Aortic Iron Overload With Oxidative Stress and Inflammation in Human and Murine Abdominal Aortic Aneurysm

Hisashi Sawada,* Hiroyuki Hao,* Yoshiro Naito, Makiko Oboshi, Shinichi Hirotani, Masataka Mitsuno, Yuji Miyamoto, Seiichi Hirota, Tohru Masuyama

Objective—Although iron is an essential element for maintaining physiological function, excess iron leads to tissue damage caused by oxidative stress and inflammation. Oxidative stress and inflammation play critical roles for the development of abdominal aortic aneurysm (AAA). However, it has not been investigated whether iron plays a role in AAA formation through oxidative stress and inflammation. We, therefore, examined whether iron is involved in the pathophysiology of AAA formation using human AAA walls and murine AAA models.

Approach and Results—Human aortic walls were collected from 53 patients who underwent cardiovascular surgery (non-AAA=34; AAA=19). Murine AAA was induced by infusion of angiotensin II to apolipoprotein E knockout mice. Iron was accumulated in human and murine AAA walls compared with non-AAA walls. Immunohistochemistry showed that both 8-hydroxy-2′-deoxyguanosine and CD68-positive areas were increased in AAA walls compared with non-AAA walls. The extent of iron accumulated area positively correlated with that of 8-hydroxy-2′-deoxyguanosine expression area and macrophage infiltration area in human and murine AAA walls. We next investigated the effects of dietary iron restriction on AAA formation in mice. Iron restriction reduced the incidence of AAA formation with attenuation of oxidative stress and inflammation. Aortic expression of transferrin receptor 1, intracellular iron transport protein, was increased in human and murine AAA walls, and transferrin receptor 1–positive area was similar to areas where iron accumulated and F4/80 were positive.

Conclusions—Iron is involved in the pathophysiology of AAA formation with oxidative stress and inflammation. Dietary iron restriction could be a new therapeutic strategy for AAA progression. (Arterioscler Thromb Vasc Biol. 2015;35:1507-1514. DOI: 10.1161/ATVBAHA.115.305586.)

Key Words: aneurysm ■ inflammation ■ iron ■ oxidative stress

Abdominal aortic aneurysm (AAA) is a common vascular disease, which occurs in approximately 1% to 8% of men aged 65 to 80 years. Aortic rupture is the most feared clinical consequence of AAA progression, resulting in mortality in ≈90% of cases. Because AAA usually progresses without symptoms, AAA is often discovered in advanced stage. At present, surgical aortic replacement and endovascular stent graft repair are performed as standard definitive therapies for AAA. However, there is no effective medical therapy to prevent aortic rupture in AAA. Although several studies have reported the pathophysiology of AAA, the mechanisms of AAA formation are largely unknown. Therefore, it is necessary to investigate the molecular pathophysiology of AAA to find noninvasive strategies for the prevention of AAA.

Iron is an essential element for maintaining physiological function. However, excess iron causes tissue damage by oxidative stress via the Fenton/Haber–Weiss reaction. Therefore, iron is involved in the pathophysiology of several diseases including cardiovascular diseases. In fact, we have previously shown that iron accumulation and superoxide production are observed in the renal tubules of a rat model of chronic kidney disease. Lee et al have shown that iron accumulation is observed in the atherosclerotic lesions of apolipoprotein E knockout mice. Most recently, Martinez-Pinna et al have demonstrated that iron is deposited in human AAA walls. Meanwhile, we have also reported that dietary iron restriction (IR) prevents the development of hypertension and proteinuria with inhibition of oxidative stress and inflammation in Dahl salt-sensitive hypertensive rats. Although oxidative stress and inflammation are well known to be involved in the development of AAA formation, it has not been investigated whether iron is associated with the pathophysiology of AAA through...
oxidative stress and inflammation. Furthermore, the effects of dietary IR on AAA formation remain unknown. In the present study, we investigated whether iron is involved in the pathophysiology of AAA. In addition, we examined the effects of dietary IR on the development of AAA through oxidative stress and inflammation in a murine model of AAA.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Iron Accumulation in Human AAA Walls

We first examined iron accumulation in human AAA walls. The patient characteristics are shown in the Table. There was no significant difference between 2 groups except the proportions of male and smoking patients. Berlin blue staining revealed that iron was markedly accumulated in AAA walls but was not detected in non-AAA walls (Figure 1A). Aortic iron content of tissue homogenate was also increased in AAA walls compared with non-AAA walls (Figure 1B). Next, we performed immunohistochemistry for 8-hydroxy-2'-deoxyguanosine (8-OHdG) to assess oxidative stress in human aortic walls. Focal 8-OHdG-positive areas were constantly detected in AAA walls, whereas few positive areas were found in non-AAA walls (Figure 1A; Figure I in the online-only Data Supplement). By serial sections of human AAA walls, the distribution of Berlin blue–positive area was similar to that of 8-OHdG and CD68-positive areas (Figure 1A). Furthermore, the extent of Berlin blue–positive area in AAA walls positively correlated with that of both 8-OHdG and CD68-positive areas (Figure 1C and 1D). Thus, these results from human aortic walls suggest that iron is involved in the pathophysiology of AAA and is associated with oxidative stress and inflammation in AAA.

