Deficiency of Adrenomedullin Induces Insulin Resistance by Increasing Oxidative Stress

Tatsuo Shimosawa, Takehide Oghara, Hiromitsu Matsui, Tomoichiro Asano, Katsuyuki Ando, Toshiro Fujita

Abstract—Hypertension, insulin resistance, and obesity are common age-related metabolic disorders that are often associated with increased oxidative stress and the resultant vascular damage. Underlying mechanisms have been suggested, and age-related overproduction of oxidative stress is one possible candidate. Since we recently found a vasoactive peptide, adrenomedullin, to be an endogenous antioxidant that potently inhibits oxidative stress–induced vascular damage, in the current study we evaluated oxidative stress–induced changes in aged mice. Insulin sensitivities in young and aged adrenomedullin-deficient mice were measured by means of the hyperinsulinemic-euglycemic clamp method; insulin resistance was apparent in aged adrenomedullin-deficient mice with increased urinary excretion of 8-iso-prostaglandin F_{2\alpha}, a marker of oxidative stress, but not in young adrenomedullin-deficient mice. Concomitantly, only aged adrenomedullin-deficient mice not only showed increased production of muscular reactive oxygen species, as demonstrated by the electron spin resonance method, but also had significantly decreased insulin-stimulated glucose uptake into the soleus muscle associated with impairment of insulin signals such as insulin receptor substrate-1,2 and phosphatidylinositol-3 kinase activities. In turn, these abnormalities could be nearly reversed by either treatment with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl, a membrane-permeable superoxide dismutase mimic, or adrenomedullin supplementation. Evidence presented in this report suggests that age-related accumulation of oxidative stress is involved in blood pressure regulation and insulin resistance in aged adrenomedullin-deficient mice, and adrenomedullin is thus an endogenous substance counteracting oxidative stress–induced insulin resistance associated with aging. (Hypertension. 2003;41:1080-1085.)

Key Words: insulin resistance ■ oxidative stress ■ aging ■ diabetes mellitus ■ adrenomedullin

Non–insulin-dependent diabetes mellitus, hypertension, and atherosclerotic cardiovascular diseases are common metabolic disorders that ultimately afflict the majority of individuals. Moreover, the incidence of these disorders increase as the population ages. There is emerging evidence that reactive oxygen species (ROS) might be involved in these metabolic disorders and the related organ damage in aged patients. ROS interact with and inactivate nitric oxide by nitric oxide synthase–dependent mechanisms that include increased generation of endothelin-1 and the effects of superoxide anions and hydrogen peroxide on vascular smooth muscle cells. As for insulin resistance, in vitro studies have shown that ROS impairs insulin signaling, decreases glucose transporter (GLUT) translocation in adipocytes, and reduces insulin internalization into endothelial cells. However, there are few in vivo studies evaluating the relationships among ROS, hypertension, and insulin resistance.

Adrenomedullin (AM), a potent vasodilator, was originally isolated from pheochromocytoma cells, but it is also produced in and secreted by vascular endothelial cells. The DNA sequence encoding the precursor of AM, proadrenomedullin, has been identified, and the first paired basic amino acid of this precursor (Lys{\textsuperscript{41}}-Arg{\textsuperscript{44}}) is a representative site for proteolytic cleavage, yielding the other product now called proadrenomedullin N-terminal 20 peptide. Its main physiological effect is inhibition of sympathetic activity, and it does not share the AM receptor.

Whereas ROS increases AM production in vascular endothelial and smooth muscle cells, AM inhibits the generation of ROS in cultured mesangial cells and macrophages. Moreover, we have generated mice with target-gene disruption of the AM gene alone and demonstrated that AM deficiency induces higher ROS and the resultant vascular damage. We can speculate, therefore, that AM deficiency would not inhibit the formation of ROS that accumulate with advancing age. Thus, the aged AM-deficient mouse is a potential animal model of high oxidative stress.

To clarify the relationship between oxidative stress and insulin resistance, in this study, we measured insulin sensitivity, by both the hyperinsulinemic-euglycemic clamp method, in vivo, and 2-deoxyglucose uptake into the soleus muscle, ex vivo, in aged AM-deficient mice.
Methods

Animals
Six-month-old (young) and 12-month-old (aged) male AM+/− mice were used. All mice analyzed in this study were F4 and F5, and littermate AM+/− mice were used as control animals. Mice were handled in our accredited facility in accordance with the institutional animal care policies of the University of Tokyo, and all research protocols conformed to the guiding principles for animal experimentation as enunciated by the Ethics Committee on Animal Research of the University of Tokyo, Faculty of Medicine.

