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Unchanged cardiac angiotensin II levels accompany losartan-sensitive cardiac injury due to nitric oxide synthase inhibition

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Abstract

Chronic nitric oxide synthase (NOS) inhibition results in hypertension and myocardial injury. In a rapid and severe model of chronic NOS inhibition, we determined the role of angiotensin II in these effects by using angiotensin II receptor blockade and by measuring cardiac angiotensin II concentrations before and during development of cardiac damage. Rats received either no treatment, the NOS inhibitor *N*ω-nitro-L-arginine (L-NNA; 500 mg/l), the angiotensin AT_1 receptor antagonist losartan (400 mg/kg chow), or L-NNA plus losartan for 21 days. In the second protocol, five groups of rats received L-NNA (500 mg/l) for 0, 4, 7, 14 and 21 days, respectively. L-NNA increased systolic blood pressure (SBP) (227 ± 8 versus 143 ± 6 mm Hg; $P < 0.01$), heart weight index (0.44 ± 0.02 versus 0.32 ± 0.01; $P < 0.01$) and induced coronary vasculitis and myocardial necrosis. Co-treatment with losartan prevented all changes. L-NNA during 4 days decreased cardiac angiotensin II (23 ± 4 versus 61 ± 15 fmol/g; $P < 0.05$). Although after 7 days, fresh infarcts and after 14 days organized infarcts were present, cardiac angiotensin II was only slightly increased after 21 days (100 ± 10 fmol/g; $P < 0.05$). In conclusion, losartan-sensitive cardiac damage due to chronic NOS inhibition is not associated with primary increase of cardiac angiotensin II, suggesting that chronic NOS inhibition increases cardiac sensitivity for angiotensin II.

Keywords: Nitric oxide synthase inhibition; Myocardial infarct; Angiotensin II cardiac; Angiotensin AT_1 receptor antagonist

1. Introduction

Endothelium-derived nitric oxide (NO) plays an important role in the regulation of cardiovascular structure and function. Inhibition of NO production causes hypertension (Baylis et al., 1992; Ribeiro et al., 1992), structural vascular changes (Baylis et al., 1992; Ribeiro et al., 1992; Numaguchi et al., 1995; Arnal et al., 1992), and a reduction in coronary artery flow and cardiac output (Amrani et al., 1992; Kassab et al., 1998). This is accompanied by myocardial lesions (Numaguchi et al., 1995; Xu et al., 1995; Moreno et al., 1996). Chronic co-treatment of nitric oxide synthase (NOS) inhibitors with angiotensin converting enzyme inhibitors or angiotensin AT_1 receptor antagonists in rats prevents hypertension (Ribeiro et al., 1992; Pollock et al., 1993; Jover et al., 1993; Hropot et al., 1994; Michel et al., 1996) and coronary vascular and myocardial remodeling (Takemoto et al., 1997a,b; Luvar et al., 1998) suggesting an important role for angiotensin II in the pathogenesis of hypertension and cardiac morphological changes induced by chronic NOS inhibition. However, this does not seem to relate to circulating renin levels, which have been reported to be increased (Ribeiro et al., 1992), decreased (Arnal et al., 1992; Pollock et al., 1993; Navarro-Cid et al., 1994), unchanged (Jover et al., 1993; Michel et al., 1996) or variable (Qiu et al., 1998) after chronic NOS inhibition.

These contradictory findings suggest that the local rather than the circulating renin–angiotensin system plays a major role in the cardiac effects of chronic NOS inhibition. Indeed, it has been shown that all the components of the renin–angiotensin system are present in the heart (Baker et al., 1992), and that cardiac angiotensin converting enzyme

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(Takemoto et al., 1997b; Arnal et al., 1992) as well as angiotensin II receptors (Katoh et al., 1998) are upregulated during chronic NOS inhibition. However, it is not known whether cardiac angiotensin II concentrations are actually increased during chronic NOS inhibition.

Our first aim was to study the effects on the heart in a rapid and severe model of chronic NOS inhibition (Verhagen et al., 1998), and to determine the role of angiotensin II in these effects. For these purposes, we used high doses of the potent NOS inhibitor *N*ω-nitro-L-arginine (L-NNA) and losartan, a selective angiotensin type 1 (AT_1) receptor antagonist, respectively. The second aim was to get more insight into the angiotensin II-dependence of the cardiac effects in this model by measuring cardiac angiotensin II concentrations before and during the development of cardiac damage.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (180–210 g; Harlan-Olac, UK) were conventionally housed, received standard natural chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and had free access to tap water unless specified otherwise. Sentinel animals, monitored regularly for infection by nematodes and pathogenic bacteria, as well as antibodies to a large number of rodent viral pathogens (ICLAS, Nijmegen, The Netherlands), tested negative for infection throughout the course of the experiment. The protocol was approved by the Utrecht University Board for studies in experimental animals and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Protocol 1: cardiac morphology