IR Inhibited Angiotensin II–Induced AAA Formation

We then examined whether iron is involved in the pathophysiology of a murine model of AAA. Berlin blue staining demonstrated that iron was accumulated in AAA walls of angiotensin II (AngII) mice as well as in human AAA walls (Figure 2A in the online-only Data Supplement). By serial sections of human AAA walls, the development of Berlin blue–positive area was similar to that of 8-OHdG and CD68-positive areas (Figure 1A). Furthermore, the extent of Berlin blue–positive area in AAA walls positively correlated with that of both 8-OHdG and CD68-positive areas in morphometric analysis (Figure 1C and 1D). Thus, these results from human aortic walls suggest that iron is involved in the pathophysiology of AAA and is associated with oxidative stress and inflammation in AAA.

Table. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Non-AAA</th>
<th>AAA</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>34</td>
<td>19</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>74±9</td>
<td>71±11</td>
<td>0.29</td>
</tr>
<tr>
<td>Male/female</td>
<td>13/23</td>
<td>15/4</td>
<td></td>
</tr>
<tr>
<td>Smoke</td>
<td>15 (44%)</td>
<td>15 (79%)</td>
<td>0.02</td>
</tr>
<tr>
<td>HT</td>
<td>24 (71%)</td>
<td>16 (84%)</td>
<td>0.33</td>
</tr>
<tr>
<td>DL</td>
<td>15 (44%)</td>
<td>12 (63%)</td>
<td>0.25</td>
</tr>
<tr>
<td>DM</td>
<td>6 (18%)</td>
<td>2 (11%)</td>
<td>0.70</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>7 (21%)</td>
<td>3 (16%)</td>
<td>1.00</td>
</tr>
<tr>
<td>ACE-I/ARB</td>
<td>22 (65%)</td>
<td>12 (63%)</td>
<td>1.00</td>
</tr>
<tr>
<td>CCB</td>
<td>14 (41%)</td>
<td>12 (63%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Statins</td>
<td>12 (35%)</td>
<td>9 (47%)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD and as numbers of patients and percentages for categorical variables. AAA indicates abdominal aortic aneurysm; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II type 1 receptor blockers; CCB, calcium channel blockers; DL, dyslipidemia; DM, diabetes mellitus; HT, hypertension; and non-AAA, patients with non–abdominal aortic aneurysm.
We next investigated the effects of dietary IR on AAA formation in this murine model. AngII mice demonstrated 67% incidence of AAA, whereas dietary IR reduced the incidence of AAA to 6% (Figure 2C). During the experimental period, 10 of 27 mice (37%) died of aortic rupture in AngII mice but not in AngII-IR mice. Maximal abdominal aortic diameter was significantly increased in AngII mice compared with control mice, while this change was attenuated by IR (Figure 2D and 2E). Systolic blood pressure was increased in both AngII and AngII-IR mice compared with control mice, and there was no significant difference between AngII and AngII-IR mice (Figure 2F). Aortic iron content was lower in AngII-IR mice than in AngII mice (Figure 2B), and aortic iron accumulation was hardly detected in the abdominal aortas of AngII-IR mice (Figure 2G). Neither aortic 8-OHdG nor F4/80 was detected in AngII-IR mice (Figure 2G). As shown in Table II in the online-only Data Supplement, red blood cell counts were not different among the 3 groups. Blood hemoglobin content was higher in AngII mice than in control mice, whereas blood hemoglobin content and hematocrit value were decreased in AngII-IR mice compared with those in AngII mice. Serum iron levels were increased in AngII mice when compared with control mice, whereas serum ferritin levels were not different between control and AngII mice. Meanwhile, serum iron and ferritin levels were decreased in AngII-IR mice when compared with those in AngII mice. Serum erythropoietin levels were not statistically different among the 3 groups. These results indicate that dietary IR inhibits the development of AAA formation with attenuation of oxidative stress and inflammation in AngII-induced AAA mice.

