Insulin Resistance With Enhanced Insulin Signaling in High-Salt Diet–Fed Rats

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Previous clinical studies showed an apparent correlation between hypertension and insulin resistance, and patients with diabetes are known to have increased blood pressure responsiveness to salt loading. To investigate the effect of high salt intake on insulin sensitivity and the insulin signaling pathway, a high-salt diet (8% NaCl) or a normal diet was given to 7-week-old SD rats for 2 weeks. High salt–fed rats developed slightly but significantly higher systolic blood pressure than controls (133 ± 2 vs. 117 ± 2 mmHg, $P < 0.001$), with no change in food intake or body weight. High salt–fed rats were slightly hyperglycemic (108.5 ± 2.8 vs. 97.8 ± 2.5 mg/dl, $P = 0.01$) and slightly hyperinsulinemic (0.86 ± 0.07 vs. 0.61 ± 0.06 ng/ml, $P = 0.026$) in the fasting condition, as compared with controls. Hyperinsulinemic-euglycemic clamp study revealed a 52.7% decrease in the glucose infusion rate and a 196% increase in hepatic glucose production in high salt–fed rats, which also showed a 66.4% decrease in 2-deoxyglucose uptake into isolated skeletal muscle and a 44.5% decrease in insulin-induced glycogen synthase activation in liver, as compared with controls. Interestingly, despite the presence of insulin resistance, high salt–fed rats showed enhanced insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1, IRS-2 (liver and muscle), and IRS-3 (liver only). Phosphatidylinositol (PI) 3-kinase activities associated with IRS and phosphotyrosine in the insulin-stimulated condition increased 2.1- to 4.1-fold, as compared with controls. Insulin-induced phosphorylation of Ser-473 of Akt and Ser-21 of glycogen synthase kinase-3 also increased 2.9- and 2-fold, respectively, in the liver of the high salt–fed rats. Therefore, in both the liver and muscle of high salt–fed rats, intracellular insulin signaling leading to PI 3-kinase activation is enhanced and insulin action is attenuated. The hyperinsulinemic-euglycemic clamp study showed that decreased insulin sensitivity induced with a high-salt diet was not reversed by administration of pioglitazone. The following can be concluded: 1) a high-salt diet may be a factor promoting insulin resistance, 2) the insulin-signaling step impaired by high salt intake is likely to be downstream from PI 3-kinase or Akt activation, and 3) this unique insulin resistance mechanism may contribute to the development of diabetes in patients with hypertension. *Diabetes* 50:573–583, 2001

R ecently, much interest has focused on the association between diabetes and hypertension. A previous pioneering study showed an association of hypertension with insulin resistance (1). A recent large clinical study, the U.K. Prospective Diabetes Study, showed that >30% of patients recently diagnosed with type 2 diabetes had hypertension, underscoring the importance of treating hypertension in patients with diabetes (2). Although the molecular mechanism underlying the coexistence of hypertension and diabetes remains unclear, multiple lines of evidence indicate that high blood pressure is among the factors contributing to the development of insulin resistance (3–5). In fact, glucose intolerance and diabetes develop more frequently in patients with hypertension at baseline than in patients with normal blood pressure (6). In a recent prospective study, type 2 diabetes was almost 2.5 times as likely to develop in patients with hypertension as in normotensive subjects (7). However, the reverse is also true. In an 8-year follow-up, hypertension reportedly developed twice as often in subjects who were initially normotensive and had either glucose intolerance or diabetes at baseline as in subjects who had normal glucose tolerance (8). Thus, it is very likely that the two mechanisms coexist: hypertension is promoted by insulin resistance and insulin resistance is promoted by hypertension.

Much remains unclear regarding the pathogenesis of hypertension in human subjects. However, it is widely and firmly believed that some unidentified genetic factors and acquired factors, such as excessive salt intake, excessive caloric intake (obesity), and alcohol consumption are likely to be involved in the occurrence of hypertension (9). Several epidemiological studies have demonstrated a clear relationship between dietary sodium intake and hypertension (10), although not all patients show a tendency for salt loading to increase blood pressure and salt depletion to decrease blood pressure (11). Recently, several clinical reports have shown that patients with insulin resistance or hyperinsulinemia tend to have salt-sensitive hypertension (12–15). In addition, excessive salt intake has been reported to decrease insulin sensitivity (16).

