Iron Overload Augments Angiotensin II–Induced Cardiac Fibrosis and Promotes Neointima Formation

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Methods and Results—Angiotensin II was continuously infused to rats at a dose of 0.7 mg/kg per day for 7 consecutive days. No iron deposits were observed in the hearts of untreated rats, whereas iron deposition was seen in the cells in the subepicardial and granulation regions after angiotensin II infusion. Concomitant administration of deferoxamine, an iron chelator, significantly reduced the extent of cardiac fibrosis, which suggests that iron deposition aggravates the cardiac fibrosis induced by angiotensin II. Iron overload caused by the administration of iron-dextran resulted in an augmentation of cardiac fibrosis and the generation of neointimal cells in the coronary artery in angiotensin II–infused rats. By contrast, neointima was not formed in the cardiac vessels in norepinephrine-infused rats with iron overload.

Conclusions—Cardiac iron deposition may be involved in the development of cardiac fibrosis induced by angiotensin II. In addition, iron overload may enhance the formation of neointima under conditions of increased circulating angiotensin II but not catecholamines. (Circulation. 2002;106:1840-1846.)

Key Words: angiotensin • stress • oxygen • catecholamines

Iron, an essential element for many critical biological functions, can potentially promote cellular damage by causing the formation of highly reactive hydroxyl radicals and by causing the peroxydation of unsaturated lipids. Deposition of iron induced during conditions of iron overload plays a role in cardiac fibrosis. Epidemiological and experimental data also suggest that there is a link between body iron stores and the development of atherosclerosis. We have previously reported that long-term administration of angiotensin II results in an abnormal iron deposition in the lysosome of renal tubular epithelial cells, which may mediate proproteinuric effects of angiotensin II. Heme oxygenase-1 (HO-1) is an inducible form of heme oxygenase that is a rate-limiting enzyme of heme degradation. Recent studies have demonstrated that HO-1 also plays a crucial role in controlling the intracellular iron content and thus the extent of oxidant-induced cellular injury.

Methods

Animal Models

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II–induced hypertension was induced in male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical), as described previously. Briefly, Val-angiotensin II (Sigma) was infused at a dose of 0.7 mg/kg per day for 7 days. Some rats were given the nonspecific vasodilator hydralazine (15 mg/kg per day) (Sigma) or the selective AT1 receptor antagonist losartan (25 mg/kg per day) (a kind gift from Merck). These antihypertensive agents effectively blocked the hypertensive effects of angiotensin II. Contrast this model with another model of hypertension, norepinephrine (Sigma) was infused at a rate of 2.8 mg/kg per day. In some experiments, rats were given daily subcutaneous injections of the iron chelator deferoxamine (DFO, a kind gift from Novartis) at a dose of 6 mg/kg per day, beginning 2 days before pump implantation and continuing until the animals were euthanized. The iron overload group received an intraperitoneal injection of an iron-dextran complex (a kind gift from Teikoku Hormone Mfg) at a dose of 240 mg of elemental iron/kg at 0, 2, 4, and 6 days of angiotensin II infusion.

Protein Purification and Western Blot Analysis

Protein was isolated by homogenizing samples in the lysis buffer including protease inhibitors: 50 mmol/L HEPES, 5 mmol/L EDTA,
and 50 mmol/L NaCl (pH 7.5), 10 μg/mL aprotinin, 1 mmol/L PMSF, and 10 μg/mL leupeptin. Polyclonal antibody against rat ferritin (Panapharm) was used at a 1/2000 dilution. The ECL Western blotting system (Amersham) was used for detection. Bands were visualized with the use of a lumino-analyzer (Fuji Photo Film). Band intensity was calculated and expressed as a percentage of the control value.