**IR Attenuated Inflammatory and Fibrotic Responses in AngII-Induced AAA Mice**

It is well known that inflammation and fibrosis are important in the mechanisms of AAA formation. Therefore, we examined the effects of dietary IR against inflammation and fibrosis in AngII-induced AAA mice. The abdominal aortas of AngII mice demonstrated an increase of inflammatory cell infiltration into media and adventitia (Figure 3A; Table II in the online-only Data Supplement), medial and adventitial fibrosis (Figure 3B; Figure III in the online-only Data Supplement), and elastin degradation and thinning of media (Figure 3C; Figure III in the online-only Data Supplement). In contrast, these changes were apparently attenuated in the abdominal aortas of AngII-IR mice (Figure 3A–3C). Aortic expression of inflammation-related genes, such as F4/80 and monocyte chemotactic protein-1, was increased in AngII mice, whereas these increases were attenuated by IR (Figure 3D). Aortic interleukin-6 gene expression tended to be increased in AngII mice but not in AngII-IR mice (Figure 3D). Similar to inflammation-related genes, aortic expression of tumor growth factor-β, Collagen I, and Collagen III was increased in AngII mice, whereas these increases were suppressed in AngII-IR mice (Figure 3E).

**IR Attenuated Aortic Matrix Metalloproteinase Activity and c-Jun N-Terminal Kinase Phosphorylation in AngII-Induced AAA Mice**

To find out the mechanism of suppressive effects of dietary IR on AAA formation, we evaluated aortic matrix metalloproteinase (MMP) activity and c-Jun N-terminal kinase (JNK) phosphorylation in AngII-induced AAA mice. Both MMP-2 and MMP-9 activities were increased in the aortas of AngII mice, whereas these increases were attenuated in AngII-IR mice (Figure 4A). JNK phosphorylation was increased in the aortas of AngII mice, and it was suppressed in AngII-IR mice (Figure 4B). Phosphorylation...
Iron was involved in MMP activity and JNK phosphorylation in macrophages. Iron chelation attenuated MMP activity and JNK phosphorylation in macrophages. Iron was involved in MMP activity and JNK phosphorylation in AAA walls, and iron accumulation was detected in macrophages of AAA walls. From these results, aortic iron...
Iron and Oxidative Stress in AAA Walls

Data Supplement).

Furthermore, these increments were observed in vehicle-treated cells (Figure VIIA in the online-only Data Supplement). Hence, we further investigated whether iron is involved in MMP activity and JNK phosphorylation in AAA walls. MMP-9 activity was increased in AngII-treated THP-1 cells compared with vehicle-treated THP-1 cells, whereas iron chelation with deferoxamine (DFO) suppressed this increment. Phosphorylation of JNK, extracellular signal–regulated kinase (ERK), and p38 did not alter with or without DFO in AngII-treated THP-1 cells (Figure 6B). Finally, we assessed TfR1 and ferroportin expression in AngII-treated THP-1 cells. We noticed that the expression of TfR1 and ferroportin was increased in AngII-treated THP-1 cells compared with vehicle-treated cells (Figure VIIA in the online-only Data Supplement). Furthermore, these increments were observed in AngII-treated macrophages (Figure VIIIB in the online-only Data Supplement).

**Discussion**

**Iron and Oxidative Stress in AAA Walls**

Oxidative stress contributes to the pathophysiology of inflammation and tissue damage.\(^{14,15}\) These features are common to many chronic diseases including cardiovascular diseases.\(^{16,17}\)

It is obvious that AAA demonstrates not only passive enlargement of aortic wall but also inflammation and tissue degradation. During the past decade, oxidative stress is reported to be one of important components in the pathogenesis of AAA.\(^{18}\)

Oxidative stress is increased in AAA walls, and it causes aortic structural disorder through activation of inflammation and

**Figure 4.** Effects of dietary iron restriction on matrix metalloprotease (MMP) activity and c-Jun N-terminal kinase (JNK) phosphorylation in abdominal aortic aneurysm (AAA) walls. A, Gelatin zymography of aortic tissue homogenates from control, angiotensin II (AngII), and AngII-iron restriction (IR) mice. Densitometric quantifications of gelatinolytic activities are shown in the lower column of zymogram. B, Western blot analysis of phosphorylated and total state of JNK, extracellular signal–regulated kinase (ERK), and p38 in the aortas of control, AngII, and AngII-IR mice. The expression of phosphorylated state is normalized with that of total state in the graph. \(n=4\) to 6 per group. *\(P<0.05\) vs control and †\(P<0.05\) vs AngII.