Based on these previous reports, we considered the possibility that a high-salt diet induces not only hypertension but also insulin resistance. Herein, we first investigated whether salt loading leads to insulin resistance in high salt–fed rats using a hyperinsulinemic-euglycemic clamp study. Then, we investigated the effect of salt loading on the insulin-signaling pathway in both liver and muscle of high salt–fed rats. We identified a novel mechanism of insulin resistance related to the high-salt diet that differs from those attributed to other factors such as obesity (17,18), high-fat diet (19), aging (20), and administration of dexamethasone (21).

RESEARCH DESIGN AND METHODS

Antibodies. The affinity-purified antibodies against insulin receptor substrate (IRS)-1, IRS-2, IRS-3, and GLUT4 were prepared as previously described (22–24). The antibody against Akt was prepared by immunizing rabbits with a synthetic peptide derived from COOH-terminal amino acids 466–479 of mouse Akt. The antibodies against phospho-Ser473 of Akt, glycogen synthase kinase (GSK)-3α, phospho-Ser21 of GSK-3α, and phosphotyrosine were purchased from Upstate Biotechnology (Lake Placid, NY).

Animals. Male SD rats were purchased from Tokyo Experimental Animals (Tokyo, Japan) at 7 weeks of age and fed standard rodent diet containing 0.3% NaCl (control group) or 8% NaCl (salt loading group) for 2 or 8 weeks. In some experiments, rats were fed with 0.3 or 8% NaCl diet with 0.02% pioglitazone HCl (Takeda Chemical Industry, Osaka, Japan). The rats were housed in a room maintained at constant humidity (60 ± 5%), temperature (23 ± 1°C), and light cycle (0700 to 1900). Food and water were available ad libitum throughout the study.

Analytical methods. The blood pressure and heart rates of the rats were measured by the tail-cuff method using an automatic sphygmomanometer (Softron BP-98A; Softron, Tokyo). Blood from the tail vein was collected and blood glucose was assayed by the glucose oxidase method, and plasma insulin was measured by radioimmunoassay. Plasma parameters were measured using an automatic analyzer (Hitachi 7350). Plasma renin activity and aldosterone levels were measured using radioimmunoassay.

Immunoprecipitation and immunoblotting. Food was withdrawn 12 h before the experiments. The rats were anesthetized with pentobarbital sodium (60 mg/kg body wt i.p.) and used in experiments 10–15 min later. The abdominal cavity was opened, the portal vein was exposed, and 4 ml normal saline (0.9% NaCl) with or without 10^{-5} mol/l insulin was injected. Livers and hindlimb muscles were removed 30 and 90 s later, respectively, and immediately homogenized in a 6× volume of homogenization buffer A with a polytron operated at maximum speed for 30 s. Homogenization buffer A was composed of 1% Triton X-100, 50 mmol/l HEPES (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin. Both extracts were centrifuged at 15,000g at 4°C for 30 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation or immunoblotting as previously described (18). Briefly, supernatants containing equal amounts of protein were incubated with anti-IRS-1, anti-IRS-2, anti-IRS-3 (liver lysate only), antiphosphotyrosine, or anti-Akt antibodies (10 mg/ml each) and then incubated with 10 ml protein A-sepharose. The samples were washed and then boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol. Total lysates were also boiled to allow detection of GLUT4, GSK-3, and phosphorylated GSK-3. Total lysates or immunoprecipitants (10 μg) were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted using one of the antibodies. Proteins were visualized with enhanced chemiluminescence (ECL) and band intensities were quantified with a Molecular Imager GS-525 using Imaging Screen-CH (Bio-Rad Laboratories, Hercules, CA).