Drug Treatments
Animals were maintained ad libitum by 10 mmol/L of 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO) (Sigma-Aldrich Japan) in drinking water, or a 300 ng/kg per hour infusion of AM given intraperitoneally by ALZET osmotic pump 1002 (Durect Co) was administered for 4 weeks.

Blood Pressure Measurements
Systolic blood pressure was measured by the tail-cuff method (BP908A, Softron Co) in the conscious and unrestricted state. Blood pressure was measured 10 times in each mouse, and averages were calculated for all mice.

Measurement of 8-Isoprostaglandin F₂α (Isoprostane)
Mice were placed in metabolic cages (KN-645, Natsume Co Ltd) to collect urine for a 24-hour period for 3 consecutive days from 10 days after osmotic pump implantation, and urine samples were kept frozen until the assay was performed. Isoprostane was measured by the EIA method, following the manufacturer’s instructions (Assay Designs, Inc). For measurement of isoprostane, urine samples were separately stored with 10 μg/mL of indomethacin to avoid degradation of synthesis of prostaglandins. The samples were diluted 100-fold. Total excretion of isoprostane (ng/d) was calculated.

Quantification of ROS
We measured O₂⁻ by using electron spin resonance (ESR) spectroscopy with the nitroxide radical hydroxy-TEMPO as a spin probe. The ESR was set as follows: a microwave power of 10 mW, an external magnetic field range of 10 mT, and a scan rate of 1 mT/s. The homogenates were subjected to centrifugation at 15 000 × g for 30 minutes at 4°C. ESR spectra were recorded, and there was a linear relation in the semilogarithmic plot of peak signal intensity versus time. The rate of signal decay was calculated from the slope of this line.

Measurement of Soleus Muscle AM Content
Mice were anesthetized with pentobarbital, and soleus muscles were dissected free. Samples were rapidly frozen and kept until assay. According to a previous report using a polyclonal antibody, AM concentrations were measured and the values were standardized with tissue weight (g).

Hyperinsulinemic-Euglycemic Clamp Study
Food was withdrawn 12 hours before the experiments. Mice were anesthetized with sodium pentobarbital administered intraperitoneally, and the left jugular vein was cannulated with 2 tip-tapered catheters, one for insulin infusion and the other for glucose infusion. The euglycemic-hyperinsulinemic clamp was performed as previously described with slight modification. Briefly, 30 mU/kg per minute of human insulin was continuously infused for 2 hours. The blood glucose concentration was clamped at 100 mg/dL, by estimating the blood glucose level at 5-minute intervals in samples taken from the tail and adjusting the rate of infusion of a 10% glucose solution delivered through the jugular cannula. The glucose infusion rate (GIR) during the second hour of the clamp study was taken as the area under the curve of whole-body insulin action.

2-Deoxy-o-[1-14C]glucose (50 μCi) and D-[U-14C]glucose (50 μCi) (NEL Life Science Products) were administered together as an intravenous bolus 75 minutes after starting the clamp study. Blood samples for determination of plasma glucose and tracer concentrations were obtained at 2, 5, 10, 15, 20, 30, and 45 minutes after administration. The glucose utilization rate (GUR) was estimated as previously described.

Insulin-Stimulated Glucose Uptake Into Isolated Soleus Muscles
Glucose uptake into isolated muscles was measured as previously described. Mice were anesthetized with pentobarbital, and soleus muscles were dissected free. Muscles were incubated in a shaking water bath at 35°C for 60 minutes in 20-mL flasks containing 2.0 mL Krebs-Henseleit bicarbonate (KHB) buffer supplemented with 8 mmol/L glucose, 32 mmol/L mannitol, and 0.1% BSA. Muscles were gassed continuously with 95% O₂–5% CO₂ throughout the experiment. The muscles were then incubated for 20 minutes in oxygenated KHB buffer in the presence or absence of human insulin at 0.02, 0.2, and 0.1 mmol/L. The muscles were then rinsed for 10 minutes at 29°C in 2 mL KHB buffer containing 40 mmol/L mannitol and 0.1% BSA. Next, the muscles were incubated for 20 minutes at 29°C in 1.5 mL of KHB buffer containing 8 mmol/L 2-deoxy-o-[1,2-14C]-glucose (2.25 mCi/mL), 32 mmol/L [14C]mannitol (0.3 mCi/mL), 2 mmol/L sodium pyruvate, and 0.1% BSA. Insulin was present throughout the wash and the uptake incubations. After the incubation, muscles were rapidly blotted, weighed, and solubilized with 1 mL of Soluene 350. Radioactivity was counted in the samples by using a liquid scintillation counter. 2-Deoxy-o-[14C]glucose uptake rates were corrected for extracellular trapping by using [14C]mannitol counts.

