Original Article

Pressure Overload–Induced Transient Oxidative Stress Mediates Perivascular Inflammation and Cardiac Fibrosis through Angiotensin II

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Oxidative stress is implicated in the pathogenesis of various cardiovascular diseases. We have shown that in Wistar rats with a suprarenal aortic constriction (AC), pressure overload–induced transient perivascular inflammation (monocyte chemoattractant protein-1 [MCP-1] induction and macrophage accumulation) in the early phase is the determinant of reactive myocardial fibrosis and resultant diastolic dysfunction in the late phase. Thus, we investigated the role of reactive oxygen species production in cardiac remodeling in AC rats. Superoxide production and the footprint of lipid peroxidation were assessed using dihydroethidium staining and immunohistostaining against 4-hydroxy-2-nonenal (4-HNE), respectively. In sham rats, dihydroethidium and 4-HNE signals were scarcely found in the heart. At day 3, AC rats showed dihydroethidium signals mainly in the intramyocardial arterial wall, whereas modest 4-HNE staining was observed diffusely in the myocardium. These signals declined to lower levels by day 14 despite sustained hypertension. Chronic administration of a subdepressor dose of an angiotensin II type 1 receptor blocker candesartan reduced the pressure overload–induced dihydroethidium and 4-HNE signals at day 3. Moreover, candesartan decreased MCP-1 induction and macrophage infiltration at day 3 and prevented myocardial fibrosis at day 14, without affecting left ventricle and myocyte hypertrophy. In conclusion, acute pressure overload induced self-limited superoxide production mainly in the vascular wall. The reactive oxygen species production would contribute to the perivascular inflammation and subsequent myocardial fibrosis. Angiotensin II was suggested to have a pressure-independent effect on the reactive oxygen species production. (*Hypertens Res* 2006; 29: 711–718)

Key Words: oxidative stress, hypertension, macrophage, fibrosis

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Introduction

Myocyte hypertrophy and myocardial fibrosis, especially reactive fibrosis that expands from the perivascular space to the intermuscular space, are typical features of hypertensive cardiac remodeling (1). In our recent series of studies employing rats with a suprarenal aortic constriction (2–4), we showed that pressure overload induced transient perivascular inflammation (*e.g.*, monocyte chemoattractant protein-1 [MCP-1] induction and macrophage infiltration) and fibroblast activation (*e.g.*, fibroblast proliferation and transforming factor- β induction) in the intramyocardial arteries in the early phase. In the late phase, pressure overload produced cardiac hypertrophy characterized by reactive fibrosis and resultant diastolic dysfunction (for review see Kai *et al.* (5)). In this model, it was demonstrated that the local angiotensin II plays a role in the pressure overload-induced perivascular inflammation through a mechanism independent of the pressor effect (6).

Among the variety of pleiotropic effects of angiotensin II, reactive oxygen species production through the activation of NADPH oxidase has been highlighted in the pathogenesis of various cardiovascular diseases (7–10). In addition, a number of studies have supported a role of reactive oxygen species in the pathophysiology of various models of hypertension (11–14). Accordingly, we investigated the role of reactive oxygen species in the pathogenesis underlying cardiac remodeling in rats with aortic constriction. For this purpose, the temporal and spatial distribution of reactive oxygen species were determined in the pressure-overloaded hearts of rats with aortic constriction. We also examined the role of angiotensin II in the production of reactive oxygen species.

Methods

Animal Preparation

All procedures were performed in accordance with institutional guidelines of animal care and treatment. After male Wistar rats (300–400 g) had been anesthetized intraperitoneally with pentobarbital (50 mg/kg), aortic constriction or sham-operation (sham) was established with a ligature at the supra-renal abdominal aorta using a 3-0 surgical silk suture tightly around the blunt end of a curved 22-G needle (2, 15, 16). On each of the 7 days before the operation, rats orally received 0.1 mg/kg/day of candesartan or vehicle. This dose of candesartan was the maximum dose that did not change blood pressure just before (day 0) and 14 days after aortic constriction, as previously reported (6, 17). Rats were randomized to the following three groups. Sham rats received the vehicle and sham operation; AC rats received the vehicle and aortic constriction; AC+Cand rats received candesartan and aortic constriction. Arterial pressure was measured in the unrestricted, conscious state through a heparinized polyethyl-

ene catheter that was indwelled into the left carotid artery 1 day before measurement (2). Unless otherwise indicated, 5 rats were studied in each group for each time point.

