High-Salt Diet Enhances Insulin Signaling and Induces Insulin Resistance in Dahl Salt-Sensitive Rats

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Abstract—A high-salt diet, which is known to contribute to the pathogenesis of hypertension, is also reportedly associated with insulin resistance. We investigated the effects of a high-salt diet on insulin sensitivity and insulin signaling in salt-sensitive (Dahl-S) and salt resistant (Dahl-R) strains of the Dahl rat. Evaluation of hyperinsulinemic-euglycemic clamp studies and glucose uptake into the isolated soleus muscle revealed that salt loading (8% NaCl) for 4 weeks induced hypertension and significant insulin resistance in Dahl-S rats, whereas no significant effects were observed in Dahl-R rats. Despite the presence of insulin resistance, insulin-induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrates, activation of phosphatidylinositol 3-kinase, and phosphorylation of Akt were all enhanced in Dahl-S rats fed a high-salt diet. The mechanism underlying this form of insulin resistance thus differs from that previously associated with obesity and dexamethasone and is likely due to the impairment of one or more metabolic steps situated downstream of phosphatidylinositol 3-kinase and Akt activation. Interestingly, supplementation of potassium (8% KCl) ameliorated the changes in insulin sensitivity in Dahl-S rats fed a high-salt diet; this was associated with a slight but significant decrease in blood pressure. Evidence presented suggest that there is an interdependent relationship between insulin sensitivity and salt sensitivity of blood pressure in Dahl-S rats, and it is suggested that supplementing the diet with potassium may exert a protective effect against both hypertension and insulin resistance in salt-sensitive individuals. (Hypertension. 2002;40:83-89.)

Key Words: hypertension, sodium-dependent ■ insulin resistance ■ potassium ■ rats, Dahl ■ kinase

It is well known that hypertension is associated with insulin resistance1–3 and that this association is clinically important because it forms the basis of insulin resistance syndrome or syndrome X.4,5 Genetic background and excessive sodium intake are considered key factors contributing to the development of hypertension,6 although the specific genetic factors determining one’s susceptibility to sodium-induced hypertension are mostly unidentified. According to blood pressure response to salt loading, patients with essential hypertension could be divided into two groups: salt-sensitive and non–salt-sensitive. Interestingly, a number of studies have also established an association between salt sensitivity and insulin resistance in both normotensive and hypertensive subjects.7–11

We previously showed that the administration of a high-salt diet to normal Sprague-Dawley rats induces insulin resistance and that such salt-induced insulin resistance is unique in that it is accompanied by an enhancement of the early steps of insulin signaling.12 It remains unclear, however, whether or not Sprague-Dawley rats are salt-sensitive because there was only a small but significant salt-induced elevation in blood pressure. The aim of the present study was to determine whether the same genetic factors responsible for salt-induced increases in blood pressure are also responsible for salt-induced insulin resistance. To address this question, we evaluated the changes in insulin signaling that occur with the development of salt-induced insulin resistance in Dahl rats.

The binding of insulin to its transmembrane receptor (IR) stimulates the receptor tyrosine kinase activity, resulting the phosphorylation of the IR itself and its substrates, which in skeletal muscle, liver, and adipose tissue include insulin receptor substrate (IRS)-1 and IRS-2. IRS-3 is also abundant in liver and adipose tissue, but it is not detected in skeletal muscle.13 Tyrosine-phosphorylated IRSs in turn associate with and activate phosphatidylinositol (PI) 3-kinase, which has been implicated in insulin-induced glucose uptake into adipocytes and skeletal muscle, and glycogen synthesis and inhibition of gluconeogenesis in liver.14 In addition, activation of PI 3-kinase leads to the activation of Akt, a serine-threonine kinase also known as protein kinase B. Those mice deficient in Akt2 exhibit a diabetic phenotype that attests to

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of Akt in insulin signaling and glucose metabolism. In the present study, we examined the effect of salt loading on insulin-induced tyrosine phosphorylation of the IR and IRSs, activation of PI 3-kinase, and phosphorylation of Akt.