**Figure 5.** Transferrin receptor 1 (TfR1) expression in human and murine abdominal aortic aneurysm (AAA) walls. A, Western blot analysis of TfR1 in human non-AAA and AAA walls. TfR1 expression was standardized on the basis of GAPDH expression in the graph. B, Representative images of TfR1 staining of human non-AAA and AAA walls and results of morphometric analysis. Box area shows high magnification view. C, Western blot analysis of TfR1 in the aortas of control and angiotensin II (AngII) mice. TfR1 expression was standardized on the basis of GAPDH expression in the graph. D, Representative images of TfR1 staining of murine aortas of control and AngII mice and results of morphometric analysis. Arrowheads indicate TfR1-positive areas. E, Representative images of double immunofluorescence staining of TfR1 (green) and F4/80 (red) in AAA walls of AngII mice. F, Representative images of immunohistochemistry for TfR1, Berlin blue staining, and immunohistochemistry for F4/80 in AAA walls of CaCl\(_2\)-induced AAA mice. Arrowheads indicate TfR1-positive areas. *\(P<0.05\) vs non-AAA or control. Scale bars, 50 \(\mu\)m for human aortas and 100 \(\mu\)m for murine aortas. A indicates adventitia; L, lumen; and M, media.
Iron and Inflammation in AAA Walls

Macrophage-mediated vascular inflammation plays a key role in the pathophysiology of AAA. For instance, macrophages secrete inflammatory cytokines and MMP in response to oxidative stress in aortic walls, which lead to aortic structural disruption. Meanwhile, iron homeostasis is reported to be associated with macrophage immune functions. In this study, we demonstrated that iron accumulation and macrophage infiltration were detected in human and murine AAA walls compared with non-AAA walls, and iron accumulated area was close to macrophage infiltrated area in AAA walls. In addition, dietary IR inhibited AAA formation with attenuation of aortic iron accumulation and macrophage infiltration in AngII-induced AAA mice. Dietary IR also suppressed inflammatory-related gene expression and MMP activity in these mice. Moreover, iron chelation with DFO suppressed MMP activity in AngII-treated THP-1 cells. Taken together, these results indicate that iron is associated with macrophage-mediated vascular inflammation in AAA walls.

Mechanism of Iron Accumulation in AAA Walls

Several studies have reported that intravascular hemorrhage and thrombus were complicated with AAA. Thus, intravascular hemorrhage and thrombus are considered to be one of the causes of aortic iron accumulation. Although we removed adherent thrombus from human AAA walls as much as possible in this study, adherent thrombus may still have remained. Therefore, it is difficult to exclude the possibility of iron from the remaining thrombus in human AAA walls. This issue is an important limitation of this study. However, iron accumulation was detected in both AngII- and CaCl2-induced AAA walls in mice. Intravascular hemorrhage and thrombus are not complicated with CaCl2-induced AAA walls in mice. In addition, Berlin blue staining is dyed in response to trivalent ferric ion (Fe3+), but not bivalent ferric ion (Fe2+). Because hemoglobin is consisted of Fe3+, iron inside hemoglobin is not stained by Berlin blue staining directly. These findings indicate that there are other mechanisms of aortic iron accumulation than intravascular hemorrhage and thrombus in AAA walls.

Iron is vital for organisms and contributes to a wide range of metabolic processes. However, because excessive iron leads to tissue damage, tissue iron concentration is tightly regulated. Intracellular iron transport occurs through Tfr1 when iron uptake is needed. Therefore, Tfr1 expression is normally upregulated under low iron condition. In contrast, high iron condition makes Tfr1 downregulation. In this study, we found increased iron content and Tfr1 expression in both human and murine AAA walls. These results suggest that dysregulated aortic Tfr1 may induce aortic iron overload. Furthermore, aortic expression of ferroportin as a key regulator of macrophage iron content was increased in AngII mice compared with control mice. Failure of iron homeostasis in AAA walls may cause aortic iron overload with oxidative stress and inflammation, which may contribute to AAA formation.

IR Attenuates MMP Activity and JNK Phosphorylation in AAA Walls

AAAs are characterized by a permanent dilation of aortic walls, which results from extracellular matrix degeneration. MMP