Morphometry and Immunohistostaining

Rats were killed with an overdose injection of intraperitoneal pentobarbital. After perfusion-fixation with 4% glutaraldehyde in Hank's solution at 100 mmHg, the left ventricle (LV) was processed for histological and immunohistological studies (2). To evaluate myocyte hypertrophy and myocardial fibrosis, 3 independent hematoxylin-eosin-stained and 3 Mallory-Azan-stained sections of each rat were scanned and analyzed using a digital image analyzer, respectively. The shortest transverse myocyte diameter was measured in 50 nucleated transverse sections of the myocytes in each tissue section (18). The percent area of myocardial fibrosis (% myocardial fibrosis area) was calculated as previously described (19).

The paraffinized sections (5 μ m in thickness) were subjected to immunohistostaining with an antibody against ED1 (Chemicon International, Temecula, USA) for monocytes/macrophages or 4-hydroxy-2-nonenal (4-HNE; Nikken SEIL Co., Fukuroi, Japan) for the footprint of oxidative stress (lipid peroxidation) and a commercially available detection system (DAKO, Carpinteria, USA) (2). The number of macrophages was counted at $\times 200$ magnification in 4 independent entire cross sections of each animal (4).

Dihydroethidium Staining

Rats were euthanized with an overdose injection of intraperitoneal pentobarbital. Immediately after perfusion with ice-cold saline at 100 mmHg, the LV was excised, snap-frozen in liquid nitrogen, and stored at -80°C until use for dihydroethidium staining and real-time reverse transcription-polymerase chain reaction (RT-PCR).

Fresh-frozen LV sections (15 μ m-slices) were incubated for 1 h at 37°C with dihydroethidium (2 mmol/l; Invitrogen Corp., Carlsbad, USA), a fluorescent dye used to assess superoxide formation (typically nuclear localization) (20). Imaging was performed on an epifluorescence microscope (Olympus, Tokyo, Japan).

Real-Time RT-PCR

Quantitative analysis of MCP-1 mRNA expression was performed by using real-time TaqMan RT-PCR with the relative standard curve method (2). Total RNA was extracted from the frozen hearts using TRIzol (GIBCO BRL, Tokyo, Japan). Aliquots (25 ng) of total RNA were reverse-transcribed and amplified in triplicate using a TaqMan EZ RT-PCR kit and a Sequence Detection System model 7700 (PE Biosystems, Foster City, USA) according to the manufacturer's instructions. The RT-PCR conditions and the nucleotide sequences

Fig. 1. Effects of 0.1 mg/kg per day candesartan on hypertension (A) and left ventricle (LV) hypertrophy (B) induced by aortic constriction (AC). Open circle, vehicle-treated AC rats; closed circle, candesartan-treated AC rats; triangle, sham-operated rats. Bar: 1 SD (n=5). *p<0.05 vs. sham.

Fig. 2. Time courses of reactive oxygen species production in pressure-overloaded hearts. A: Dihydroethidium (DHE) staining was performed in AC rats at days 3 and 14. Red fluorescence (fluorescopy) was typically found in the nuclei of the superoxide-generating cells in DHE staining. DHE signals were observed in the arterial wall (microscopy). B: Immunohistochemistry against 4-hydroxy-2-nonenal (4-HNE) was performed in AC rats at days 3 and 14. 4-HNE was stained pink. AC, aortic constriction. Four independent experiments showed similar results.

of PCR primers and TaqMan probe for MCP-1 were as previously described (2). The MCP-1 expression level was normal-

ized by the GAPDH levels in each sample.