Five groups of rats were studied. The first group ($n = 8$) received regular chow and water. The second group ($n = 9$) received the NOS inhibitor L-NNA (Sigma, USA) dissolved in drinking water (500 mg/l) for 21 days. This resulted in an L-NNA intake of approximately 40 mg/kg/day. Previously, we found that this dose resulted in a maximum increase in systolic blood pressure (SBP) (Verhagen et al., 1998). The third group ($n = 6$) was treated with the angiotensin AT_1 receptor antagonist losartan mixed with finely ground chow (400 mg/kg chow) during 21 days. This resulted in an intake of approximately 30 mg/kg/day losartan. The fourth group ($n = 8$) was treated with L-NNA (500 mg/l) together with losartan (400 mg/kg chow) during 21 days. This resulted in an intake of approximately 40 mg/kg/day L-NNA and 30 mg/kg/day losartan. Losartan was kindly provided by R.D. Smith, PhD (DuPont-Merck Pharmaceuticals). To determine whether the observed morphological effects were related to NOS inhibition in general or specifically to L-NNA, a fifth group ($n = 6$) received *N*ω-nitro-L-arginine methyl ester (L-NAME) (Sigma) (5 g/kg chow) for 21 days. This resulted in an L-NAME intake of approximately 400 mg/kg/day. In a preliminary experiment, treatment with this dose of L-NAME resulted in a similar increase in blood pressure as observed with L-NNA (500 mg/l). At the end of the treatment periods, SBP was measured in the awake rats by the tail-cuff method (IITC, San Diego, USA). The rats were weighed and anaesthetized with pentobarbital 60 mg/kg i.p. The hearts were rapidly excised, dotted free of blood and weighed. The heart weight index was calculated as heart weight/body weight × 100. The hearts were processed for histology and immunohistochemistry.

2.3. Protocol 2: cardiac Ang II concentrations

Five groups of rats were studied (each $n = 12$). The first group received tap water (control). Groups 2, 3, 4 and 5 received L-NNA (500 mg/l) for 4, 7, 14 and 21 days, respectively. At the end of the treatment periods, SBP was measured, rats were anaesthetized and the heart weight index was determined. Hearts of 6 rats/group were processed for angiotensin II measurement. Hearts of the remaining rats (6/group) were processed for histology and immunohistochemistry.

2.4. Morphologic studies

From each group, one or two hearts were serially sliced from base to apex and stained by hematoxylin-eosin. The remaining hearts were cut into 1 mm slices that were either immersion-fixed in phosphate-buffered saline formaldehyde (4%, pH 7.35) and embedded in paraffin or immersion-fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) for 24 h, stored in 70% ethanol, and subsequently embedded in paraffin. Light microscopy was done on 3 μm sections of the formaldehyde-fixed tissue stained by hematoxylin-eosin or goldner trichrome elastica stain. The infarcts that were observed were characterized as fresh or organized, depending on the presence of granulation tissue that had replaced the necrotic myocardium. The extent of infarction in the right and left ventricle was determined as transmural (3 points), intramural (2 points) or microscopic (1 point) and the total extent of infarction was calculated as the sum of all infarcts. The number of vessels with perivascular infiltration and the number of necrotic vessels per section were also counted. Immunhistochemistry was carried out on 3 μm sections of methacarn-fixed tissue. Monocytes/macrophages were detected by the mouse monoclonal antibody ED-1 using the alkaline phosphatase anti-alkaline phosphatase detection method (Stojanovic et al., 1996).

2.5. Measurement of angiotensin II

The hearts were homogenized in 10 ml 95% pre-chilled methanol. The homogenates were centrifuged immediately for 10 min at 2500 rpm at 4°C and the supernatants were stored at −20°C. Heart angiotensin II was extracted by solid phase chromatography and determined by radioimmunoassay (RIA) according to the procedure of Fox et al. (1992), as described previously in more detail (Boer et al., 1997). The recovery of angiotensin II added prior to