proteolytic enzyme. These processes progressively weaken aortic walls and lead to AAA formation. In addition, previous studies have reported that antioxidant subjects inhibit AAA formation via attenuation of MMP activity in murine AAA models. Therefore, oxidative stress is considered to be one of the therapeutic targets for AAA. However, it is well known that iron is a powerful mediator of oxidative stress. Excess iron promotes reactive oxygen species production through the Fenton/Haber–Weiss reaction, thereby leading to tissue damage. Hence, iron is reported to be associated with various diseases. We have previously demonstrated that iron is involved in the pathophysiology of hypertension and chronic kidney disease. Moreover, these reports have shown that dietary IR attenuates the progression of cardiovascular remodeling and oxidative stress. We, therefore, hypothesized that iron is also involved in the pathophysiology of AAA and dietary IR could inhibit AAA formation via suppression of oxidative stress. In the present study, iron accumulation was increased in human and murine AAA walls compared with non-AAA walls, and iron accumulated area was close to 8-OHdG-positive area in AAA walls. Furthermore, dietary IR attenuated the incidence of AAA along with suppression of aortic iron accumulation and oxidative stress in AngII-induced AAA mice. Collectively, these results suggest that iron is involved in the pathophysiology of AAA through oxidative stress.
activation is known as a major cause of aortic wall disruption, and its activation is reported to be regulated by JNK in AAA walls. Consistent with these report findings, MMP-2 and MMP-9 activities and JNK phosphorylation were increased in AAA walls of AngII mice. Dietary IR attenuated these increments and inhibited AAA formation in AngII mice. Of interest, aortic MMP-9 activity and JNK phosphorylation were increased in AngII mice at 5 days after AngII infusion before AAA formation; however, aortic oxidative stress and inflammation were not increased in AngII-5d mice compared with control mice. Meanwhile, dietary IR attenuated these increases of aortic MMP-9 activity and JNK phosphorylation in AngII-5d mice. These results suggest that iron is associated with aortic MMP-9 activity and JNK phosphorylation without involvement of oxidative stress and inflammation in an acute phase (5 days) of AngII-induced AAA formation. However, in a chronic phase (4 weeks) of AngII-induced AAA formation, iron may contribute to aortic MMPs activities and JNK phosphorylation along with the increments of oxidative stress and inflammation. In addition, iron chelation with DFO inhibited MMP activity and JNK phosphorylation in AngII-treated THP-1 cells. Because macrophages are reported to secrete mainly MMP-9, DFO may not affect MMP-2 activity in THP-1 cells. However, these results suggest that iron may be associated with MMP activity and JNK phosphorylation of macrophages in AAA walls. Therefore, iron accumulation in macrophages could be involved in AAA formation through increment of aortic MMP activity and JNK phosphorylation.

In this study, phosphorylation of ERK and p38 was not increased in AngII mice and AngII-treated THP-1 cells. It is generally reported that phosphorylation of ERK and JNK is increased in AngII-induced AAA tissues, whereas p38 phosphorylation is not changed in AAA walls. However, it is reported that phosphorylation of ERK and JNK vary by the region of aorta. Because we used whole aortic tissues to examine protein expression, this apparent discrepancy may have occurred because of these concerns. Meanwhile, AngII is known to induce mitogen-activated protein kinase (MAPK) phosphorylation in THP-1 cells. Many in vitro studies examined MAPK phosphorylation at 24 hours after AngII treatment. To assess the effects of iron chelation on MAPK phosphorylation after AngII treatment, we examined MAPK phosphorylation at 48 hours after AngII treatment. Thus, some discrepancies in THP-1 cells may have been caused by the differences in experimental protocol.

IR for Patients With AAA

Several previous reports have documented that active reduction of iron stores by phlebotomy can reduce the risk for future cardiovascular events and new onset solid organ cancer as well. We have previously reported that dietary IR prevents the development of hypertension and proteinuria with inhibition of oxidative stress and inflammation in Dahl salt-sensitive hypertensive rats. Dietary IR is currently beginning to be performed as an amlatory therapy against patients with chronic hepatitis C. Iwasa et al reported that dietary IR improves aminotransferase levels in patients with chronic hepatitis C. Hepatic iron deposition is increased in patients with chronic hepatitis C, which leads to hepatic tissue damages. Meanwhile, total body iron stores in adult men are several folds higher than present in younger men or similarly aged (or younger) women. In this study, serum iron levels were increased in AngII mice compared with control group, whereas serum ferritin levels were not different between control and AngII mice. Although future studies looking at underlying mechanisms how serum iron levels were not different between control and AngII-IR mice. Our study revealed that oxidative stress and inflammation accompanied by iron overload were detected in human and murine AAA walls, and dietary IR inhibited AAA formation with suppression of oxidative stress and inflammation in AngII mice. Therefore, dietary IR may be a new therapeutic strategy for AAA.

Conclusions

In this study, we demonstrated iron accumulation with oxidative stress and inflammation in human and murine AAA walls and how dietary IR inhibits AAA formation in mice. Our results would indicate the involvement of iron in the pathophysiology of AAA with oxidative stress and inflammation, and thus dietary IR could be a new therapeutic strategy for AAA.

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Disclosures

None.