Fig. 3. Effects of a subdepressor dose of candesartan (Cand) on the pressure overload–induced reactive oxygen species production at day 3. Representative microphotographs of dihydroethidium (DHE) staining (A) and anti-4-hydroxy-2-nonenal (4-HNE) immunohistostaining (B) are shown. AC, aortic constriction. Four independent experiments showed similar results.

Fig. 4. Effects of a subdepressor dose of candesartan (Cand) on perivascular inflammation and myocardial fibrosis. Representative microphotographs of immunohistochemistry against ED1 (macrophages) at day 3 (A) and Mallory-Azan staining (fibrosis) at day 14 (B). AC, aortic constriction. Four independent experiments showed similar results.

Statistical Analysis

Each quantitative analysis was performed by a single observer in a blind fashion. One-way ANOVA followed by Scheffé's *F* test was performed for the statistical comparisons. A value of $p < 0.05$ was considered significant.

Results

Systolic arterial pressure was similar in the sham, AC, and AC+Cand rats at day 0. Aortic constriction induced a rapid and huge rise in arterial pressure within 3 days, followed by a small gradual elevation (Fig. 1A). In AC rats, the ratio of LV weight over body weight (LVW/BW), an indicator of LV hypertrophy, progressively increased along with the elevation of arterial pressure (Fig. 1B). In AC+Cand rats administered 0.1 mg/kg per day of candesartan, arterial pressure levels at days 3 and 14 were similar to those in AC rats. Also, the increase in LVW/BW did not differ between AC and AC+Cand rats. Arterial pressure and LVW/BW did not change over the 14 days in sham rats.

Time Course of Pressure Overload–Induced Reactive Oxygen Species Production

To determine the superoxide production, dihydroethidium staining was performed (Fig. 2A). In sham rats, dihydroethidium signals were scarcely found in the heart. AC rats showed evident dihydroethidium signals mainly in the intramyocardial arterial wall at day 3. At day 14, the dihydroethidium-labeled cells were markedly reduced in the vessel wall. At day 28, the dihydroethidium signals returned to levels similar to those of sham rats (data not shown).

Next, to confirm the footprints of oxidative stress, immunohistostaining against 4-HNE was performed (Fig. 2B). Sham rats did not show 4-HNE signals. In AC rats, modest 4-HNE staining was found diffusely in the myocardium at day 3. The 4-HNE induction decreased to the sham levels by day 14.

Effects of Candesartan on Reactive Oxygen Species Production

To investigate the effects of a subdepressor dose of candesartan on the production of reactive oxygen species, dihydroethidium staining and immunohistostaining against 4-HNE were performed in the candesartan- and the vehicle-treated AC rats at day 3 (Fig. 3A). Candesartan remarkably decreased dihydroethidium-labeled cells in the arterial wall in rats with the aortic constriction to nearly the sham levels. The aortic constriction–induced induction of 4-HNE–labeled cells was also not evident in the candesartan-treated rats (Fig. 3B).

Effects of Candesartan on Early Inflammatory Responses and Myocardial Remodeling

Aortic constriction transiently upregulated myocardial MCP-1 expression and induced perivascular macrophage infiltration with a peak at day 3 in AC rats (4). The subdepressor dose of candesartan significantly reduced the aortic constriction–induced MCP-1 induction and macrophage infiltration at day 3 (Figs. 4A and 5A). Furthermore, myocardial fibrosis was prevented by candesartan at day 14, whereas myocyte hypertrophy was not affected (Figs. 4B and 5B).

Discussion

The present study has demonstrated that acute pressure overload transiently induces superoxide production mainly in the intramyocardial arterial wall. The time course of the superoxide production was transient, in spite of the sustained arterial pressure elevation. The footprint of reactive oxygen species production (4-HNE formation) was also observed in the myocardium with a similar time course. Moreover, a subdepressor dose of candesartan reduced the aortic constriction–induced reactive oxygen species production associated with the inhibition of MCP-1 induction and macrophage infiltration at day 3. Also, candesartan prevented myocardial fibrosis, but not LV and myocardial hypertrophy, at day 14. Taken together, these results suggested that the pressure overload–induced transient reactive oxygen species production mediated perivascular inflammation and the subsequent cardiac fibrosis.

