAS is demonstrated by increased mRNA expression for ACE and AT1R in FFR aortic tissue. The aorta is composed of different tissue types, and our results do not distinguish which cell type or types contributed to this increase. Cardiac tissue RAS was also upregulated in the FFR, as shown by increased AGT and AT1R gene expression. These results suggest a generalized increase of vascular tissue RAS expression in this model of fructose-induced hypertension and insulin resistance. The observation that aortic AT1R mRNA expression is increased in FFR confirms a previous study of fructose-fed mice (*5*). However, that previous study did not measure ACE or AGT expression, nor did it measure RAS gene expression in heart tissue.

As these gene expression changes were also associated with increased blood pressure and impaired vascular relaxation in response to acetylcholine, an ACE inhibitor could be expected to significantly reduce AT1R expression reduce blood pressure and restore arterial vasodilatory response to acetylcholine. These results demonstrate that, in the FFR model of insulin resistance, it may be the upregulation of the vascular tissue AT1R that mostly contributes to hypertension

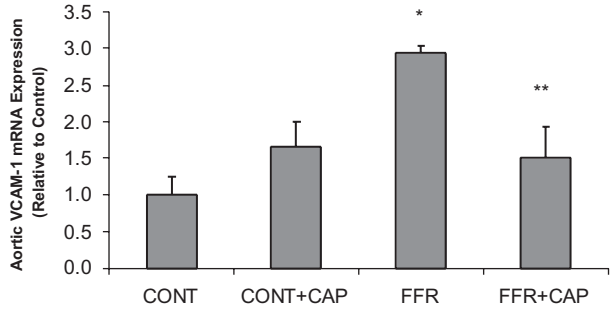


**Fig. 5.** *Aortic VCAM-1 mRNA expression measured by qRT-PCR. Aortic VCAM-1 expression was significantly upregulated in FFR. Captopril prevented this upregulation. *p < 0.05 vs. CONT; **p < 0.05 vs. FFR. Each bar represents mean ± SEM of 6 samples.*

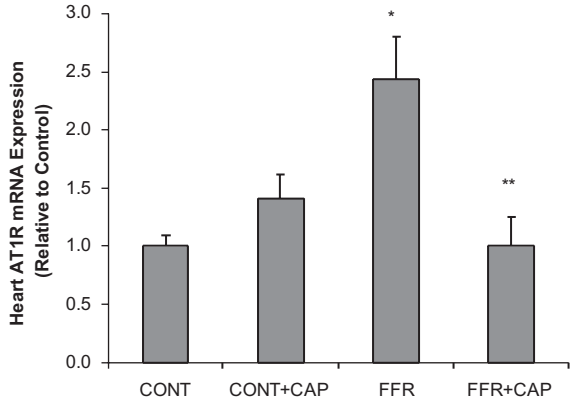


**Fig. 6.** *Heart tissue AT1R mRNA expression measured by qRT-PCR. Heart AT1R mRNA expression was upregulated in FFR. Captopril treatment prevented this increased expression. *p < 0.05 vs. CONT; **p < 0.05 vs. FFR. Each bar represents mean ± SEM of 6 samples.*

and vascular dysfunction. In the present study, captopril insignificantly increased AT1R gene expression in both aorta and heart. The mechanism underlying this increase is not known, but it may be a compensatory increase to help maintain blood pressure under these conditions. Blood pressure's dependence on the functional interaction of vascular AT1R and AT2R has been demonstrated in FFR, yet that study was indirect in that they used AT1R blockers rather than gene expression to show effects on blood pressure these rats (*12*). Increased expression of renal AT1R has also been associated with hyperinsulinemia in the Zucker obese rat, another model of insulin resistance (*14*). Thus, a common mechanism exists whereby insulin resistance can be linked to increased AT1R expression as a potential mechanism for hypertension in these insulin-resistant models. However, since plasma triglyceride concentrations are also increased in these models, this and other lipid factors must also be considered as a potential mechanism for the increased expression of AT1R.