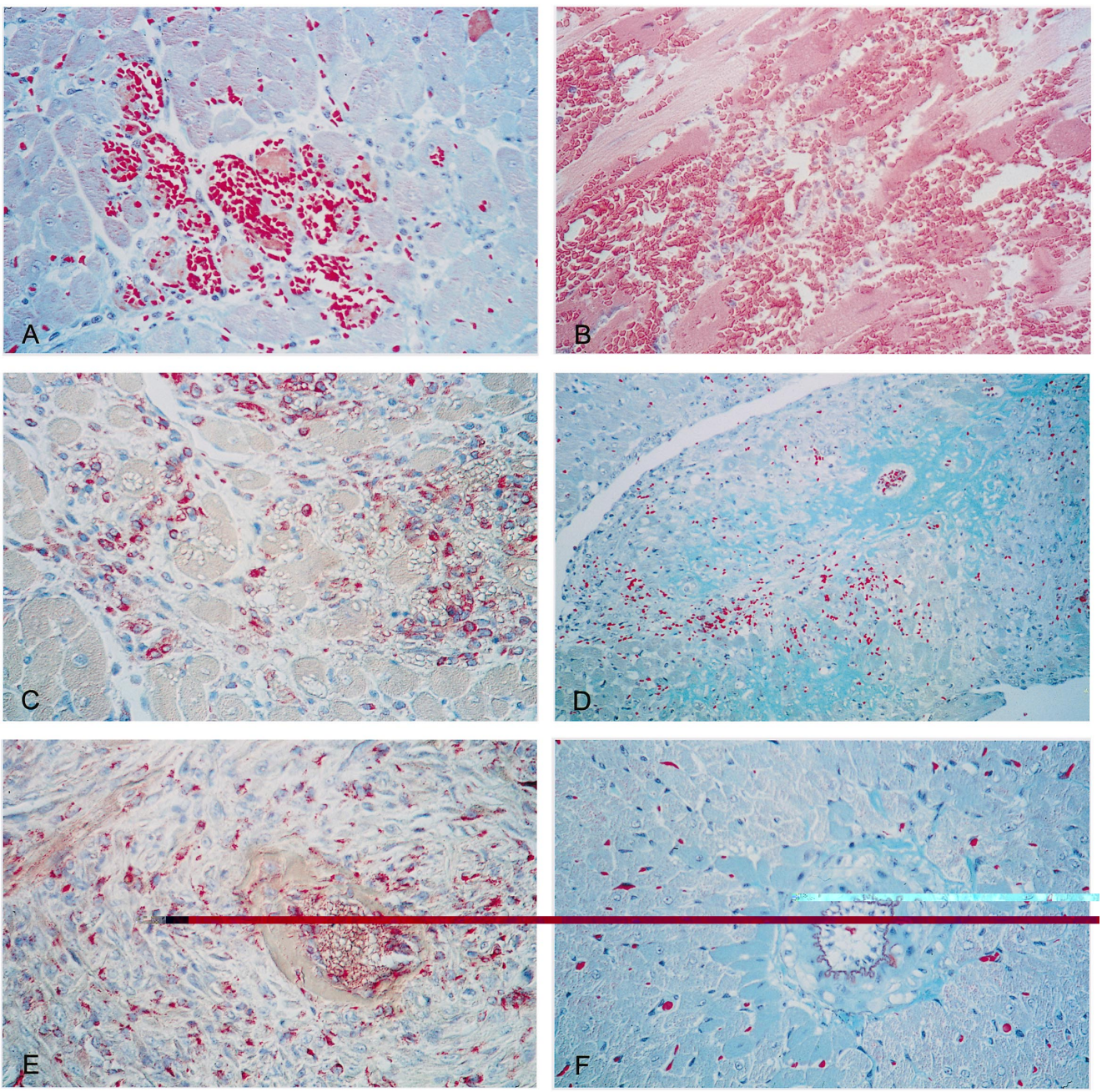

Fig. 1. Myocardial infarcts at different stages of organization after treatment of rats with L-NNA (500 mg/l; A, C, D, E) or L-NAME (5 g/kg chow; B) during 21 days. Prevention by co-treatment of L-NNA with losartan (400 mg/kg chow; F). (A) A small focus of hemorrhage into the myocardium, almost without participation of mononuclear cells; a few necrotic cardiomyocytes within the center of hemorrhage. (B) Large transmural area of hemorrhagic necrosis with necrotic cardiomyocytes without cross striations and without nuclei. (C) In hemorrhagic necrotic areas, monocytes/macrophages constitute the majority of infiltrating cells. (D) Infarct area with granulation and scar tissue. (E) In a fully developed lesion, monocytes/macrophages could be seen in the endothelium and media of necrotic vessels, and prominently in the surrounding granulation tissue that had replaced the necrotic myocardium. (F) Co-treatment with losartan prevented the arteritis, capillaritis and infarcts. The small intramural artery has a normal appearance, there is no edema of the interstitium. Cardiomyocytes are closely situated next to each other without interstitial mononuclear infiltration. (A, D, F) goldner trichrome elastica stain; (B) hematoxylin–eosin stain; (C, E) ED-1 staining. (A–F) magnification 620×.

extraction (40 fmol) amounted to 76%. The sensitivity of the assay was 0.5 fmol/assay tube. Angiotensin II concentrations were corrected for blank values (generally < 0.5 fmol) and procedural losses. A specific binding of radiolabeled angiotensin II in the RIA was less than 2%, and the initial binding was 30–35%. The cross-reaction of the angiotensin II antibody with angiotensin I was less than 1%.