References


Significance

Although iron is an essential element for maintaining of physiological function, excess iron leads to tissue damage caused by oxidative stress and inflammation. Therefore, iron is known to be involved in the pathophysiology of several cardiovascular diseases such as atherosclerosis. We show here for the first time the iron accumulation with oxidative stress and inflammation in human and murine abdominal aortic aneurysm (AAA) walls. Furthermore, dietary iron restriction inhibited AAA formation with attenuation of oxidative stress and inflammation in angiotensin II–induced AAA mice. In addition, we found increased expression of transferrin receptor 1, a key regulator of iron homeostasis, in both human and murine AAA walls. These findings suggest that failure of iron homeostasis in AAA walls may cause aortic iron overload with oxidative stress and inflammation, which may contribute to AAA formation. Dietary iron restriction could be a new therapeutic strategy for AAA formation.
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Materials and Methods

Human aortic walls
AAA walls were obtained from patients undergoing aortic replacement for AAA (AAA, n=19). Non aneurysmal walls were extracted from ascending aortas of patients undergoing aortic valve replacement for aortic valve stenosis or aortic valve regurgitation at the time of establishment of cardiopulmonary bypass (non-AAA, n=34). Patients with thoracic aortic aneurysm were excluded from this study. Aortic wall was quickly fixed in buffered 4% paraformaldehyde and embedded in paraffin. Informed consent was obtained from each patient. The institutional ethics committee reviewed the protocol and approved this study.

Angiotensin II-induced AAA model mice
Ten-week-old apolipoprotein E-knockout male mice (C57BL/6J background) were used for this study. An osmotic minipump (Model 2004; DURECT Corporation, CA, USA) was implanted subcutaneously to infuse vehicle or angiotensin II (1000ng/kg/min, Peptide Institute, Inc., Osaka, Japan) as previously described. Angiotensin II-infused mice were divided into two groups; one fed a normal diet and the other fed an iron-restricted diet for 5 days (AngII-5d, n=3 and AngII-5d-IR, n=3) or 4 weeks (AngII, n=27 and AngII-IR, n=16). Vehicle-infused mice given a normal diet were served as controls (Control, n=14). All the mice were maintained on a 12-h light/dark cycle and had free access to food and water. All our experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine. The nutrients of a normal diet consisted of cornstarch 33%, casein 22%, cellulose 5%, sucrose 30%, corn oil 5%, mineral mixture 4%, and vitamin mix 1%. The mineral mixture contained CaHPO₄·2H₂O 0.43%, KH₂PO₄ 34.31%, NaCl 25.06%, FeC₆H₅O₇·5H₂O 0.623%, MgSO₄ 4.8764%, ZnCl₂ 0.02%, MnSO₄·5H₂O 0.121%, CuSO₄·5H₂O 0.156%, KI 0.0005%, CaCO₃ 29.29%, (NH₄)₆Mo₇O₂₄·4H₂O 0.0025%, and microcrystal line cellulose 5.11%. An iron-restricted diet was based on a normal diet with a mineral mixture free of FeC₆H₅O₇·5H₂O, and added microcrystal line cellulose instead of FeC₆H₅O₇·5H₂O as previously described (Supplemental Table I)². Systolic blood pressure was measured by a noninvasive computerized tail-cuff system (Muromachi Kikai Co., Ltd., Tokyo, Japan). At the end of study, blood sample was obtained from the heart, and the aorta was irrigated with cold phosphate buffered saline at physiologic pressure through the left ventricle. The peri-adventitial tissue was carefully removed from the aortic walls, and then maximal aortic diameter was measured. A >50% increment in external diameter of the abdominal aorta was used to define the occurrence of AAA. The aorta was snap-frozen in liquid nitrogen or fixed in buffered 4% paraformaldehyde and embedded in paraffin.
**CaCl\_2 induced AAA model mice**

AAA was induced with periaortic application of 0.5M CaCl\_2 as described previously \(^3\). Briefly, 8-10 week old male C57BL/6J mice were used (n=4). Mice were anesthetized with 1-2% isoflurane, the aorta was isolated from the inferior vena cava, and sterile gauze soaked in 0.5M sterile CaCl\_2 was applied on its external surface for 15 minutes. At six weeks after CaCl\_2 application, the aorta was removed and fixed in buffered 4% paraformaldehyde.

**Cell culture**

Human monocytic THP-1 cells were purchased from American Type Culture Collection (ATCC, VA, USA). Cells were cultured in RPMI1640 (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, CA, USA) under standard conditions (humidified atmosphere of 5% CO\_2 at 37°C). The medium was changed every 2 or 3 days. Cells were seeded into a 6-well plate at 3x10\(^5\) cells/ml. Iron chelation for THP-1 cells were performed by treating with deferoxamine (DFO; Novartis Pharma K.K. Tokyo, Japan, 50µM). Following 24 hours DFO treatment, THP-1 cells were exposed to angiotensin II (10µM) for 48 hours. Cells were harvested using 100 µl lysis buffer (Cell Signaling Technology, MA, USA) after treatment with angiotensin II. To differentiate into macrophages, THP-1 cells were exposed to phorbor myristate acetate (10ng/ml, Sigma-Aldrich, MO, USA) for 48 hours. Then, angiotensin II (10µM) was added to the RPMI1640 medium for 48 hours. Cells were harvested using 100 µl lysis buffer (Cell Signaling Technology) after treatment with angiotensin II.

