Lipid Accumulation and Transforming Growth Factor-β Upregulation in the Kidneys of Rats Administered Angiotensin II

Kan Saito, Nobukazu Ishizaka, Masumi Hara, Gen Matsuzaki, Masataka Sata, Ichiro Mori, Minoru Ohno, Ryozo Nagai

Abstract—Abnormal lipid metabolism may play a role in progressive renal failure. We studied whether lipid accumulation occurs and whether lipid deposits are colococalized with transforming growth factor-β1 (TGF-β1) in the kidney of angiotensin II–infused animals. Oil red O staining showed marked lipid deposition in the tubular epithelial and vascular wall cells of angiotensin II–treated but not in norepinephrine-treated rats. Histological analyses showed that increased amounts of superoxide and intense TGF-β1 mRNA expression were present in lipid-positive tubular epithelial cells in angiotensin II–infused animals. Protein expression of sterol regulatory element-binding protein 1 (SREBP-1) and mRNA expression of fatty acid synthase in the kidney were ∼3 times and 1.5 times, respectively, higher in angiotensin II–treated rats than in controls. Treatment of angiotensin II–infused animals with an iron chelator, deferoxamine, attenuated the angiotensin II–induced increases in renal expression of SREBP-1 and fatty acid synthase and normalized the lipid content in the renal cortical tissues. Abnormal lipid metabolism may be associated with upregulation of TGF-β1 expression and aberrant iron homeostasis in the kidneys of angiotensin II–infused animals. (Hypertension. 2005; 46:1180-1185.)

Key Words: angiotensin II  ■ transforming growth factors  ■ lipids

Previous studies showed that accumulation of lipids in nonadipose tissues occurs in certain diseased conditions, and that it plays a crucial role in the pathogenesis of tissue damage, a phenomenon referred to as lipotoxicity. For example, lipid deposition in the arterial wall is postulated to represent an early event in the development of atherosclerosis. The accumulation of triglycerides (TGs) in cardiomyocytes may be associated with contractile dysfunction, and excess TGs in the liver may promote hepatic fibrosis. Lipid accumulation in the kidney may represent features of glomerular and tubulointerstitial damage.

We found that long-term administration of angiotensin II (Ang II) to rats caused marked deposition of iron and upregulation of the expression of transforming growth factor-β1 (TGF-β1) in renal tubular epithelial cells. We also found that iron chelation attenuated the Ang II–induced upregulation of TGF-β1 in the kidney, suggesting a possible link between aberrant iron homeostasis and TGF-β1 upregulation. However, unexpectedly, histological analysis showed that upregulation of TGF-β1 mRNA and deposition of iron occurred in different tubular cells in most cases. The aims of the present study were to investigate whether accumulation of lipids occurs in the kidney of Ang II–infused animals and to investigate the relationship between lipid accumulation and TGF-β1 in terms of localization. We also investigated whether Ang II infusion alter the expression of genes that have relation to the lipid metabolism, such as ATP-binding cassette transporter subfamily A-1 (ABCA1), scavenger receptor class B type 1 (SR-B1), sterol regulatory element-binding protein 1 (SREBP-1), SREBP-2, 3-hydroxy-3-methylglutaryl–coenzyme A reductase, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1), CoA:diacylglycerol acyltransferase-1 (DGAT-1), and LDL receptor. Furthermore, we investigated whether iron chelation affects the degree of lipid accumulation in the kidney of Ang II–infused animals.

Materials and 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. Ang II–induced hypertension was induced in male Sprague-Dawley rats (250 to 300 g) by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical) as described previously. Briefly, Val^3-Ang II (Sigma Chemical) and norepinephrine (Sigma Chemical) were infused at doses of 0.7 mg/kg per...
day and 2.8 mg/kg per day, respectively, for 7 days. An iron chelator, deferoxamine (a kind gift from Novartis, Basel, Switzerland) was subcutaneously administered at a dose of 200 mg/kg per day while rats were receiving Ang II. Deferoxamine did not significantly affect the blood pressure of Ang II–infused rats.