2.6. Statistical methods

Results are expressed as mean ± S.E.M. (quantitative data) or median and range (semiquantitative morphological data). Quantitative data were analysed by two-way analysis of variance (ANOVA) for protocol 1 or by one-way ANOVA for protocol 2. Semiquantitative data were tested by the nonparametric Kruskal–Wallis ANOVA. If the variance ratio (F) reached statistical significance ($P < 0.05$), differences between groups were analysed with the Student–Newman–Keuls test for multiple comparisons (quantitative data), or with Dunn's test for multiple comparisons (semiquantitative data).

3. Results

3.1. Protocol 1: cardiac morphology

3.1.1. Blood pressure and heart weight index

Treatment of rats with L-NNA during 21 days resulted in an increase in SBP (227 ± 8 versus 143 ± 6 mm Hg in control; $P < 0.01$) and an increase in heart weight index (0.44 ± 0.02 versus 0.32 ± 0.01 in control; $P < 0.01$). Treatment of rats with losartan during 21 days resulted in a SBP of 128 ± 4 mm Hg and a heart weight index of 0.29 ± 0.004. These were not significantly different from control. Treatment of rats with L-NNA + losartan during 21 days resulted in a SBP of 159 ± 7 mm Hg. This was not significantly different from control, although it was significantly higher than SBP in rats treated with losartan alone ($P < 0.05$). Heart weight index of rats treated with L-NNA + losartan (0.29 ± 0.01) was not significantly different from heart weight index of control rats or rats treated with losartan alone. Treatment of rats with L-NAME (5 g/kg chow) during 21 days resulted in a SBP (216 ± 7 mm Hg) that was not different from L-NNA (500 mg/l).

3.1.2. Cardiac morphology

After 21 days of L-NNA treatment, 89% (8/9) of the rats showed myocardial infarcts at different stages. Fresh myocardial infarcts with and without hemorrhage as well as infarcts in different stages of organization with granulation and scar tissue were observed (Fig. 1; Table 1). Cardiomyocytes showed loss of nuclei and homogenous cell cytoplasm with loss of striation (Fig. 1B). Intramyocardial arteries and arterioles showed focal swelling of endothelial cells and adherent mononuclear cells (Fig. 2A). In some vessels, intimal cellular infiltration partly occluded the arterial lumen (Fig. 2B). Advanced lesions demonstrated infiltration of the vascular wall by monocytes/macrophages (Fig. 2C) and focal fibrinoid necrosis of the artery (Fig. 2D; Table 1). Serial sections of whole heart did not reveal thrombosis of epicardial arteries. Venules, like small intramural arteries, demonstrated mononuclear cells arranged along and beneath the endothelium (Fig. 2E). In some venules and capillaries, breaks of the wall with variably large hemorrhage into the surrounding interstitium and myocardium were observed (Fig. 2E). In arteries affected by transmural inflammation, monocytes/macrophages were observed in all layers of the vascular wall (Fig. 2C) and prominently in the surrounding granulation tissue that had supplemented the necrotic my-

Table 1
Semiquantitative evaluation of cardiac histology in control rats and rats treated with L-NNA (500 mg/l), losartan (400 mg/kg chow) or L-NNA + losartan for 21 days

	Infarct					Vessel			
	N	Fresh	*n*	Organized	*n*	Periv. Inf.	*n*	Necrosis	*n*
Left ventricle									
Control	8	0 (0–0)	0	0 (0–1)	1	0 (0–0)	0	0 (0–0)	0
L-NNA	9	1 (0–5)[a]	5	5 (0–11)[a]	8	0 (0–3)[a]	4	2 (0–12)[a]	5
Losartan	6	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0
L-NNA + losartan	8	0 (0–0)[b]	0	0 (0–4)[b]	1	0 (0–0)[b]	0	0 (0–0)[b]	0
Right ventricle									
Control	8	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0
L-NNA	7[c]	0 (0–2)	2	8 (3–12)[a]	7	0 (0–2)	2	1 (0–12)[a]	6
Losartan	6	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0	0 (0–0)	0
L-NNA + losartan	8	0 (0–0)	0	0 (0–1)[b]	1	0 (0–0)	0	0 (0–0)[b]	0

Data are expressed as median (range). *N*: number of rats; *n*: number of affected rats; Periv. Inf.: perivascular infiltration.
[a] $P < 0.05$ versus control.
[b] $P < 0.05$ versus L-NNA.
[c] Right ventricle only sectioned in 7 out of 9 rats in this group.