Histological and Immunohistochemical Analyses
Quantification of the fibrous areas was performed by the operator without knowledge of the treatment group. After Mallory-Azan staining, heart sections were photographed and digitalized and the number of pixels of blue color was counted with the use of a photoimaging system (Canon). The ratios of the area affected by fibrosis to total cardiac area in the samples were expressed as percent fibrosis. Immunohistochemistry was performed as described previously.6 Primary antibodies against rat macrophage/monocyte (ED-1; Chemicon International), rat HO-1 (StressGen), human α-SM actin (Sigma) and rat ferritin (Panapharm) was used at a 1/2000 dilution. The ECL Western blotting system (Amersham) was used for detection. Bands were visualized with the use of a lumino-analyzer (Fuji Photo Film). Band intensity was calculated and expressed as a percentage of the control value.

Electron Microscopy
Samples were first fixed in 2.5% glutaraldehyde, subsequently fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series, and then embedded in EPON. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a transmission electron microscope (JEM 1200 EX; JEOL).

Statistical Analysis
Data are expressed as mean±SEM. We used ANOVA followed by a multiple comparison test for comparisons on initial data before expressing results as a percentage of the control value, using the statistical analysis software Statistica version 5.1 J for Windows (StatSoft Inc). A value of P<0.05 was considered to be statistically significant.

Results

Cardiac Deposition of Iron After Angiotensin II Administration
Continuous administration of angiotensin II for 7 days resulted in a significant increase in blood pressure (control 133±3 mm Hg, n=9, angiotensin II 197±14, n=9, P<0.01). We first assessed the iron deposition in the hearts of angiotensin II–infused rats. ED-1–positive cells, that is, monocytes/macrophages, were observed in the subepicardial (Figure 1a) and the granulation (Figure 1b) regions. These cells stained positively for iron (arrows in a through i). Similar section in subepicardial regions is shown in j through l, j, differential interference contrast image with ferritin signals (green fluorescence); k, confocal imaging of ferritin staining; l, Prussian blue staining. Some ferritin-positive cells were also positive for iron (arrowheads in j through l). Original magnifications ×660 (a through f), ×800 (i and l), and ×1000 (g, h, j, and k). Scale in h and k = 10 μm. Immunoblot analysis of ferritin expression at various time points after angiotensin II infusion; n, data from 4 to 6 experiments for each time point are summarized in the graph; o, effects of norepinephrine and antihypertensive agents on ferritin expression. Data from 4 to 6 experiments in each group are summarized. All, H, L, and Ne indicate angiotensin II, hydralazine, losartan, and norepinephrine, respectively. In the graphs, ferritin expression is expressed as a percentage of the control value. *P<0.05, †P<0.01 vs untreated control.
The iron-laden cells stained positively for ferritin in the granulation (arrows in Figure 1, g through i) and subepicardial (arrowheads in Figure 1, j through l) regions. Ferritin protein expression was induced markedly by angiotensin II infusion (Figure 1, m and n) but was subsequently blocked completely by losartan and partially by hydralazine (Figure 1o). Norepinephrine, which caused hypotensive effects comparable to those of angiotensin II (200±3 mm Hg, n=8), did not increase cardiac expression of ferritin (Figure 1o) or cause cardiac iron deposition (data not shown). Ferritin expression (Figure 1o) roughly correlated with the extent of iron deposition, as demonstrated histologically (data not shown). No positive staining for iron could be seen in the heart of rats not infused with angiotensin II (data not shown).

**Effect of Iron Chelation on Angiotensin II–Induced Cardiac Fibrosis**

We examined whether deposition of cardiac iron is involved in the cardiac fibrosis induced by angiotensin II. DFO treatment did not significantly alter hemodynamic values in nontreated or angiotensin II–treated rats (DFO alone, 131±2 mm Hg, n=6, DFO plus angiotensin II, 197±8 mm Hg, n=9, NS versus control and angiotensin II groups, respectively). However, it reduced the extent of the fibrous areas induced by angiotensin II (Figure 2a). Treatment of rats with DFO also suppressed angiotensin II–induced upregulation of ferritin (Figures 2b) and deposition of cardiac iron (data not shown). Losartan, which completely blocked the angiotensin II–induced upregulation of cardiac ferritin (Figure 1o), also completely suppressed the fibrotic effects of angiotensin II (fibrotic area; right ventricle 0.74±0.13%, left ventricle 0.12±0.03%, n=4, P<0.05 versus angiotensin II group).