**Measurement of Lipid Contents in the Serum and Renal Cortex**

Serum levels of total cholesterol (TC) and TGs in each sample were measured by enzymatic methods (SRL) using Total Cholesterol E Test Wako and Triglycerides E Test Wako (Wako Pure Chemicals), respectively.

**Western Blot Analysis, Immunohistochemistry, and In Situ Hybridization**

Western blot analysis was performed as described previously. Polyclonal antibodies against ABCA1 (Novus Biologicals), SR-B1 (Novus Biologicals), SREBP-1 (Santa Cruz Biotechnology), and SREBP-2 (Santa Cruz Biotechnology) were used at dilutions of 1/1000, 1/1000, and 1/500, respectively. Immunohistochemistry was performed as described previously. For this purpose anti–SREBP-1 antibody was used at a dilution of 1/200. In situ hybridization was performed as described previously.

**Oxidative Fluorescent Microscopy**

Staining with the oxidative fluorescent dye dihydroethidium (DHE) performed as described previously. Fluorescence intensity was obtained for ≥6 fields for each section. The fluorescent intensity in Ang II–treated group was presented as the percentage of that of untreated control group.

**Real-Time RT-PCR**

Real-time quantitative PCR was performed by LightCycler (Roche Diagnostics). Gene expression was normalized to the endogenous control GAPDH mRNA in each sample, and then the amount of target gene expression in the sample from treated animals was expressed as the percentage of that in the untreated control. The following primers and probes were used: for HMG-CoA reductase; forward 5'-GACACITCAACTCTGTATGTG-3'; reverse 5'-CTTGAGAGGTAATACGTGCA-3'; for ACC: forward 5'-GATT-TGTCCACCCGCTTTTG-3', reverse 5'-TGGAGGCGATCCACTTGA-3'; for SCD-1; forward 5'-CTCTTACACCCGATGCGCA-3'; for LDL receptor, forward: 5'-TCTTCTCAGGGGATGCTAAATC-3'; reverse 5'-TCCCTGCAGACACAGCTTGG-3'; for plasminogen activator inhibitor-1 (PAI-1), forward: 5'-TCTTCATCGCTCGTAAGT-3', reverse 5'-TGATGTCGCTGCTACAGG-3'; for LDL receptor, forward: 5'-TCTTTCACCCGCTTTTG-3', reverse 5'-TGGAGGCGATCCACTTGA-3'; for ACC: forward 5'-GATT-TGTCCACCCGCTTTTG-3', reverse 5'-TGGAGGCGATCCACTTGA-3'; for GAPDH: forward 5'-GAACCCATTAAAGATTTTCGCAA-3'; for DSGAT-1, forward: 5'-TCTTCTCACGGGATGCTCAAATC-3'; reverse 5'-TCCCTGCAGACACAGCTTGG-3'; for LDL receptor, forward: 5'-TCTTCATCGCTCGTAAGT-3', reverse 5'-TGATGTCGCTGCTACAGG-3'; for plasminogen activator inhibitor-1 (PAI-1), forward: 5'-TCTTCATCGCTCGTAAGT-3', reverse 5'-TGATGTCGCTGCTACAGG-3'; and for GAPDH: forward 5'-GAACCCATTAAAGATTTTCGCAA-3'; for DSGAT-1, forward: 5'-TCTTCTCACGGGATGCTCAAATC-3'; reverse 5'-TCCCTGCAGACACAGCTTGG-3'.

**Statistical Analysis**

Data are expressed as the mean±SEM. We used ANOVA followed by a multiple comparison test to compare raw data before expressing the 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**

**Serum and Tissue Lipids Contents**

Administration of Ang II for 7 days slightly but significantly increased the serum levels of TC and TGs compared with the respective levels in control rats. These increases in the serum TC and TG levels were suppressed by treatment with an iron chelator (Table 1). Similarly, Ang II increased the contents of TC and TGs in the renal cortical tissues, and these increases were suppressed by treatment with an iron chelator (Table 2). A pressor dose of norepinephrine did not increase the serum levels of TC or TGs nor the content of TC or TGs in renal cortical tissues.