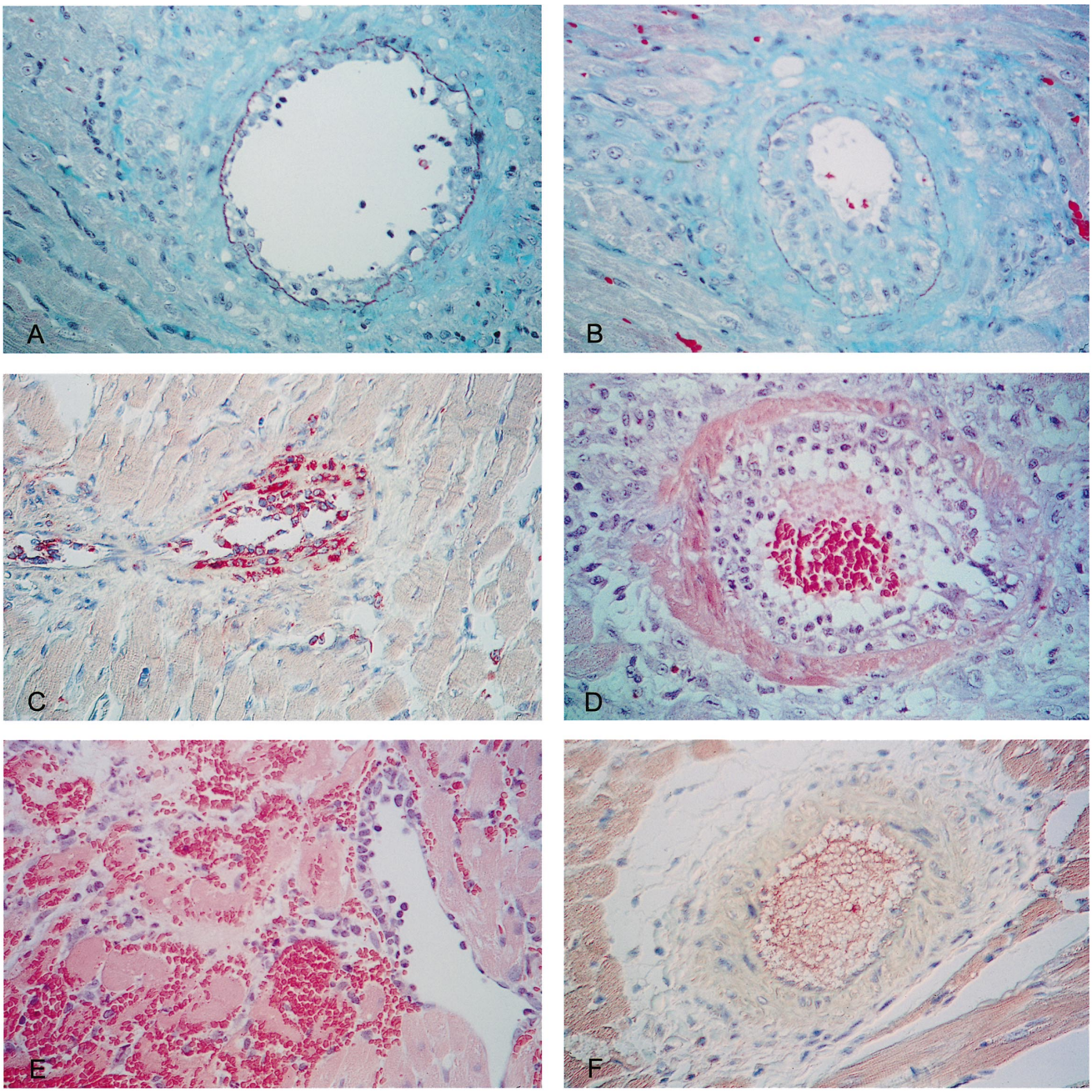

Fig. 2. Inflammation of cardiac intramural arteries, capillaries and venules after treatment with L-NNA (500 mg/l; A–E) during 21 days. Prevention by co-treatment with losartan (400 mg/kg chow; F). (A) Artery with intact lamina elastica interna and vacuolated endothelium infiltrated by mononuclear cells. (B) In a later stage pronounced broadening of the intima with luminal obstruction; lamina elastica interna focally ruptured. Mononuclear cells were also present in the periadventitial space. Viable cardiomyocytes can be observed at the periphery of the photograph. (C) Monocytes/macrophages attached to endothelium and infiltrated in the media of a small intramyocardial artery. (D) In its most severe form, the intramural arteritis was characterized by pronounced infiltration of intima by mononuclear cells. Fibrinoid necrosis of media, that was transgressed by mononuclear cells. The latter could also be found in high density in the periadventitial area. (E) In capillaries and venules, the endothelium was covered and infiltrated by many mononuclear cells; breaks of the vessel wall led to hemorrhage into the surrounding myocardium. (F) During simultaneous administration of L-NNA and losartan, only a few monocytes/macrophages could be seen within the blood-filled lumen of an intramural artery. No monocytes/macrophages were observed in the intima, media or adventitia of the vessel, or in the surrounding myocardium. (A, B) goldner trichrome elastica stain; (D, E) hematoxylin–eosin stain; (C, F) ED-1 staining. (A–C, E) magnification 620×; (D) magnification 200×; (F) magnification 400×.