Phosphatidylinositol 3-kinase activity. After injection of insulin into the portal vein, portions of the liver and hindlimb muscles were removed and immediately homogenized as previously described (18). The homogenates were subjected to centrifugation at 15,000g for 30 min at 4°C, and the supernatants were used as samples. IRS-1, IRS-2, IRS-3 (liver lysate only), and tyrosine-phosphorylated proteins were immunoprecipitated from aliquots of the supernatant containing 10 mg protein with anti-IRS-1, anti-IRS-2, anti-IRS-3, and antiphosphotyrosine antibodies, respectively, followed by protein A-Sepharose 6MB. The assays of phosphatidylinositol (PI) 3-kinase activity in the immunoprecipitants were performed as previously described (18).

Hyperinsulinemic-euglycemic clamp study. The rats were anesthetized with pentobarbital sodium administered intraperitoneally and the left jugular and left femoral veins were catheterized. The jugular and femoral catheters were used for blood sampling and infusion, respectively, during the clamp study. The euglycemic-hyperinsulinemic clamp was performed as previously described (19). Briefly, 30 mU · kg^{-1} · min^{-1} human insulin (Novolin R; Novo Nordisk, Denmark) was continuously infused for 2 h. The blood glucose concentration was clamped at 100 mmol/l by estimating the blood glucose level at 5-min intervals in samples taken from the jugular vein and adjusting the rate of infusion of a 10% glucose solution delivered via the femoral cannula. The glucose infusion rate during the second hour of the clamp study was taken as the parameter of the potency of whole-body insulin action. Blood samples were obtained for insulin determination in all studies at 0, 60, and 120 min.

In vivo insulin action in individual tissues. 2-deoxy-D-[1-^{3}H]glucose (50 μCi) and D-[U-^{14}C]glucose (50 μCi) (NEN Life Science Products, Boston, MA) were administered together as an intravenous bolus 75 min after the start of the study. Blood samples for determination of plasma glucose and tracer concentrations were obtained at 2, 5, 10, 15, 20, 30, and 45 min after bolus administration. At the end of the clamp study, soleus muscles were removed, weighed, and solubilized with 1 ml Soluene 350 (Packard, Meriden, CT). The muscle samples were counted in a liquid scintillation counter. An estimate of muscle glucose uptake (defined as the glucose metabolic index, Rg′), the glucose utilization rate, and hepatic glucose production were calculated as previously described (25).

Glucose uptake in isolated skeletal muscle. Glucose uptake in isolated muscles was measured as previously described (26). Rats were anesthetized and soleus muscles were dissected out and rapidly cut into 40- to 80-mg strips. The rats were then sacrificed by intracardiac injection of pentobarbital. Muscle strips were incubated in a shaking water bath at 35°C for 60 min 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% bovine serum albumin (BSA) (radioimmunoassay grade). Flasks were gassed continuously with 95% O_2:5% CO_2 throughout the experiment. The muscles were then incubated for 20 min in oxygenated KHB buffer in the presence or absence of human insulin (Novolin R; Novo Nordisk) at two mU/ml. This concentration of insulin induces maximal glucose transport in the soleus muscles (data not shown). The muscles were then rinsed for 10 min at 29°C in 2 ml KHB buffer containing 40 mmol/l mannitol and 0.1% BSA. Next, the muscles were incubated for 20 min at 29°C in 1.5 ml of KHB buffer containing 8 mmol/l 2-deoxy-D-[1,2-^{3}H(N)]glucose (2.25 μCi/ml), 32 mmol/l [^{14}C]mannitol (0.3 μCi/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 (Packard). Radioactivity was counted in the samples using a liquid scintillation counter. 2-Deoxy-[^{3}H]glucose uptake rates were corrected for extracellular trapping using [^{14}C]mannitol counts (26).