Insulin-Stimulated Insulin Receptor Substrate (IRS) Phosphorylation and Phosphatidylinositol (PI)-3 Kinase Activation in Muscle
The affinity-purified antibodies against IRS-1 and IRS-2 were prepared as previously described. Food was withdrawn 12 hours before the experiments. The mice were anesthetized with pentobarbital, and insulin (10 μmol/L) was injected into the portal vein. Hindlimb muscles were removed and immediately homogenized. The homogenates were subjected to centrifugation at 15 000 g for 30 minutes at 4°C, and the supernatants were used as samples. Immunoprecipitants were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted using one of the antibodies. Proteins were visualized with enhanced chemiluminescence. The assay of PI-3 kinase activity in the immunoprecipitants was performed as previously described.

Statistical Analysis
All values were expressed as mean ± SEM. Comparisons among groups were made using ANOVA followed by the Scheffé method, and probability values <0.05 were considered to indicate statistical significance.

Results

Oxidative Stress and AM Contents in AM+/− Mice
Urinary excretion of isoprostane was significantly greater (P<0.01) in aged AM+/− mice (43.58±3.58 ng/d) than in aged AM−/− mice (22.66±2.26 ng/d) (Figure 1) (n=5 for each group). However, there was no significant difference in urinary isoprostane excretion between young AM+/− mice and AM−/− mice. The rate of ESR signal decay, an index of the amount of ROS, was significantly (P<0.02) greater in the
soleus muscles of aged AM+/− mice (−0.027±0.005/min, n=4) than in those of aged AM+/+ mice (−0.007±0.001/min, n=5), suggesting increased ROS in the soleus muscles of aged AM−/− mice.

Soleus muscle AM contents in young and aged AM−/− mice were half those of young and aged AM+/+ mice: 0.225±0.013 fmol/mg tissue weight (young AM+/+ mice) versus 0.128±0.0109 fmol/mg per mg tissue wt (young AM−/− mice) (P<0.05) and 0.199±0.014 fmol/mL per mg tissue wt (aged AM+/+ mice) versus 0.108±0.0159 fmol/mL per mg tissue wt (aged AM−/− mice) (P<0.059) (n=6 for each group).

Body Weight, Blood Pressure, and Insulin Resistance in Aged AM−/− Mice

Body weights of each group of mice were comparable in young mice but in aged mice, AM−/− mice were heavier than AM+/+ mice (Table 1). Systolic blood pressures in young mice were comparable (89±7 mm Hg, 88±6 mm Hg in AM+/+ and AM−/− mice; n=10) but tended to be higher in aged AM−/− mice (105±8 mm Hg; n=20) than in AM+/+ mice (95±9 mm Hg; P<0.1, n=15). Fasting blood glucose was significantly higher in aged AM−/− mice than in AM+/+ mice (120±6 mg/dL versus 91±5 mg/dL, n=12; P<0.02) but was comparable in young AM−/− and AM+/+ mice (89±6 mg/dL versus 90±5 mg/dL, n=10; NS)

We further estimated, in vivo, insulin sensitivity by the hyperinsulinemic-euglycemic clamp method. GIR and GUR were significantly decreased in aged AM−/− mice as compared with aged AM+/+ mice (Table 2).

To study insulin resistance precisely, we examined insulin-induced 2-deoxyglucose uptake into isolated soleus muscle. Insulin-induced 2-deoxyglucose uptake into soleus muscle was clearly impaired in aged AM−/− mice (4.56±0.23 nmol/mg tissue wt in AM+/+ mice and 3.08±0.33 nmol/mg tissue wt in AM−/− mice by 0.2 mU insulin) but not in young AM+/+ mice (4.910±0.532 nmol/mg tissue wt in AM+/+ mice and 4.874±0.593 nmol/mg tissue wt in AM−/− mice by 0.2 mU insulin) (Figure 2) (n=6 for each experiments).

Concurrently, insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2 in skeletal muscle was decreased in aged AM−/− mice as compared with AM+/+ mice (Figures 3A and 3B, lower panels), whereas the expression levels of these proteins were comparable (Figures 3A and 3B, upper panels). PI-3 kinase activities associated with IRS-1 and IRS-2 in skeletal muscle were significantly decreased in aged AM−/− mice as compared with AM+/+ mice (Figures 3C and 3D) (n=5 for each experiments).