**Iron Overload in Hypertensive Animals**

Next, we examined whether iron overload affects cardiac damage induced by vasopressors. Iron loading by itself resulted in a slight increase in blood pressure (Fe alone, 142±2 mm Hg, n=10, P<0.01 versus control) and in cardiac fibrosis in the right ventricle. Iron loading did not significantly change the blood pressure of angiotensin II–infused rats (Fe plus angiotensin II, 196±11 mm Hg, n=11, NS versus angiotensin II group) or that of norepinephrine-infused rats.

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**Figure 2.** Iron chelation and cardiac fibrosis. a, Fibrotic area quantified by Mallory-Azan–stained samples. Data are summarized from 6 to 8 rats in each group. b, Ferritin protein expression. Data are summarized from 4 to 6 experiments in each group. *P<0.05, †P<0.01 vs untreated control; ‡P<0.01 between indicated groups. All and DFO indicate angiotensin II and deferoxamine.

**Figure 3.** Iron overload and cardiac fibrosis. a through f, Mallory-Azan staining. a through c, Angiotensin II–infused rat. d through f, Angiotensin II–infused rat with iron overload. g through i, Norepinephrine-infused rat with iron overload. In each group, images from the right ventricle (a, b, d, e, g, and h) and the left ventricle (c, f, and i) are shown. Original magnification ×250 for a, c, d, f, g, and i and ×800 for b, e, and h. j, Fibrotic area quantified by Mallory-Azan–stained samples. Data from 6 to 8 rats in each group are summarized. *P<0.05, †P<0.01 vs untreated control; ‡P<0.01 between indicated groups.
rats (Fe plus norepinephrine, 193±8 mm Hg, n=12, NS versus norepinephrine group). Iron loading significantly increased cardiac fibrosis in both ventricles of rats given angiotensin II and in the left ventricle of rats given norepinephrine (Figure 3). Histological examination showed that there was no apparent iron deposition in the vascular wall of rats given angiotensin II (Figure 4a) or norepinephrine (Figure 4b). Iron overload by itself resulted in an iron deposition in the interstitial and perivascular regions but again, not in the vessel wall (Figure 4c). In the heart of angiotensin II–infused rats with iron loading, robust iron deposition could be observed both in the arterial wall and in the interstitial cells (Figure 4, d and e). In contrast, iron deposition was not seen in the vascular wall in the heart of norepinephrine-infused rats with iron overload (Figure 4f). Some endothelial cells stained positively for iron. Elastica–van Gieson staining of the serial sections showed that there was apparent neointima formation and iron deposition in neointimal cells (Figure 4, g through i). In the heart of angiotensin II–infused rats with iron loading, robust iron deposition could be observed both in the arterial wall and in the interstitial cells (Figure 4, a through d). Staining for both α-smooth muscle actin and iron showed that iron was deposited in neointima (Figure 5, a through c) and in myofibroblast-like cells (Figure 5, d through g). Some iron-laden cells were ED-1–positive (Figure 5, h and i) and thus judged to be monocytes/macrophages.

**Electron Microscopy**

Toluidine blue staining showed thickening of the vascular wall (Figure 6a) in the heart of angiotensin II–infused rats with iron overload. Electron microscopy showed that there were electron-dense deposits, that is, iron particles, both within and outside the vascular cells (Figure 6b). These iron deposits were observed in granules with limiting membranes and thus lysosomes (Figure 6c, arrows). Occasionally, iron deposition was seen between the plasma membrane and basement membrane (Figure 6c, arrowheads). Iron deposits were also observed in the interstitial cells surrounding the vascular cells (Figure 6d). Notably, no iron deposits were apparent in cardiomyocytes (Figure 6d). Iron deposits were found in vascular endothelial cells, as observed by Prussian blue staining (Figure 6e).