Glycogen synthase activity. Normal saline (4 ml) with or without 10^{-5} mol/l insulin was injected into the portal veins of anesthetized rats. Livers were removed 15 min later, immediately homogenized in homogenization buffer B (50 mmol/l Tris-HCl, pH 7.8, 10 mmol/l EDTA, 100 mmol/l NaF, 1 mmol/l dithiothreitol, and 1 mmol/l phenylmethylsulfonyl fluoride), and sonicated for 5 min. The insoluble matter was removed by centrifugation at 10,000g for 15 min and the supernatant was used for glycogen synthase assay. Insulin stimulation of glycogen synthase activity was determined as previously described (27) with modification. Supernatant (32.5 μl) and 17.5 μl assay buffer (33.5 mmol/l Tris-HCl, pH 7.8, 3.35 mmol/l EDTA, 6.5 mmol/l glycogen, 0.75 mmol/l uridine diphosphate [UDP]-glucose, 1.19 μCi/mol UDP-[^{14}C]glucose, in the presence or absence of 10 mmol/l glucose-6-phosphate [G6P]) were mixed by pipetting and incubated at 30°C for 20 min. Then, 60% KOH was added to stop the reaction. The resultant solution (100 μl) was spotted onto prelabeled Whatman filter papers (GF/A, 24 cm), which were immediately immersed in 500 ml 70% ethanol at 4°C, mixed for 40 min, and then washed two more times in 250 ml 70% ethanol for 30 min to remove unincorporated substrate from precipitated glycogen. Filters were air-dried, and radioactivity was counted with 3.5 ml Aquasol 2 scintillant (Packard).

Statistical analysis. Data are expressed as means ± SE. Comparisons were made using the unpaired Student's t test; $P < 0.05$ was considered a statistically significant difference.

RESULTS

Characterization of rats studied. Male Sprague-Dawley rats, at 7 weeks of age, were fed normal diet or a high-salt (8% NaCl) diet for 2 or 8 weeks ($n = 6$ for each group). The body weight, blood pressure, heart rate, food intake, and serum parameters of the rats studied are summarized in

TABLE 1
Characterization of high salt–fed rats at 2 weeks of salt loading

	Controls	High salt–fed rats	P
n	6	6	
Body weight (g)	246.9 ± 5.3	244.2 ± 5.6	0.37
Systolic pressure (mmHg)	116.5 ± 2.2	132.5 ± 2.2	<0.001
Diastolic pressure (mmHg)	57.4 ± 2.5	65.3 ± 3.9	0.11
Heart rate (min^{-1})	373.6 ± 6.1	353.5 ± 10.1	0.10
Food intake per rat (g/day)	24.4 ± 0.5	24.2 ± 0.6	NS
Fasting blood glucose (mg/dl)	97.8 ± 2.5	108.5 ± 2.8	0.010
Fasting plasma insulin (ng/dl)	0.61 ± 0.06	0.86 ± 0.07	0.026
Total cholesterol (mg/dl)	69.3 ± 0.3	71.3 ± 3.4	0.58
Triglyceride (mg/dl)	77.0 ± 2.1	46.7 ± 6.9	<0.05
Plasma renin activity ($ng \cdot l^{-1} \cdot h^{-1}$)	13.2 ± 3.4	2.5 ± 1.1	<0.05
Plasma aldosterone (pg/ml)	230 ± 20	72 ± 15	<0.001
Plasma norepinephrine (pg/ml)	112.8 ± 7.5	113.8 ± 7.1	NS

Data are means $\pm$ SE.

Tables 1 and 2. Food intake per rat did not differ between the two groups (control versus high-salt diet). Compared with control rats, high salt–fed rats showed slightly but significantly higher systolic blood pressure, whereas the diastolic pressures of each group did not differ between groups at 2 weeks (Table 1). Blood glucose and plasma insulin levels were slightly but significantly higher in the high salt–fed rats than in the control rats. Total cholesterol levels of the two groups were similar, but the triglyceride level of the high salt–fed rats was significantly lower than that in the controls (77.0 ± 2.1 vs. 46.7 ± 6.9 mg/dl). Plasma renin activity was significantly lower in the high salt–fed rats than in the controls (13.2 ± 3.4 vs. 2.5 ± 1.1 $ng \cdot ml^{-1} \cdot h^{-1}$). Plasma aldosterone was significantly lower in the high salt–fed rats than in the controls (230 ± 20 vs. 72 ± 15 pg/ml). Plasma norepinephrine concentration was not significantly different between the both groups (112.8 ± 7.5 vs. 113.8 ± 7.1 pg/ml). After 8 weeks of salt loading (Table 2), body weight was lower, although not significantly, in the high salt–fed rats than in the controls. Systolic and diastolic blood pressures and heart rates were significantly higher in the high salt–fed rats than in controls. Neither the fasting blood glucose level nor the plasma insulin level differed significantly between the two groups at 8 weeks.