Recently, we introduced the AC rat as a model of cardiac hypertrophy with preserved systolic but impaired diastolic function without overt congestive heart failure (2, 5). The histological features of this model are a transient induction of perivascular inflammatory changes in the early phase corresponding to the initial rapid rise in arterial pressure and reactive myocardial fibrosis in the late phase with stable hypertension (2–4). Thus, we used this model to investigate the temporal and spatial changes in the reactive oxygen species production and the role of reactive oxygen species in hypertensive cardiac remodeling. In this study, we evaluated reactive oxygen species production by using two independent histological methods: dihydroethidium staining for detecting cellular superoxide generation and anti-4-HNE-immunohistostaining for detecting the footprint of the oxidative stress.

Reactive Oxygen Species Production in Pressure-Overloaded Hearts

The first novel finding of this study is that arterial pressure overload induces superoxide production mainly in the intramyocardial arterial wall (Fig. 2). Moreover, the superoxide production was self-limiting within the early phase, despite the sustained hypertension and cardiac remodeling in the late phase. These findings may indicate that the major

Fig. 5. A: Pooled data showing the effects of a subdepressor dose of candesartan (Cand) on MCP-1 mRNA expression and macrophage infiltration in AC rats at day 3. B: Pooled data presenting the effects of a subdepressor dose of candesartan on myocyte diameter and myocardial fibrosis in AC rats at day 14. Bar: 1 SD (n=5).

determinant of the superoxide production is the acute rise in arterial pressure rather than the level of arterial pressure *per se*. In AC rats, the initial rapid arterial pressure elevation provokes transient perivascular inflammation, which is crucial for reactive fibrosis and diastolic dysfunction in the late phase (3, 4). The finding that the vascular superoxide production and the perivascular inflammation were observed concurrently suggests that the pressure overload-induced vascular oxidative stress has a close relation with the mechanism of the perivascular inflammation, and contributes to the subsequent cardiac fibrosis.

A transient induction of 4-HNE, the footprint of oxidative stress, was the supportive evidence that acute pressure overload transiently induced reactive oxygen species production in the heart, despite sustained hypertension (Fig. 2). However, the immunoreactivity of 4-HNE was modest and diffuse in the myocardium. Currently, the reason for the discrepancy between the distribution patterns of dihydroethidium staining and 4-HNE staining remains uncertain. One of the possible explanations may be that reactive oxygen species other than superoxide would contribute to the 4-HNE formation in the myocardium. This issue should be addressed in a future study.

Roles of Angiotensin II in Reactive Oxygen Species Production

In AC rats, cardiac angiotensin-converting enzyme activity was rapidly and transiently elevated within day 1 with a peak at day 3 (6). In contrast, AC rats did not show significant changes in plasma renin activity, serum cardiac angiotensin-converting enzyme activity, or the circulating levels of angiotensin II and aldosterone during the observation period (day 1 or later) (2, 5, 6), although we cannot deny the possibility that they were transiently activated within 24 h after the operation. Accordingly, it was suggested that the local angiotensin II, but not the systemic renin-angiotensin-aldosterone system, participated in the fibro-inflammatory changes in AC rats. In this regard, it has been shown that both the early perivascular inflammation and the late reactive fibrosis are prevented by blocking the angiotensin II type 1 (AT1) receptors (6, 17). Angiotensin II is known to activate NADPH oxidase, which plays a central role in the reactive oxygen species production in vascular wall cells (21, 22). Also, angiotensin II can produce reactive oxygen species *via* NADPH oxidase in the cardiac myocytes (23). Thus, we hypothesized that angiotensin II mediates the aortic constriction-induced reactive oxygen species production. As expected, candesartan markedly reduced dihydroethidium signals in the vascular wall, as well as the inflammatory changes (Fig. 3). The effect of candesartan was not dependent on the depressor effect because arterial pressure was not changed.