**Lipid Staining**

Oil red O staining of kidney sections showed no apparent lipid deposition in the kidneys of untreated rats (Figure 1A). On the other hand, in Ang II–infused rats, marked accumulation of oil red O–stainable lipid was observed in tubular epithelial cells in kidney sections (Figure 1B). Lipid deposition was not apparent in kidney sections from the norepinephrine-infused rats (Figure 1C). Staining of serial sections showed that lipid deposits that were positively stained by oil red O (Figure 1D) could also be stained positively by Sudan black (Figure 1E). In Ang II–infused rats, the percentage of cells that were positive for iron deposition decreased after the treatment with an iron chelator (Figure 1F).

**Staining for Superoxide, Lipids, and Iron**

We previously found that superoxide production was increased in tubular epithelial cells that contain granular materials in the kidneys of Ang II–infused rats, and that majority of the cells that contained these granular materials were not positive for iron staining. In the current study, staining of serial sections showed that tubular cells found to contain granular material on differential interference contrast (DIC) images were positive for oil red O staining, indicating that

**TABLE 1. Body Weight, Blood Pressure, and Serum Levels of TC and TGs**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Body Weight (g)</th>
<th>Systolic Blood Pressure (mm Hg)</th>
<th>TC (mg/dL)</th>
<th>TGs (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>309±3</td>
<td>131±3</td>
<td>50.9±2.6</td>
<td>20.5±2.1</td>
</tr>
<tr>
<td>Ang II</td>
<td>10</td>
<td>230±14†</td>
<td>192±4†</td>
<td>65.2±4.7†</td>
<td>42.7±4.4†</td>
</tr>
<tr>
<td>Ang II+ deferoxamine</td>
<td>10</td>
<td>232±5†</td>
<td>196±7†</td>
<td>55.0±2.4</td>
<td>25.6±4.9</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>9</td>
<td>292±2†</td>
<td>196±6†</td>
<td>52.8±2.8</td>
<td>16.7±1.7</td>
</tr>
</tbody>
</table>

*P<0.05 and †P<0.01 vs untreated control.

**TABLE 2. Lipid Contents in the Renal Cortex**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TC (μg/mg wet weight)</th>
<th>TGs (μg/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.3±0.6</td>
<td>5.6±0.8</td>
</tr>
<tr>
<td>Ang II</td>
<td>10</td>
<td>6.4±0.7†</td>
<td>8.3±0.8†</td>
</tr>
<tr>
<td>Ang II+ deferoxamine</td>
<td>10</td>
<td>4.2±1.1</td>
<td>5.1±1.3</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>9</td>
<td>3.8±1.1</td>
<td>5.0±1.5</td>
</tr>
</tbody>
</table>

*P<0.05 vs untreated control.
observed granular materials are lipid droplets (Figure 2A and 2B). Cells with granular materials (ie, lipid droplets) had increased superoxide production (Figure 2B through 2G), which is consistent with a previous report. The levels of superoxide detected by DHE oxidation were increased after Ang II administration (control 100±10%, n=4 versus Ang II 209±30%, n=4; P<0.01). Only a small fraction of lipid-positive cells were found to be positive for iron deposition (Figure 2H and 2I, asterisks), which is again consistent with previous findings.

In Situ Hybridization for TGF-β1
The antisense but not sense (Figure 3A) probes for TGF-β1 mRNA showed positive staining in tubular epithelial cells (Figure 3B and 3D). Cells that expressed increased levels of TGF-β1 mRNA contained oil red O–stainable lipid deposits (Figure 3C and 3E). Higher magnification revealed that cells with increased levels of TGF-β1 mRNA contained lucid granular materials, which were presumably lipid droplets (Figure 3F). Lipid deposition was occasionally observed in the vascular wall cells, in which increased TGF-β1 mRNA expression was
observed (Figure 3G and 3H). On the other hand, vascular wall cells that did not contain lipid deposition did not express increased levels of TGF-β1 mRNA (Figure 3I and 3J).