**Localization of Cardiac HO-1 After Iron Overload and Angiotensin II Infusion**

Finally, we investigated the expression of HO-1 in the heart of angiotensin II–infused rats with iron loading. Confocal microscopy revealed that only a fraction of the ED-1–positive cells expressed high levels of HO-1 (arrows in Figure 7, a through c). Some cells that were positive for both ED-1 and HO-1 did not contain iron (arrowheads in Figure 7, f through...
h), whereas some ED-1-positive cells with apparent iron deposition did not express HO-1 (arrows in Figure 7, f, h, and j). Costaining of the sections for α-smooth muscle actin and HO-1 showed that most of the neointima cells with iron deposition did not express HO-1 (Figure 7, k through o), and the myofibroblast-like cells in the granulation regions did not express high levels of HO-1 (Figure 7, p through t). These results indicate that high expression levels of HO-1 do not necessarily represent a marker of iron deposition in the heart of rats treated with both angiotensin II infusion and iron overload.

Discussion

In this study, we first demonstrated that angiotensin II infusion induced cardiac iron deposition. The iron-laden cells were ED-1-positive and were thus identified as macrophages/monocytes. Ferritin expression, which is presumably a marker of iron deposition, was increased by angiotensin II infusion but blocked partially by hydralazine and completely by losartan. This pattern parallels the angiotensin II–induced cardiac damage that we had reported previously, which suggests the possibility that iron has a role in cardiac damage. Indeed, treatment of angiotensin II–infused rats with the iron chelator deferoxamine reduced the extent of cardiac fibrosis.

Although there could be several possible sources of the iron deposits observed in the heart of the angiotensin-infused rats, two sources seem more likely than others. First, because angiotensin II causes myocardial damage, heme protein released by the damaged muscular cells may be the source of the iron. Second, similar to what happens in ischemia-reperfusion, angiotensin II may cause destabilization of the intracellular mitochondrial and microsomal heme proteins, such as cytochromes, resulting in an increase in intracellular heme.

In humans, iron overload and subsequent tissue iron deposition can be seen in some disorders such as idiopathic hemochromatosis and transfusion-related siderosis. As in the liver, iron deposition in the heart is postulated to induce cardiac dysfunction, in part, by inducing reactive fibrosis. In some animal models of iron overload, the occurrence of both cardiac iron deposition and fibrosis have been reported. Our data suggest that iron deposition may exacerbate the cardiac fibrosis induced by angiotensin II. The findings that the myocardial fibrosis that occurs after myocardial ischemia...
can be attenuated by angiotensin-converting enzyme inhibitor and angiotensin receptor antagonist\(^{19}\) and that less cardiac fibrosis is observed in the AT\(_1\) receptor knockout mouse than in wild-type\(^{20}\) suggest that angiotensin II has a crucial role in the development of cardiac fibrosis. Several factors that possibly mediate the fibrogenic effects of angiotensin II have been reported, such as transforming growth factor-\(\beta\) and cardiac aldosterone. Whether iron deposition causes cardiac upregulation of transforming growth factor-\(\beta\), as it does in the liver,\(^{21}\) should be elucidated in future studies.

Increased generation of reactive oxygen species is thought to be involved in the development of cardiac fibrosis, because in certain disorders, antioxidants can attenuate cardiac fibrosis.\(^{22}\) Iron is known to play a crucial role in the generation of highly toxic hydroxyl radicals via the Haber-Weiss and Fenton reactions. Although the reactive oxygen species that are most potent in the formation of fibrotic regions in the heart remains to be determined, generation of hydroxyl radicals through these reactions may have a role in the development of cardiac fibrosis by mechanisms similar to those proposed for other organs.\(^ {23-25}\)