Finally, we tested the effect of adding potassium to the high-salt diet, because this reportedly reverses salt-induced hypertension in hypertensive subjects. Moreover, potassium supplementation has a protective effect in salt-loaded Dahl rats with renal and cerebral lesions that is independent of changes in blood pressure. We now present evidence that potassium has a protective effect on both blood pressure and insulin resistance in salt-induced hypertension.

Methods

Materials

Affinity-purified antibodies against IRS-1, IRS-2, IRS-3, and GLUT4 were prepared as previously described. Antibodies against phosphotyrosine, Akt, and phospho-Ser473 Akt were purchased from Upstate Biotech Inc. Human insulin (Novolin R) was from Novo Nordisk.

Animals

Four-week-old male Dahl salt-sensitive (Dahl-S) and salt-resistant (Dahl-R) rats (Japan SLC Inc, Shizuoka, Japan) were fed a standard rodent diet containing 0.3% NaCl (normal diet group) or 8% NaCl (high-salt group) during a 4-week experimental period. In some experiments, Dahl-S rats were fed a diet containing 8% NaCl and 8% KCl for 4 weeks (high-sodium/high-potassium group). The rats were housed in a room maintained at constant humidity (60 ± 5%), temperature (23 ± 1°C), and light cycle (12 hours: 0700 to 1900). Food and tap water were available ad libitum throughout the study. All experimental procedures were approved and carried out in accordance with the guidelines of the University of Tokyo and the Institute for Adult Diseases, Asahi Life Foundation, for the care and use of laboratory animals.

Analytical Methods

The blood pressures of the rats were measured by the tail-cuff method using a Softron BP-98A automatic sphygmomanometer. Blood glucose was assayed using the glucose oxidase method; plasma insulin was assayed by radioimmunoassay.

Immunoprecipitation and Immunoblotting

Food was withdrawn 12 hours before experimentation. The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight); within 10 to 15 minutes, the abdominal cavity was opened, the portal vein exposed, and 4 mL of normal saline (0.9% NaCl), with or without 10^{-7} mol/L human insulin, was injected. Livers, hindlimb muscles, and epididymal adipose tissue were respectively removed 30 s, 90 s, and 120 s later and immediately homogenized as previously described. The extracts were centrifuged at 15 000g for 30 minutes at 4°C to remove insoluble material, after which the supernatants were used for immunoprecipitation or immunoblotting as described previously.

PI 3-Kinase Activity

After insulin injection into the portal vein, portions of the liver and hindlimb muscles were removed and immediately homogenized as previously described. IRS-1, IRS-2, IRS-3 (liver and adipose tissue lysates only), and the corresponding tyrosine phosphorylated proteins were immunoprecipitated from aliquots of supernatant containing 10 mg of protein using anti-IRS-1, anti-IRS-2, anti-IRS-3, and anti-phosphotyrosine antibodies, respectively, and then incubated with protein A-Sepharose 4FF. PI 3-kinase activity in the immunoprecipitates was then assayed as previously described.

Hyperinsulinemic-Euglycemic Clamp Analysis and In Vivo Insulin Action in Individual Tissues

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium, after which the left jugular and femoral veins were cannulated for blood sampling and infusion, respectively. Euglycemic-hyperinsulinemic clamp analysis was performed as described previously.

Glucose Uptake Into Isolated Skeletal Muscle

Glucose uptake into isolated soleus muscle was measured as previously described.

Statistical Analysis

Data are expressed as mean±SE. Comparisons were made using unpaired t tests. Values of P<0.05 were considered significant.