ocardium (Fig. 1E). In hemorrhagic necrotic areas, monocytes/macrophages constituted the majority of infiltrating mononuclear cells (Fig. 1C). Treatment with losartan alone had no effect on cardiac morphology (Table 1). Losartan given concomitantly with L-NNA completely prevented vasculitis and myocardial infarcts (Table 1; Fig. 1F); only

Table 2
SBP, heart weight index (HWI) (% of body weight) and cardiac angiotensin II concentrations (fmol/g) in rats treated with L-NNA (500 mg/l) for 0, 4, 7, 14 or 21 days

L-NNA (days)	0	4	7	14	21
SBP (mm Hg)	135 ± 4	161 ± 4[a]	192 ± 6[b]	220 ± 6[b]	230 ± 9[b]
HWI (%)	0.34 ± 0.01	0.34 ± 0.01	0.32 ± 0.01	0.45 ± 0.03[b]	0.44 ± 0.02[b]
angiotensin II (fmol/g)	61 ± 15	23 ± 4[a]	90 ± 12	46 ± 10	100 ± 10[a]

Data are expressed as mean ± S.E.M.
[a] $P < 0.05$ versus 0 days.
[b] $P < 0.01$ versus 0 days.

very few monocytes/macrophages could be detected (Fig. 2F). All rats treated with L-NAME showed similar lesions in intramural vessels and the myocardium as described for L-NNA (Fig. 1B).

3.2. Protocol 2: cardiac angiotensin II concentrations

3.2.1. Blood pressure, heart weight index and angiotensin II concentrations

L-NNA treatment resulted in an increase in SBP from day 4 (161 ± 4 versus 135 ± 4 mm Hg in control; $P < 0.05$). Heart weight index was increased from day 14 (0.45 ± 0.03 versus 0.34 ± 0.01 in control; $P < 0.01$). L-NNA treatment during 4 days resulted in a decrease in cardiac angiotensin II (23 ± 4 versus 61 ± 15 fmol/g in control; $P < 0.05$). After 7 and 14 days of NOS inhibition, cardiac angiotensin II was not significantly different from control. After 21 days of NOS inhibition, cardiac angiotensin II was slightly increased when compared to control (100 ± 10 fmol/g; $P < 0.05$) (Table 2).

3.2.2. Cardiac morphology

After 4 days of NOS inhibition, there were no significant cardiac morphological changes. The only difference as compared to control was the adhesion of mononuclear cells to the activated endothelium. After 7 days of NOS inhibition, fresh infarcts with hemorrhage, anemic infarcts as well as vasculitis were noticed. With increasing duration of NOS inhibitor therapy (14 and 21 days), the extent and number of infarcts rose. At later time points (14 and 21 days) fresh infarcts and infarcts in different stages of organization were present.

4. Discussion

This study shows that angiotensin AT_1 receptor blockade could prevent myocardial infarcts and coronary vasculitis in a rapid and severe model of chronic NOS inhibition. Although this suggests a major role for angiotensin II in the pathogenesis of chronic NOS inhibition, this study demonstrates for the first time that cardiac angiotensin II concentrations were not increased before the occurrence of hypertension and cardiac injury. These findings strongly suggest that during chronic NOS inhibition, the sensitivity for angiotensin II is increased in the heart.

Treatment with the potent NOS inhibitor L-NNA for 21 days resulted in hypertension, cardiac hypertrophy, vasculitis and myocardial infarcts. Treatment with this dose L-NNA resulted in the extremely rapid development of severe hypertension, in a pattern similar to that observed previously (Verhagen et al., 1998). It is conceivable that such severe hypertension accounts for the more pronounced cardiac hypertrophy observed in this study when compared to other studies with chronic NOS inhibition (Arnal et al., 1992, 1993). However, cardiac hypertrophy observed after NOS inhibition seems to be related to a disturbed balance between angiotensin II and NO and not to hypertension, because it has been shown that co-treatment with hydralazine, which normalized blood pressure, could not prevent hypertrophy. Furthermore, co-treatment with low dose angiotensin converting enzyme inhibitor that did not affect blood pressure could reduce cardiac hypertrophy (Takemoto et al., 1997b).