**Effects of Superoxide Dismutase Mimetics and AM Supplementation**

Since ROS production was increased in aged AM−/− mice, to clarify the involvement of oxidative stress and AM deficiency in the development of insulin resistance, we administered hydroxy-TEMPO, a superoxide dismutase mimetic21 to aged mice. The result was that the urinary excretion of isoprostane decreased significantly (1.32±0.10 ng/d in AM+/+ mice and 1.28±0.69 ng/d in AM−/− mice) (n=5 for each group). Concordant with the normalization of ROS, the fasting glucose level normalized in AM−/− mice (90±4 mg/dL). Moreover, an ex vivo study of insulin sensitivity in skeletal muscle revealed that 2-deoxyglucose uptake to recover to the young AM+/+ level in response to treatment with hydroxy-TEMPO (5.08±0.46 nmol/mg tissue wt in aged AM−/− mice by 2 mU of insulin) (Figure 4) (n=5). Systolic blood pressure also decreased significantly in AM−/− mice (88±11 mm Hg; P<0.05, n=5) but not in AM+/+ mice (90±12 mm Hg, NS, n=5).

Consistently, AM supplementation not only restored toward normal the urinary excretion of isoprostane

**TABLE 1. Body Weight (g)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Young (n=15)</th>
<th>Aged (n=20)</th>
<th>Hydroxy-TEMPO (n=10)</th>
<th>AM Supplementation (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM+/+</td>
<td>23±3</td>
<td>31±3</td>
<td>31±4</td>
<td>30±4</td>
</tr>
<tr>
<td>AM−/−</td>
<td>24±4</td>
<td>37±4*</td>
<td>38±5*</td>
<td>37±5*</td>
</tr>
</tbody>
</table>

*P<0.05 vs AM+/+ mice.

**TABLE 2. Measurement of Insulin Sensitivity in AM-Deficient Aged Mice by the Hyperinsulinemic-Euglycemic Clamp Method**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>GIR, mg/kg per minute</th>
<th>GUR, mg/kg per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM+/+</td>
<td>5</td>
<td>22.73±3.30</td>
<td>23.38±3.28</td>
</tr>
<tr>
<td>AM−/−</td>
<td>4</td>
<td>11.58±0.71</td>
<td>12.99±1.04*</td>
</tr>
</tbody>
</table>

GIR indicates glucose infusion rate; GUR, glucose utilization rate.

*P<0.05 vs AM+/+ mice.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Urinary excretion of isoprostane, an oxidative stress marker in young and aged mice (n=5). Bars indicate mean±SEM.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Insulin-induced 2-deoxy glucose uptake into isolated soleus muscle of young and aged mice (n=6). Bars indicate mean±SEM. *P<0.05 vs age-matched AM+/+ mice.
(24.38±0.67 ng/dL) (Figure 1) but reduced the fasting glucose level (92±6 mg/dL) and improved the decreased insulin-induced glucose uptake into isolated soleus muscle in AM-deficient aged mice (6.27±0.83 nmol/mg tissue wt in aged AM−mice by 2 mU of insulin) (Figure 4) (n=5). Systolic blood pressure decreased in both AM+/+ and AM−/− mice (85±10 mm Hg and 86±11 mm Hg; P<0.05 versus mice without AM supplementation, n=5 for each group)

On the other hand, body weight did not differ with either hydroxy-TEMPO or AM (Table 1).

Discussion

Insulin resistance is a key cause of syndrome X, and the incidence of syndrome X increases with age.22 In the current study, aged AM-deficient mice showed increased body weights, developed insulin resistance, and tended to have higher blood pressure, phenotypic features very similar to those of syndrome X. Oxidative stress is well known to increase with advancing age and might be a causative factor of syndrome X.23 In the current study, systemic stress and local oxidative stress were evaluated by measuring urinary excretion of an ROS marker, isoprostane,24 and real-time ROS production in the soleus muscle by ESR.16 Although ROS production was comparable in young AM+/+ and AM−/− mice, in aged AM−/− mice, moderate ROS accumulation occurred with advancing age. There is a growing body of evidence suggesting that aging induces the accumulation of oxidative stress by both increasing ROS production and decreasing its clearance.25 Since AM reportedly inhibits ROS production in mesangial cells or macrophages, it is possible that oxidative stress was significantly greater in the aged AM−/− mice than in the AM+/+ mice, in part because of increased production of ROS, a speculation supported by the ESR results of this study.