Results

Characterization of the Rats Studied

Table 1 summarizes the body weights, food intakes, blood pressures, and plasma parameters of the rats studied. Although their food intake was similar, Dahl-S rats fed a high-salt diet had lower body weights than those on a normal diet. The body weights and food intake of Dahl-R rats fed a high-salt diet did not differ significantly from those fed a normal diet. Dahl-S rats fed a high-salt diet also had signifi-

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<th>TABLE 1. Characterization of Rats</th>
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<td><strong>Dahl-S</strong></td>
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<td><strong>Dahl-R</strong></td>
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<td>Normal</td>
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<tr>
<td>Body weight, g</td>
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<tr>
<td>250.4±3.0</td>
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<td>202.0±8.1*</td>
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<tr>
<td>Food intake, g/d</td>
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<tr>
<td>18.2±3.4</td>
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<td>17.2±2.1</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
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<td>130.2±2.4</td>
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<td>181.9±3.8*</td>
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<td>Diastolic blood pressure, mm Hg</td>
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<td>86.6±5.7</td>
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<td>114.6±2.7*</td>
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<td>Heart rate, bpm</td>
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<td>382.7±10.7</td>
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<td>411.3±9.3</td>
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<tr>
<td>Fasting blood glucose, mg/dL</td>
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<td>120.0±2.3</td>
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<td>121.8±2.0</td>
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<tr>
<td>Fasting plasma insulin, ng/dL</td>
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<td>0.88±0.02</td>
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<td>0.89±0.05</td>
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<td>Data are means±SE; n=6 rats in each group. *P&lt;0.01 compared with rats fed a normal diet.</td>
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significantly higher systolic and diastolic blood pressures than those on the normal diet, whereas salt loading had no effect on blood pressure in Dahl-R rats. Salt loading did not affect the fasting blood glucose or plasma insulin levels in either Dahl-S or Dahl-R rats.

Hyperinsulinemic-Euglycemic Clamp Analysis

Whole-body insulin sensitivity was evaluated using the hyperinsulinemic-euglycemic clamp technique. We found that the glucose infusion rate and glucose utilization rate, respectively, were 68.0% and 73.3% lower in Dahl-S rats fed a high-salt diet than in those fed a normal diet (Figures 1A and 1B). Hepatic glucose production did not change with salt loading, but glucose uptake into skeletal muscle during the clamp (glucose metabolic index) was reduced by 53.5% (Figures 1C and 1D). In contrast to Dahl-S rats, salt loading did not significantly affect glucose infusion rate, glucose use rate, hepatic glucose production, or glucose metabolic index in Dahl-R rats. Thus, salt loading induced whole-body and muscle insulin resistance in Dahl-S rats but not in Dahl-R rats.

Insulin-Induced 2-Deoxy Glucose Uptake in Isolated Skeletal Muscle

Insulin stimulation increased 2-deoxy glucose uptake into isolated muscle by 3.1-fold in Dahl-S rats fed a normal diet (Figure 2A); however, the response was reduced by 65.3% in those fed a high-salt diet. Insulin-induced glucose uptake into isolated muscle from Dahl-R rats was unaffected by diet.

Western blot analysis showed that soleus muscle and adipose tissue GLUT4 content was similar among those groups (Figure 2B), indicating that the impairment of insulin-induced glucose uptake by skeletal muscle from Dahl-S rats fed a high-salt diet was not due to diminished expression of GLUT4 protein, but due to the impairment of insulin signaling.

Insulin-Induced Tyrosine Phosphorylation of the IR and IRS Proteins

To assess insulin-induced tyrosine phosphorylation of IR and IRSs, saline, with or without insulin, was injected into the portal veins, after which livers, hindlimb muscles, and epididymal fat were removed, homogenized, and immunoprecipitated with anti-IRS or anti-phosphotyrosine antibodies. In muscle from Dahl-S rats fed a high-salt diet, insulin-induced tyrosine phosphorylation of IR (indicated by a band at 90 kDa immunoprecipitated by anti-phosphotyrosine) was 4.8-fold greater than in muscle from rats fed a normal diet (Figure 3A). In addition, although expression of muscle IRS-1 and IRS-2 was similar in Dahl-S rats, regardless of diet (Figures 3B and 3C, upper panels), insulin-induced tyrosine phosphorylation of those proteins was enhanced 4.2- and 3.1-fold, respectively, in the salt-loaded animals (Figures 3B and 3C, lower panels). Virtually identical results were obtained from both liver and adipose tissue (Figures 3D to 3K). Among Dahl-R rats, neither IRS expression nor phosphorylation of IR and IRSs were significantly affected by salt intake. As with induction of insulin resistance, salt loading enhanced insulin-induced tyrosine phosphorylation of IR and IRSs only in Dahl-S rats.