Another intriguing finding of the present study was that coadministration of iron dextran plus angiotensin II but not iron dextran plus catecholamines induced neointima formation with marked deposits of iron. A link between iron and the development of atherosclerosis has been the subject of debate for more than two decades.\(^ {4,26}\) Recent studies have shown that administration of an iron-chelating agent suppresses neointimal formation in the balloon-injured porcine artery\(^ {27}\) and that an iron-deficient diet blocks atherogenesis in atherosclerosis-prone animal models.\(^ {5}\) These findings indicate that iron is involved in the generation of vascular lesions. Daemen et al\(^ {28}\) showed that a 2-week infusion of angiotensin II infusion, at a rate comparable to that used in our model, enhanced neointimal formation in the rat carotid artery induced by balloon injury, whereas angiotensin II infusion alone did not form

**Figure 7.** Confocal microscopic analyses of localization of HO-1. a through j, Staining for HO-1, ED-1, and iron. a through e, Granulation regions. a, ED-1 staining. b, HO-1 staining. c, Costaining of ED-1 and HO-1. d, Nomarski differential interference contrast (DIC) imaging. e, Prussian blue staining. Some ED-1-positive cells stained positively for HO-1 (arrows in a through c). f through j, Subepicardial regions. f, ED-1 staining. g, HO-1 staining. h, Costaining of ED-1 and HO-1. i, DIC imaging. j, Prussian blue staining. Some cells positive for iron and ED-1 were apparently negative for HO-1 (arrows in f, h, and j). By contrast, some cells with positive staining for both HO-1 and ED-1 were negative for iron (arrowheads in f, g, and h). k through o, Staining for \(\alpha\)-smooth muscle actin, HO-1, and iron in neointima. k, \(\alpha\)-Smooth muscle actin staining. l, HO-1 staining. m, Costaining of \(\alpha\)-smooth muscle actin and HO-1. n, DIC imaging. o, Prussian blue staining. p, q, and r, Confocal imaging of \(\alpha\)-smooth muscle actin and HO-1 in granulation regions. p, \(\alpha\)-Smooth muscle actin staining. q, HO-1 staining. r, Costaining of \(\alpha\)-smooth muscle actin and HO-1. s and t, Costaining of \(\alpha\)-smooth muscle actin and HO-1 (s) and DIC imaging of the same region (t). Original magnifications \(\times 1000\) (a through d, f through i, k through n, and s), \(\times 400\) (p through r), and \(\times 800\) (e, j, and o). Scale=20 \(\mu\)m except in p through r, in which scale=50 \(\mu\)m.
neointima. Carthew et al 18 showed that in a rodent model of iron overload, with a larger dose and for a longer period than used in our protocol, cardiac fibrosis and iron deposition occurred in the myocardium, although neointimal formation was not noted. Consistent with these studies, we found that neointima did not form in the hearts of rats treated by iron overload alone or by angiotensin II infusion alone. Thus, iron may act to promote arteriosclerosis and neointimal formation when other proatherogenic stimuli, such as endothelium removal or increased circulatory levels of angiotensin II, are present.

We previously reported the high expression of HO-1 in the renal cells containing iron deposits after angiotensin II infusion, 6 which suggested that HO-1 induction might be a possible marker of intracellular iron deposition. However, confocal microscopic analysis revealed that unlike in the kidney, HO-1 expression does not appear to be a good marker for iron deposition in cardiac cells under our experimental conditions.

In summary, continuous administration of angiotensin II but not catecholamines induced cardiac deposition of iron. Iron chelation ameliorated and iron overload exacerbated the extent of angiotensin II–induced cardiac fibrosis, which suggests that iron may be involved in the development of cardiac fibrosis induced by angiotensin II. In addition, iron overload caused prominent neointimal formation in the heart angiotensin II–treated but not norepinephrine-treated rats. Iron overload may therefore enhance the proliferative properties of intimal cells induced by angiotensin II. Our rat model of angiotensin II and iron–dextran administration may represent a new tool for identifying the pathway of neointima hyperplasia and atherosclerosis in the context of the activated renin angiotensin system.

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