Multiple lines of study have shown diabetic patients to have increased oxidative stress and the resultant organ damage.2 In turn, it is hypothesized that oxidative stress can induce diabetes, since in vitro study has demonstrated ROS to impair insulin internalization in endothelial cells,5 block IRS phosphorylation, and impair PI-3 kinase activity in hepatocytes3 and reduce the translocation of GLUT4 in adipocytes.4,26 In vivo study also showed administration of ROS to aggravate diabetes in diabetes-prone obese Zucker rats.6 It has been shown that the incidence of diabetes increases with advancing age in Western countries.1 These lines of evidence led us to the plausible hypothesis that increased oxidative stress might cause insulin resistance in aged AM-deficient mice, since AM deficiency did not inhibit the accumulation of oxidative stress with advancing age.

We next studied, in vivo, insulin sensitivity in AM+/+ mice by using the hyperinsulinemic-euglycemic clamp method, which revealed, on the basis of the decreased glucose infusion rate, aged AM+/+ mice to be significantly insulin-resistant. Concomitantly, insulin-stimulated glucose uptake into the isolated skeletal muscle was significantly attenuated in aged AM+/+ mice. These findings are consistent with the results of

**Figure 3. Expression levels and insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 (A and B) and their associated PI-3 kinase activities (C and D) in skeletal muscle of aged mice (n=5). Representative immunoblots are shown in A and B. IP indicates immunoprecipitation; IB, immunoblotting; αIRS-1, anti-IRS-1; αIRS-2, anti-IRS-2; and αPY, antiphosphotyrosine antibodies. *P<0.05, **P<0.01 vs insulin-stimulated AM+/+ mice.**

**Figure 4. Changes in insulin sensitivity in response to either treatment with hydroxy-TEMPO, a membrane-permeable superoxide dismutase (n=5), or AM supplementation (n=5) in aged mice. Bars indicate mean±SEM. *, **P<0.05 vs AM+/+ with neither AM supplementation nor hydroxy-TEMPO treatment.**
previous studies indicating muscle and liver to be the main organs storing glycogen and that skeletal muscle–specific inhibition of insulin signaling is adequate to cause insulin resistance in hypertension as well as diabetes.

Next, we investigated the molecular mechanism underlying insulin resistance induced by ROS. We focused on important steps of insulin-induced glucose uptake into skeletal muscle: insulin-stimulated phosphorylation of IRS-1 and IRS-2 and the associated PI-3 kinase activation. Insulin-resistant animal models as well as human subjects have been shown to exhibit an impairment of insulin-induced PI-3 kinase activation in skeletal muscle. Consequently, the present findings indicate that in soleus muscle, phosphorylation of IRS-1 and IRS-2 apparently is decreased, in association with the resultant decrease in PI-3 kinase activities with anti–IRS-1 and anti–IRS-2. This is consistent with the results of a study using iNOS-disrupted mice, showing that iNOS induction causes muscle-specific insulin resistance. NO itself increases blood flow and may improve insulin resistance, but when NO is abundant such as in an iNOS-induced state, it is easily converted to ONOO⁻, a ROS, and this could impair insulin signaling.

To clarify the involvement of ROS in AM deficiency–induced insulin resistance, we administered hydroxy-TEMPO, a superoxide dismutase mimetic, and provided AM supplementation to AM-deficient aged mice. Treatment with hydroxy-TEMPO decreased urinary excretion of isoprostane, a marker of oxidative stress, associated with both the blood glucose level and improved insulin-stimulated glucose uptake into skeletal muscle. Concurrently, AM supplementation could not only decrease urinary isoprostane but also the fasting blood glucose level and insulin-stimulated glucose uptake into skeletal muscle in AM-deficient aged mice.

Systolic blood pressure also decreased to a greater extent in AM⁻/⁻ mice given hydroxy-TEMPO, suggesting that in aged mice, blood pressure is maintained at a higher level partly by oxidative stress. On the other hand, a high dose of AM decreased systolic blood pressure to the same level in AM⁻/⁻ and AM⁻/+ mice. The vasodilating effect of AM could explain the additive effect on blood pressure. Aged AM⁻/⁻ mice were more obese, and obesity is a key factor inducing insulin resistance. However, in the current study, either hydroxy-TEMPO treatment or AM supplementation reversed insulin resistance without reducing body weight. Taken together, these observations suggest that oxidative stress might be involved in the development of insulin resistance in aged AM-deficient mice.

**Perspectives**

Based on the results of the present study, we conclude that long-term deficiency of AM increases oxidative stress, resulting in insulin resistance possibly through impaired insulin signaling in aged AM-deficient mice. The precise mechanism underlying AM-induced inhibition of oxidative stress remains unknown, and further investigation is required. This is the first report to clearly demonstrate that naturally occurring oxidative stress with advancing age can induce insulin resistance resembling human type 2 diabetes and syndrome X. Therefore, it is anticipated to open the way to the clarifying the pathophysiology of and future treatment strategies for aged diabetic patients.

**References**


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