Regulation of Genes Related to Lipid Metabolism and PAI-1

Western blot analysis showed that the expression of SREBP-1 and ABCA1 but not AR-B1 was increased in the kidneys of Ang II–treated animals (Figure 4A and 4B). Administration of norepinephrine did not significantly alter the expression of SREBP-1, and Ang II–induced upregulation of SREBP-1 was suppressed by iron chelation. (Figure 4C and 4D). Immunohistochemical and histological analysis showed that Ang II increased protein expression of SREBP-1 in the tubular cells, where deposition of oil red O–stainable lipid was positive (Figure 5).

Expression of HMG-CoA reductase, ACC, SCD-1, DGAT-1, LDL receptor, and fatty acid synthase (FAS) in the control kidney were examined by quantitative RT-PCR. In the kidney of control (n=10) and Ang II–infused (n=11) animals, expression of these genes were as follows: HMG-CoA reductase 100±7% versus 129±18%, respectively (P=NS); ACC 100±7% versus 84±5%, respectively (P=NS); SCD-1 100±41% versus 99±50%, respectively (P=NS); DGAT-1 100±17% versus 83±8, respectively (P=NS); LDL receptor 100±30% versus 165±20%, respectively (P<0.05); and FAS 100±10% versus 153±25%, respectively (P<0.05). Deferoxamine suppressed Ang II–induced upregulation of LDL receptor (76±9%; n=5; P=NS versus control) and FAS mRNA (70±19%; n=6; P=NS versus control). Ang II infusion increased PAI-1 mRNA expression in the kidney (control 100±22%; n=11 versus Ang II 191±24%, n=10; P<0.01), which was again suppressed by deferoxamine (137±30%; n=5; P=NS versus control).

Discussion

In the present study, we showed that long-term administration of Ang II but not norepinephrine caused robust accumulation of oil red O–stainable lipid in tubular epithelial cells and vascular wall cells in the rat kidney. Increased expression of

Figure 3. Lipid staining and in situ hybridization for TGF-β1 mRNA. Sections of unfixed frozen kidney samples were used. A, In situ hybridization using the TGF-β1 sense probe (background). B, D, F, H, and J, In situ hybridization using the TGF-β1 antisense probe. A through C, D and E, G and H, and I and J are serial sections. Tubular epithelial cells with increased expression of TGF-β1 mRNA are also positive for lipid deposition (B and C, ×200; D and E, ×100). Higher magnification shows granular materials in the cells with increased expression of TGF-β1 mRNA (F). Vascular wall cells with lipid deposits (G) expressed increased levels of TGF-β1 mRNA (H), and those without lipid deposits (I) did not express increased levels of TGF-β1 mRNA (J). Also note that tubular cells that contain lipid droplets (I) expressed an increase level of TGF-β1 mRNA (J). Original magnifications ×200 (A through C and G through J); ×100 (D and E); and ×400 (F).

Figure 4. Western blot analysis of genes related to lipid metabolism. A and B, Expression of ABCA1, SR-B1, SREBP-1, and SREBP-2 in the kidney of Ang II–infused animals. A, Representative Western blots. The size of the SREBP-1 bands shown in the figures was ~70 kDa and thus judged to correspond to the mature form. B, Summary of data from 5 to 7 experiments in each group. C and D, Effects of iron chelation and norepinephrine on SREBP-1 expression. C, Representative Western blots. D, Summary of data from 5 to 7 experiments in each group.
TGF-β1 mRNA, shown by in situ hybridization, and increased amount of superoxide, shown by DHE staining, were observed in cells with lipid deposition.

We showed previously that administration of Ang II to rats induces the expression of ferritin and heme oxygenase-1, an enzyme for heme degradation, in the renal tubular cells, and that tubular epithelial cells with increased expression of these proteins were positive for Prussian blue–stainable iron. We found that Ang II–induced aberrant iron homeostasis may have modulatory effects on the extent of urinary protein excretion and on the expression of genes that have relation to anti-aging and fibrogenesis. However, notably, in situ hybridization showed that the localization of upregulation of TGF-β1 mRNA and deposition of iron were found to occur in different renal tubular cells. In addition, cells that contain granular materials observed by DIC were also negative for iron staining. In the current study, the granular materials observed in the tubular cells were identified as lipid droplets, and cells with lipid deposition were found to have intense expression of TGF-β1 mRNA and increased levels of superoxide.