**Western blot analysis**

The aortic walls, THP-1 cells, and macrophages were homogenized on ice with 100µl lysis buffer (Cell Signaling Technology), and the lysates were spun at 15000×g for 30 minutes at 4°C to collect the supernatants. Protein concentration was specified using the Lowry method. The total protein homogenate (25µg) from the aorta was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The expression levels of proteins were detected by an enhanced chemiluminescence kit (Thermo Scientific, IL, USA). The antibodies used were against rabbit antiphospho-c-Jun N-terminal kinase (JNK) (Thr183/tyr185), JNK, phospho-extracellular signal-regulated kinase (ERK) (Thr202/Tyr204), ERK, phospho-p38 (Thr180/Tyr182), p38 (Cell Signaling Technology; dilution 1:1000), transferrin receptor1 (TfR1) (Zymed Laboratories, CA, USA; dilution 1:1000), ferroportin (Alpha Diagnostic International, TX, USA, dilution 1:1000), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology; dilution 1:1000). Expression of TfR1 and ferroportin was standardized on the basis of GAPDH expression.
Matrix metalloproteinase (MMP)-2 and MMP-9 Activity Assays

MMP-2 and MMP-9 activities in homogenates of the aortic walls and THP-1 cells were determined by gelatin zymography. Twenty micrograms of protein was measured in each experiment. Gelatin zymography was performed using Gelatin Zymo-Electrophoresis Kit (COSMO BIO co., ltd. Tokyo, Japan) according to the manufacturer’s directions.

Gene expression analysis

Total RNA was isolated from the aortic walls using TRIzol reagent (Invitrogen). Real-time reverse transcription polymerase chain reactions (RT-PCR) were performed using ABI PRISM 7900 with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, CA, USA). Real time RT-PCR were performed using mouse specific primers and probes for Emr1 (also known as F4/80; Mm00802529_m1), CCL2 [also known as monocyte chemotactic protein-1; Mm00441242_m1], interleukin-6 (Mm00446190_m1), tumor growth factor-β (Mm03024053_m1), collagen I (Mm0080166_g1), and collagen III (Mm00802331_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1) was used as internal control.

Histological analysis

The aortic walls were quickly fixed in buffered 4% paraformaldehyde, embedded in paraffin, and cut into 4-µm-thick sections. Hematoxylin and eosin, Masson’s trichrome, Elastica van Gieson, and Berlin blue staining were performed using standard protocols. Immunohistochemical and immunofluorescence staining were performed using standard protocols. Immunohistochemical and immunofluorescence staining were performed with a primary mouse anti-8-Hydroxy-2’-deoxyguanosine (8-OHdG) antibody (JaICA, Shizuoka, Japan; dilution 1:50), a primary mouse anti-CD68 antibody (DAKO, Glostrup, Denmark; dilution 1:100), a primary rat anti-F4/80 antibody (AbD Serotec, Oxford, UK; dilution 1:15000), and a primary mouse anti-TfR1 antibody (Zymed Laboratories; dilution 1:200). Heat-induced epitope retrieval was always performed. As a negative control antibody staining, species- and isotype-matched IgG were used in place of primary antibody. Positive control antibody staining of TfR1 was performed with a primary mouse anti-TfR1 antibody (Zymed Laboratories; dilution 1:200) using the spleen of apoliprotein E-knockout mice. The stained areas were quantified using ImageJ software by scanning 10 non-overlapping fields in each 6 aortic serial sections and expressing the positive areas as a percentage of the total area.
**Assessments of aortic iron content, blood cell count, serum iron, ferritin, and erythropoietin levels.**

Human and murine aortic iron contents were assessed by atomic absorption spectrometry (SRL Inc, Tokyo, Japan). Peripheral blood cell count was measured using an automatic cell count analyzer (Pentra 60 LC-5000, Horiba, Kyoto, Japan). Serum iron levels were determined as previously described. Serum ferritin levels were determined using ELISA Kit (Immunology Consultants Laboratory, OR, USA). Serum erythropoietin levels were measured using Mouse Erythropoietin Quantikine ELISA Kit (R&D systems, MN, USA).

**Statistical analysis**

Data are presented as the mean ± SD for human experiments or the mean ± SEM for murine and *in vitro* experiments. The assumption of normality and equal variance were assessed by Shapiro-Wilk test and Levene’s test, respectively. Statistical analysis was performed using Student’s *t* test for homogeneous variance normal data, Welch’s *t* test for heterogeneous normal data, and Mann-Whitney *U* test for non-normal data. One way ANOVA was performed to detect differences between groups, and Tukey-Kramer test was used for multiple pairwise comparisons of means among groups. Correlations were assessed with Spearman’s rank correlation coefficient. Values of *p*<0.05 were considered statistically significant.