Insulin-Induced PI 3-Kinase Activation

An assay of the immunoprecipitated solutions revealed that insulin-stimulated PI 3-kinase activity associated with IRS-1, IRS-2, and phosphotyrosine was increased 3.8-, 3.7-, and 1.8-fold, respectively, in muscle samples from Dahl-S rats fed a high-salt diet compared with those fed a normal diet (Figures 4A to 4C). Moreover, in liver and adipose tissue from salt-loaded Dahl-S rats, insulin-stimulated PI 3-kinase activities associated with IRS-1, IRS-2, IRS-3, and phosphotyrosine were all significantly increased 1.4- to 2.4-fold (Figures 4D to 4K).
The kinase activity of Akt is regulated by PI 3-kinase products, which mediate its serine/threonine phosphorylation,23 in part on Ser-473.24 Although the levels of Akt protein in Dahl-S rats were comparable regardless of diet, insulin-stimulated phosphorylation of Akt Ser-473 was enhanced 4.7-, 5.2-, and 3.3-fold, respectively, in skeletal muscle, liver, and adipose tissue from Dahl-S rats fed a high-salt diet (Figure 5). Therefore, despite the presence of insulin resistance, early insulin-signaling steps leading to PI 3-kinase activation and Akt phosphorylation do not seem to be impaired in salt-loaded Dahl-S rats; indeed, they seem to be enhanced. Among Dahl-R rats, neither insulin-stimulated PI 3-kinase activity nor Akt phosphorylation was affected by diet.

**Improvement of Insulin Sensitivity With Potassium Loading in Dahl-S Rats Fed a High-Salt Diet**

To assess the extent to which adding dietary potassium would exert a protective effect on insulin activity in salt-loaded Dahl-S rats, some animals were fed a high sodium/high potassium (8% NaCl and 8% KCl) diet for 4 weeks. These animals were slightly smaller than those fed a high-sodium diet, although their food intake was similar (Table 2). Their systolic and diastolic blood pressures were slightly, but significantly, lower than those of rats fed a high-sodium diet. Potassium supplementation did not affect fasting blood glucose or plasma insulin levels in salt-loaded Dahl-S rats. However, hyperinsulinemic-euglycemic clamp analysis showed glucose infusion rate and glucose utili-
glucose output. However, our results clearly show that Dahl-S rats fed a high-salt diet were more insulin resistant than those fed a normal diet. The reason for the apparent discrepancy between those earlier studies and ours is unknown, but it may reflect differences in the methods used to evaluate insulin resistance. In the earlier studies, only fasting insulin, fasting glucose, glucose uptake into isolated adipocytes, and insulin levels after glucose loading were measured. The hyperinsulinemic-euglycemic clamp analysis and assays of glucose uptake into isolated soleus muscle used in the present study are more sensitive techniques that should yield more reliable results.