A recent publication has shown that blood pressure, when measured by radiotelemetry, does not increase in FFR and that the apparent increase of blood pressure observed using tail-cuff methods, such as we use, may be an artifact arising from the stress of using the animal restraint (*15*). In the present study, efforts were made to minimize stress during the blood pressure measurements. However, it remains possible that some of the differences in blood pressure that we observed may be due to stress-induced mechanisms that induced higher systolic blood pressure in the FFR. Captopril was able to prevent this stress-induced hypertension, implying that the RAS is responsible for a component of this blood pressure increase. Also, since captopril can increase bradykinin concentrations by inhibiting ACE, the possibility that some of the blood pressure changes were due to increases in bradykinin must also be considered. However, previous studies have demonstrated that increased bradykinin is not responsible for the blood pressure–reducing effects of ACE inhibitors in FFR (*16*, *17*).

The current study also demonstrates a potential mechanism whereby increased vascular RAS expression is associated with increased expression of the vascular NADPH oxidase subunit, NOX4, and increased expression of VCAM-1. Others have shown that vascular NOX4 can be upregulated by angiotensin II *in vitro*, or by increased expression of renin *in vivo* (*18*). Shinozaki *et al*. showed that aortic homogenates from FFR produced more superoxide than those from CONT, and that this could be inhibited with AT1R blockade (*5*). Also, this same group showed that fructose-fed mice expressed more NADPH oxidase subunit protein in their aortas (*5*). It has also been shown that AT1R blockade and ACEI can prevent oxidative stress in injury-elicited neointima formation (*19*). In the present study, the increased gene expression of the NADPH oxidase subunit, NOX4, is associated with increased plasma indicators of oxidative stress. Since RAS blockade prevented the increase of NOX4 mRNA expression, plasma hydrogen peroxide and plasma 8-isoprostane, oxidative stress as seen in FFR may be mediated by the vascular RAS. Thus, in conditions of high tissue RAS activity, NOX4 upregulation and increased generation of reactive oxygen species can occur, subsequently stimulating VCAM-1 expression and factors that enhance inflammation and atherosclerosis. Insulin resistance has been shown to be a risk factor in the progression of atherosclerotic disease (*20*). The blockade of vascular NO production has also been reported to increase VCAM-1 expression in the vascular walls of rats through an RAS-dependent mechanism (*21*). Other studies have shown that angiotensin II stimulates VCAM-1 production in cultured vascular endothelial cells (*22*). Along the same lines, the ability of calcium channel blockers to prevent increased expression of aortic VCAM-1 in $N^G$-nitro-L-arginine methyl ester (L-NAME)–induced hypertension has been shown to be attributable to a reduction of ACE expression in

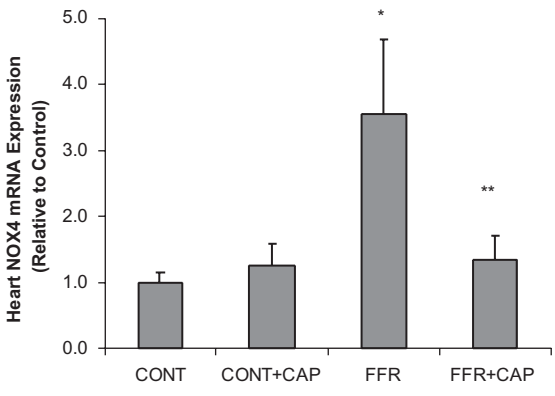


**Fig. 7.** *Heart tissue NOX4 mRNA expression measured by qRT-PCR. Heart NOX4 mRNA expression was increased in FFR. Captopril treatment prevented this increased expression. *$p < 0.05$ vs. CONT; **$p < 0.05$ vs. FFR. Each bar represents mean ± SEM of 6 samples.*

the aorta (*23*).

Our results do not eliminate the possibility that the vascular RAS, NOX4 and VCAM-1 were affected by increases in or inhibition of the systemic RAS. However, it has been shown that plasma angiotensin II in FFR is not elevated after 4 weeks of fructose feeding, nor is plasma renin activity increased at 4 or 7 weeks of fructose feeding (*24*, *25*). Since we studied our rats at 8 weeks of fructose feeding, we assume that plasma angiotensin II had normalized and was not a factor. This reduces the likelihood that the tissue changes in gene expression we observed were due to systemic RAS stimulation.

Thus, the present study provides substantive evidence that the vascular RAS mediates the cardiovascular changes leading to inflammation in the FFR model. Further proof of this relationship is the demonstration that many of the effects of insulin resistance in this model can be prevented by RAS inhibition using the ACE inhibitor captopril. Thus, our results further enhance the concept that RAS inhibitors attenuate hypertension and vascular damage through additional mechanisms that reduce inflammation, which is often seen in conditions accompanying insulin resistance.

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