**References**

## Supplemental Table I. Composition of diet

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<th>Normal diet (%)</th>
<th>Iron restricted diet (%)</th>
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<td>Cornstarch</td>
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<tr>
<td>Casein</td>
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<tr>
<td>Cellulose</td>
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<td>Corn oil</td>
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<td>Vitamin mixture</td>
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<td>CaHPO₄·2H₂O</td>
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<td>NaCl</td>
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<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
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<td>Microcrystal line cellulose</td>
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<td>total</td>
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Supplemental Table II. Hematological parameters in angiotensin II-induced AAA model mice

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<th>Control</th>
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<th>AngII-IR</th>
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<tr>
<td>Red blood cells (10^4/mm³)</td>
<td>953.0 ± 21.6</td>
<td>1114.3 ± 40.5</td>
<td>1001.1 ± 64.7</td>
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<td>Hemoglobin (g/dL)</td>
<td>13.5 ± 0.3</td>
<td>16.5 ± 0.6*</td>
<td>13.7 ± 0.9†</td>
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<td>Hematocrit (%)</td>
<td>46.2 ± 1.0</td>
<td>54.7 ± 2.4</td>
<td>44.1 ± 3.2†</td>
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<tr>
<td>Serum iron levels (µg/dL)</td>
<td>114.8 ± 2.7</td>
<td>163.5 ± 3.1*</td>
<td>84.5 ± 9.0†</td>
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<tr>
<td>Serum ferritin levels</td>
<td></td>
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<tr>
<td>(ng/mL)</td>
<td>28.9 ± 2.0</td>
<td>34.9 ± 2.9</td>
<td>25.6 ± 2.1†</td>
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<tr>
<td>Serum erythropoietin</td>
<td></td>
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<tr>
<td>levels (pg/mL)</td>
<td>231.3 ± 56.0</td>
<td>165.6 ± 27.6</td>
<td>529.2 ± 196.6</td>
</tr>
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</table>

Values are expressed as means ± SEM. Control, vehicle-infused mice fed a normal diet; AngII, angiotensin II-infused mice fed a normal diet; AngII-IR, angiotensin II-infused mice fed an iron-restricted diet. *p<0.05 compared with Control mice, †p<0.05 compared with AngII mice.
Supplemental Figure I
Representative negative control antibody staining of 8-OHdG and CD 68 in human AAA walls. Scale bars: 50µm.
Supplemental Figure II

(A) Representative low magnification images of Berlin blue staining and immunohistochemistry for 8-OHdG and F4/80 in AAA walls of AngII mice. Scale bars: 200µm. (B) Representative negative control antibody staining of 8-OHdG and F4/80 in AAA walls of AngII mice. Scale bars: 100µm.
Supplemental Figure III
Representative low magnification images of HE, MTc, and EVG staining in AAA walls of AngII mice. Scale bars: 200µm.
**Supplemental Figure IV**

(A) Representative images of HE staining and immunohistochemistry for 8-OHdG and F4/80 staining in the abdominal aortas of AngII-5d mice. Scale bars: 100µm. (B) Gelatin zymography of aortic tissue homogenates from Control, AngII-5d, and AngII-5d-IR mice. Densitometric quantifications of gelatinolytic activities are shown in the lower column of zymogram. n=3 per group. *p<0.05 vs Control and †p<0.05 vs AngII-5d.
Supplemental Figure IV

(C) Western blot analysis of phosphorylated and total state of JNK, ERK, and p38 in the aortas of Control, AngII-5d, and AngII-5d-IR mice. The expression of phosphorylated state is normalized with that of total state in the graph, respectively. n=3 per group. *p<0.05 vs Control and †p<0.05 vs AngII-5d.
Supplemental Figure V

(A) Representative negative control antibody staining of TfR1 in AAA walls of human and AngII mice. (B) Representative positive and negative control antibody staining of TfR1 in the spleens of apoe^- mice. Scale bars: 100µm. (C) Representative low magnification images of TfR1 staining in AAA walls of AngII mice. Scale bars: 200µm. (D) Representative image of triple staining of TfR1 (red), Berlin blue (blue), and F4/80 (brown) in AAA walls of AngII mice. Scale bars: 100µm.
Supplemental Figure VI
Western blot analysis of ferroportin in the aortas of Control and AngII mice. Ferroportin expression was standardized on the basis of GAPDH expression in the graph. n=3 per group. *p<0.05 compared with Control mice, †p<0.05 compared with AngII mice.
Supplemental Figure VII
Western blot analysis of TfR1 and ferroportin in angiotensin II-treated (A) THP-1 cells and (B) macrophages. The expression of TfR1 and ferroportin was standardized on the basis of GAPDH expression in the graph, respectively. n=3 per group. *p<0.05 vs Vehicle.