Insulin resistance plays a critical role in the development of type 2 diabetes. Moreover, type 2 diabetes is almost 2.5 times more likely to develop in subjects with hypertension than in normotensive subjects. Although it is still unclear precisely why hypertensive subjects tend to be diabetic, our results suggested that a high-salt diet induces both insulin resistance and hypertension, provided the possibility that there is a genetic background of salt sensitivity. Salt-sensitivity, a genetic factor, and excessive salt intake, an environmental factor, may thus be a link connecting hypertension and diabetes, and we suggest that salt-sensitivity and excessive salt intake are important elements contributing to the development of syndrome X. This plausible hypothesis was supported by several lines of evidence indicating a definite association between insulin resistance and salt-sensitivity of blood pressure in hypertensives and normotensives. To test this hypothesis, however, it will be necessary to further clarify whether subjects with syndrome X are significantly more likely to be salt-sensitive, as well as how important salt sensitivity and salt intake are to the pathogenesis of syndrome X.

Previous studies reported that the tissue angiotensin-converting enzyme (ACE) activity of Dahl-S rats increased after high-salt loading compared with those rats on the normal diet and Dahl-R rats on either diet. Because the kinin-kallikrein system is involved in sensitizing insulin action in peripheral tissues, it is possible that the increased activity of tissue ACE leads to insulin resistance through the decrease of kallikrein levels. However, Dahl-S rats fed a normal diet demonstrated lower plasma and tissue angiotensin II than Dahl-R rats, but salt loading did not cause significant changes in angiotensin II levels in either strain. Thus, it is unlikely that angiotensin II is involved in the pathogenesis of insulin resistance in the Dahl-S rat on a high-salt diet.

Our findings further indicate that, despite the presence of insulin resistance, the early steps in insulin signaling are not impaired but are actually enhanced in Dahl-S rats fed a high-sodium/high-potassium diet.
high-salt diet. There have been a number of reports indicating that the early steps in insulin signaling are impaired in insulin resistance. For instance, insulin-induced IRS phosphorylation and PI 3-kinase activation are attenuated in obese rodents and dexamethasone-treated rats. Because PI 3-kinase activation is critical for insulin’s metabolic actions, including the translocation of the GLUT4 glucose transporter to the cell surface, and suppression of gluconeogenesis, the idea that attenuation of PI 3-kinase activity results in a deficiency of insulin action seems reasonable. Moreover, the fact that Akt is situated downstream of PI 3-kinase lends additional support to the idea that insulin sensitivity is regulated by a PI 3-kinase pathway, because compelling evidence indicates Akt is a key mediator of glucose metabolism. Akt is clearly involved in insulin-induced translocation of GLUT4 to the cell surface in 3T3-L1 adipocytes, and glycerogen synthesis, and suppression of gluconeogenesis in the liver. In the present study, we observed what seems to be a different form of insulin resistance in which IRS phosphorylation, PI 3-kinase activation, and Akt phosphorylation are actually enhanced. Although the mechanism responsible for the enhanced insulin signaling remains unclear, it is apparently quite different from that related to obesity or dexamethasone and is perhaps a compensatory response to the impairment of step(s) downstream of PI 3-kinase and Akt activation.

Finally, we examined the effect of adding potassium to the high-salt diet fed the Dahl-S rats. There is substantial evidence that modern urban people eat more sodium and less potassium than prehistoric or primitive humans. Preclinical epidemiological studies have shown that people on high sodium/low potassium diets have a greater incidence of hypertension and cardiovascular complications and that there is a positive correlation between the urinary potassium or sodium/potassium ratio and blood pressure. In our previous study, moreover, supplementation of potassium attenuated salt-induced increases in blood pressure in salt-sensitive hypertensives. Several investigators have suggested that the antihypertensive effects of potassium are related to the amount of salt intake and salt sensitivity, because potassium is most effective in decreasing blood pressure in salt-sensitive rats. There have been a number of reports indicating that potassium supplementation reduced insulin resistance in Dahl-S rats fed a high-salt diet. Although the precise mechanism for the beneficial effect of potassium supplementation on insulin resistance is still controversial, a high-potassium diet could be an effective approach to the normalization of insulin resistance and the control of blood pressure, resulting in the prevention of cardiovascular events in hypertensives. Further basic and clinical studies will be needed to clarify the role of sodium/potassium in insulin resistance and the link between hypertension and diabetes.

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