Angiotensin II induces the expression of pro-inflammatory genes, including MCP-1 and inflammatory cytokines, through the activation of nuclear factor- κ B in a reactive oxygen species-dependent manner (24, 25). Of note, aortic constriction induced MCP-1 expression mainly in the intramyocardial arterial wall (4), where the reactive oxygen species induction was documented (Fig. 2). Thus, the reduction of the reactive oxygen species production may participate in the mechanism whereby candesartan attenuates the inflammatory changes in AC rats. However, candesartan reduced dihydroethidium and 4-HNE signals nearly to the sham levels (Fig. 3), whereas it decreased macrophage infiltration and MCP-1 induction to a lesser extent (Fig. 4A), suggesting the presence of the reactive oxygen species-independent inflammatory mechanism(s).

Roles of Reactive Oxygen Species in Myocardial Remodeling

Myocardial fibrosis was prevented remarkably by a subdepressor dose of candesartan in AC rats at day 14 (Fig. 4B). We have previously shown that macrophage accumulation is crucial for the perivascular fibroblast activation (*e.g.*, fibroblast proliferation and transforming factor- β induction) in the early phase in AC rats (4). And, when the early fibro-inflammatory changes were inhibited, the pressure overload-induced myocardial fibrosis was prevented in the late phase

(2, 17). Reactive oxygen species have been shown to mediate the cell-to-cell interaction between macrophages and fibroblasts, which in turn activate the fibro-inflammatory changes (26). Thus, the candesartan-induced reactive oxygen species reduction may contribute to the prevention of myocardial fibrosis by inhibiting the early fibro-inflammatory changes.

Reactive oxygen species have been shown to promote hypertrophy of cultured cardiac myocytes (27). However, a subdepressor dose of candesartan had no effect on LV and myocyte hypertrophy in AC rats (Figs. 1 and 4B). Our previous studies have demonstrated that myocyte hypertrophy is not affected by the inhibition of the early perivascular inflammation (2–4, 6), consistent with the hypothesis that the major determinant of myocyte hypertrophy is the mechanical load onto the LV wall (28). Moreover, in the present study the myocardial production of reactive oxygen species disappeared in the late phase in spite of the progression of cardiac hypertrophy. Considering these results together, it is unlikely that reactive oxygen species have the major impact on myocyte hypertrophy in this model.

Limitations of This Study

NADPH oxidase and mitochondria electron transport leakage are attributable to the major sources of reactive oxygen species in the vascular wall cells and the cardiac myocytes, respectively (21, 22, 29, 30). Also, mechanical and humoral factors other than angiotensin II can be the inducers of reactive oxygen species in AC rats. Thus, future studies should address the source and the regulation mechanism of reactive oxygen species production in this model. Second, we focused exclusively on the production of reactive oxygen species in this study. However, it has been suggested that oxidative stress is a consequence of either an increase of the reactive oxygen species formation or a decrease of the intrinsic antioxidant reserve (23, 31, 32). Recently, Tanaka *et al.* have shown that an AT1 receptor blocker upregulates the antioxidant enzyme, Cu/Zn-containing superoxide dismutase, in the hearts of stroke-prone spontaneously hypertensive rats (33). Thus, the intrinsic antioxidant levels may be altered in this model. We are now in the process of comprehensively screening the gene expression changes in reactive oxygen species inducers and intrinsic antioxidants by using the gene-chip technique.

In conclusion, the present study has shown that acute pressure elevation induces transient vascular reactive oxygen species production, which contributes to perivascular inflammation in the early phase and myocardial fibrosis in the late phase in AC rats. In addition, by inhibiting reactive oxygen species generation in the hypertensive heart, AT1 receptor blockers were suggested to have effects on myocardial fibrosis beyond their reduction of blood pressure. Finally, we investigated the acute reaction of the heart in response to sudden increase in arterial pressure. Our results showed that this phenomenon may not be a reaction to chronic hypertension.

However, it is possible that in hypertensive patients with exaggerated arterial pressure variability, repeated, abrupt pressure elevation induces repetitive and chronic perivascular reactive oxygen species production, which in turn promotes cardiovascular damage.

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