Damage to the endothelium was a prominent feature of NOS inhibition, independent of the type of vessel. Specifically, the loss of endothelial integrity in intramural venules and capillaries apparently was instrumental for hemorrhage into and consecutive necrosis of the myocardium. Mononuclear cells were frequently observed in hemorrhagic necrotic areas, as has been reported by others (Tomita et al., 1998). The myocardial infarcts were partly due to rupture of capillaries and veins and also due to occluding fibrocellular broadening of the intima and thrombosis in intramural arteries which showed inflammation of the whole vascular wall. Thrombosis of epicardial arteries, a common cause of myocardial infarcts, was not observed. It has been shown that blockade of NO synthesis resulted in an increase in myocardial oxygen consumption (Bernstein et al., 1996). This increased oxygen demand in combination with a severe reduction in coronary flow (Amrani et al., 1992; Kassab et al., 1998; Moreno et al., 1996; Hropot et al., 1994) may have contributed to the development of myocardial infarcts. Myocardial infarcts have also been shown by others after 7 or 8 weeks of treatment with L-NAME at different dosages (0.1–1.0 g/l) (Numaguchi et al., 1995; Xu et al., 1995; Moreno et al.,

1996). Comparison of the histology revealed that the injury was more extensive in our model, even though the time of exposure to L-NNA was only 3 weeks. The acute and pronounced vasculitic phenomena of intramural vessels, as well as the early stage of myocardial infarcts with breaks in capillaries and hemorrhage observed in our model, have not been described previously. The increase in blood pressure and the morphological effects were related to NOS inhibition in general and not specifically to L-NNA, since treatment with L-NAME resulted in a very similar increase in blood pressure and similar cardiac lesions, although a tenfold higher dose was necessary. Thus, L-NNA at this dose (500 mg/l) appears to be a very potent NOS inhibitor.

It could be postulated that the severe hypertension played a role in the pathogenesis of the myocardial infarcts. However, it has been shown that renovascular hypertensive rats with a similar increase in blood pressure than rats with chronic NOS inhibition showed no significant alterations in heart morphology (Moreno et al., 1996). Moreover, co-treatment of L-NAME with hydralazine, which attenuated the increase in blood pressure, reduced myocardial fibrosis associated with myocyte necrosis, but had no effect on microvascular thickening of the media and perivascular fibrosis (Numaguchi et al., 1995). Angiotensin II seems to have a decisive role in the pathogenesis of infarcts due to NOS inhibition, since low dose angiotensin AT_1 receptor blockade that did not affect blood pressure could prevent myocardial reparative fibrosis associated with myocyte necrosis (Takemoto et al., 1997a). Therefore, it is unlikely that the preventive effect of co-treatment with losartan on cardiac damage in our model is solely due to reduction in blood pressure.

Treatment with the angiotensin AT_1 receptor antagonist losartan alone resulted in a decrease insystolic blood pressure of 10 mm Hg and had no effect on cardiac morphology. Co-treatment of L-NNA + losartan completely prevented the hypertension, cardiac hypertrophy, vasculitis and myocardial infarcts induced by L-NNA, although blood pressure remained significantly higher than in rats treated with losartan alone. A similar constellation has been observed previously, when L-NAME + losartan resulted in slightly higher blood pressure than losartan alone (Ribeiro et al., 1992). This means that losartan specifically prevents the effects of chronic NOS inhibition. This is in accordance with other studies that showed that co-treatment with angiotensin converting enzyme inhibitors or angiotensin AT_1 receptor antagonists in rats prevented the hypertension (Ribeiro et al., 1992; Pollock et al., 1993; Jover et al., 1993; Hropot et al., 1994; Michel et al., 1996), cardiac hypertrophy (Hropot et al., 1994; Qiu et al., 1998; Arnal et al., 1993; Verhagen et al., 1998) and coronary vascular and myocardial remodeling associated with chronic NOS inhibition (Qiu et al., 1998; Arnal et al., 1993; Katoh et al., 1998). In one study, it was found that angiotensin converting enzyme inhibition could not prevent myocardial ischemic alterations such as necrosis and fibrosis induced by 8 weeks of treatment with L-NAME, while it could prevent hypertension and left ventricle hypertrophy (Moreno et al., 1995). However, it should be noted that in the cited study only 25% of the rats treated with L-NAME showed extensive myocardial injury whereas this was practically a universal finding in other studies, including our own.