Lipid deposition in the kidney may not be a rare phenomenon in certain animal models and in humans. Several investigators advocated that it may play a crucial role in the development of renal damage, which might be, in part, mediated by the peroxynitrite molecules. Guirjarro et al reported that dietary-induced hyperlipidemia increased the renal expression of collagen and TGF-β. Eddy et al showed that diet-induced hypercholesterolemia induced renal lipid deposition and upregulation of TGF-β expression in the kidney of uninephrectomized rats. To the best of our knowledge, the present study is the first to demonstrate the colocalization of lipid deposits and increased TGF-β1 mRNA expression in animal models of human diseases.

Several previous studies suggested that Ang II may affect circulating lipid content. Hanefeld et al showed that treatment of hypertensive patients with Ang II type 1 (AT1) receptor blocker significantly reduced the plasma TC level. The AT1 receptor blocker was potent in reducing the plasma TG level, and conversely, Ang II infusion increased the plasma TG levels by stimulating hepatic TG production in rats. In our animal models, the plasma levels of TC and TGs also increased after Ang II infusion. Thus, it is possible that lipid deposition in the kidney occurred in response to the increased level of circulating lipids.

In the present study, we also found that expression of SREBP-1 and FAS was increased in the kidney of Ang II–infused animals. Previous studies showed the possible link between Ang II and FAS expression in the adipocyte. Our data suggested that Ang II may also increase FAS expression in the kidney, which might result in the increased production of lipids. Sun et al reported that SREBP-1 expression is upregulated in the kidney of diabetic animals. Interestingly, Sun et al also showed that induction of SREBP-1 caused upregulation of not only FAS but also of TGF-β1 in the kidney. Together with our findings, it is suggested that altered renal expression of lipogenesis-related genes may play a crucial role in the regulation of TGF-β1 expression. We also found that Ang II infusion increased the renal expression of LDL receptor. Whether this phenomenon is the underlying mechanism of increase in the cortical TC content after Ang II infusion should be investigated in future experiments.

Although underlying mechanisms that link between iron and lipid dysregulation in the kidney remain unknown, there are several possibilities. First, tubular cells in proteinuric states carry large amounts of lipids, and fatty elements may be correlated with the degree of proteinuria. Thus, iron chelation may have suppressed renal lipid deposition by decreasing the urinary excretion of protein. Second, in the current study, iron chelation reduced the increase in plasma lipid content in Ang II–infused animals. Because hyperlipidemia may cause lipid accumulation in the renal tubular cells, deferoxamine may have suppressed renal lipid accumulation by lowering circulating lipids. Third, Zager et al reported the possible relationship between (heme) iron overload and regulation of lipogenesis-related genes in the kidney. We found that expression of Ang II–induced upregulation of FAS mRNA was inhibited by iron chelation. Thus, aberrant iron homeostasis may directly modulate expression of lipogenesis-related genes in the kidney. However, if so, whether certain paracrine substances would be released from the iron-positive cells that may alter the expression of lipogenesis-related genes should be investigated because iron deposition and lipid accumulation occurred in the different tubular cells.
In the present study, Ang II infusion also induced lipid deposition in the vascular wall cells, in which TGF-β1 mRNA expression was found to be upregulated. It has been shown that Ang II plays a crucial role in the lipid deposition in the arterial wall, an early event of atherogenic processes, and TGF-β upregulation in the aortas. We are now examining whether upregulation of TGF-β1 mRNA in the kidney is a downstream event of lipid deposition and whether lipid deposition also occurs in the aorta in the Ang II-infused rat.

In conclusion, long-term administration of Ang II caused robust accumulation of lipids in the rat kidney. TGF-β1 mRNA expression and superoxide production were found to be increased in renal tubular cells and vascular wall cells that contained oil red O-stainable lipids. Iron chelation suppressed the increases in serum lipid levels and in the lipid content of the renal cortical tissues in Ang II-infused rats. These findings collectively suggest the existence of a relationship among altered lipid metabolism, aberrant iron homeostasis, and the extent of renal injury in the kidneys of Ang II-infused rats.

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References

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