The preventive effect of co-treatment with losartan suggests that angiotensin II plays an important role in the pathogenesis of vasculitis and myocardial infarcts observed in our severe model of chronic NOS inhibition. All components of the renin–angiotensin system have been demonstrated in the heart (Baker et al., 1992) and studies in the isolated perfused heart have demonstrated the significance of a cardiac renin–angiotensin system (Dietz et al., 1993). Thus, it is conceivable that the intracardiac renin–angiotensin system is involved in the effects of chronic NOS inhibition. In the heart, angiotensin II can be regarded as the physiological antagonist of NO. Angiotensin II may be involved in the development of cardiac fibrosis and left ventricular hypertrophy, because it acts as a growth factor for intimal hyperplasia, myocyte growth and fibroblast growth and collagen deposition in the cardiac interstitium (Sadoshima and Izumo, 1993). In addition, angiotensin II may have contributed to endothelial dysfunction, a pronounced feature of chronic NOS inhibition, by several mechanisms. Angiotensin II is known to increase vascular permeability, thus allowing the passage of fibrin and platelets through the vessel wall (Williams et al., 1995). Angiotensin II also favors mononuclear cell adhesion on endothelial cells (Grafe et al., 1997) and it induces apoptosis of endothelial cells (Dimmeler et al., 1997). Angiotensin II may also have contributed to cardiac injury via stimulation of vascular superoxide release by NAD(P)H oxidase (Rajagopalan et al., 1996), or generation of cyclooxygenase metabolites (Zerronk et al., 1998). Thus, during pronounced inhibition of NO synthesis, unopposed action of angiotensin II on the cardiac vasculature apparently results in vasculitis and infarcts via multiple pathways.

Surprisingly, the important role of angiotensin II in the cardiac effects of chronic NOS inhibition was not related to an increase of angiotensin II concentrations in the heart. In fact, cardiac angiotensin II was slightly decreased after 4 days of NOS inhibition. After 7 and 14 days of NOS inhibition, when marked hypertension and cardiac injury were already present, cardiac angiotensin II concentrations were not different from control, while after 21 days of NOS inhibition, cardiac angiotensin II concentrations were slightly increased. This suggests that during chronic NOS inhibition, the sensitivity for angiotensin II was increased. Indeed, it has been shown that during chronic NOS inhibition, the sensitivity for exogenous angiotensin II can be increased: angiotensin II infused by osmotic pump for 3 days at a dose which induced no blood pressure elevation and fibrosis, caused significant fibrosis when given to a rat

pretreated for 2 weeks with L-NAME (Hou et al., 1995). It is not entirely clear why the sensitivity for angiotensin II is increased during chronic NOS inhibition. Because NO and angiotensin II can be regarded as physiological antagonists, it is possible that in the absence of NO, even normal angiotensin II concentrations exert detrimental effects. In addition, upregulation of the number of cardiac angiotensin AT_1 receptors during chronic NOS inhibition may have contributed to the increased sensitivity for angiotensin II (Katoh et al., 1998). The finding that cardiac angiotensin II concentrations were not significantly increased was unexpected, because it has been shown that cardiac angiotensin converting enzyme was upregulated during chronic NOS inhibition (Takemoto et al., 1997b; Arnal et al., 1993). Apparently, angiotensin converting enzyme is not the determining parameter in cardiac angiotensin II levels during chronic NOS inhibition. Although cardiac angiotensin II concentrations after 7 days of NOS inhibition were not significantly different from control, they were significantly higher than after 4 days NOS inhibition. Also after 21 days of NOS inhibition, cardiac angiotensin II concentrations were higher than after 0 or 4 days of NOS inhibition. Interestingly, the upregulation of the number of cardiac angiotensin AT_1 receptors during chronic NOS inhibition was only observed after 3 and 7 days, and the number of angiotensin AT_1 receptors had returned to normal after 4 weeks of NOS inhibition (Katoh et al., 1998). This suggests that upregulation of angiotensin AT_1 receptors during chronic NOS inhibition may be a direct effect of NO deficiency. Indeed, concomitant administration of L-arginine corrected this increase, without affecting the rise in blood pressure, while hydralazine corrected the blood pressure but had no effect on upregulation of angiotensin AT_1 receptors (Katoh et al., 1998). Increased angiotensin II generation at later time points may have been secondary to ischemia, since it has been shown that angiotensin II release was increased after coronary occlusion (Noda et al., 1993) and at sites of myocardial repair after necrosis (Sun et al., 1998).

In summary, marked inhibition of NOS resulted in severe hypertension, cardiac hypertrophy, vasculitis of intramural vessels and myocardial infarcts. Co-treatment with the angiotensin AT_1 receptor antagonist losartan prevented the rise in blood pressure and the harmful actions of angiotensin II on the endothelium of the intramyocardial vasculature: during losartan therapy vasculitis and myocardial infarcts were not observed. Although this strongly suggests an important role for angiotensin II in the pathogenesis of cardiac changes during chronic NOS inhibition, cardiac angiotensin II concentrations were not increased before the development of cardiac morphological changes. This suggests that during chronic NOS inhibition, the sensitivity for angiotensin II is increased. These experiments may serve as a model for the myocardial consequences of a dysbalance between the NO– and renin–angiotensin system.

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