Hyperinsulinemic-euglycemic clamp study. In vivo insulin action was measured using the hyperinsulinemic-euglycemic clamp technique (Table 3). The insulin infusion rate was designed to achieve submaximal stimulation of the overall glucose disposal rate and suppression of hepatic glucose production. During the steady state, glu-cose levels were clamped at euglycemic levels. With the infusion of insulin, the glucose infusion rate was considerably lower in high salt–fed rats than in the controls (control versus high-salt group; 29.4 ± 3.8 vs. 15.5 ± 5.8 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$, with 2 weeks of salt loading; 16.5 ± 0.6 vs. 11.2 ± 1.2 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.01$, with 8 weeks of salt loading). The glucose disposal rate during the submaximal infusion of insulin in the high salt–fed rats was less than that in the controls (28.6 ± 2.8 vs. 20.0 ± 1.8 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$, at 2 weeks; 21.6 ± 2.0 vs. 16.3 ± 1.5 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$, at 8 weeks). During submaximal insulin infusion, the ability of insulin to suppress hepatic glucose production was impaired in the high salt–fed rats as compared with the controls (3.1 ± 1.8 vs. 6.1 ± 2.0 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$, at 2 weeks; 2.1 ± 0.4 vs. 3.2 ± 0.6 $mg \cdot kg^{-1} \cdot min^{-1}$, $P < 0.05$, at 8 weeks). The glucose metabolic index in soleus muscle was also impaired in the high salt–fed rats as compared with the controls (14.6 ± 1.2 vs. 11.0 ± 1.6 $\mu mol/l \cdot 100 \ g^{-1} \cdot min^{-1}$, $P < 0.05$, at 2 weeks; 12.4 ± 0.4 vs. 8.7 ± 0.6 $\mu mol/l \ 100 \ g^{-1} \cdot min^{-1}$, $P < 0.05$, at 8 weeks). These results suggest that salt loading induces insulin resistance in liver and skeletal muscle.

Insulin-induced 2-deoxy glucose (2DG) uptake in isolated skeletal muscle. Next, we compared the glucose uptakes in isolated skeletal muscle. Soleus muscles that had been removed from anesthetized rats were incubated with insulin and 2DG, and 2DG uptakes into the muscle were assayed. In soleus muscles from controls with 2 weeks of salt loading, insulin stimulation increased the 2DG uptake by 6.6-fold. However, insulin-induced 2DG

TABLE 2
Characterization of high salt–fed rats at 8 weeks of salt loading

	Controls	High salt–fed rats	P
n	6	6	
Body weight (g)	451.3 ± 11.1	422 ± 11.4	0.11
Systolic pressure (mmHg)	113.4 ± 1.9	147.8 ± 1.9	<0.001
Diastolic pressure (mmHg)	76.4 ± 2.2	91.8 ± 2.3	<0.001
Heart rate (min^{-1})	310.2 ± 1.2	349.7 ± 4.2	<0.001
Fasting blood glucose (mg/dl)	126.3 ± 2.2	125.4 ± 3.9	NS
Fasting plasma insulin (ng/dl)	2.03 ± 0.14	1.99 ± 0.23	NS

Data are means $\pm$ SE.

TABLE 3
Hyperinsulinemic euglycemic clamp data and effects of salt loading on hepatic glucose production and glucose uptake into skeletal muscle

	2 weeks salt loading		8 weeks salt loading	
	Controls	High salt–fed rats	Controls	High salt–fed rats
n	5	5	5	5
Plasma insulin (ng/dl)	325 ± 34	321 ± 35	315 ± 35	345 ± 37
Blood glucose (mmol/l)	5.60 ± 0.34	5.53 ± 0.38	5.53 ± 0.29	5.49 ± 0.36
Glucose infusion rate $(mg \cdot kg^{-1} \cdot min^{-1})$	29.4 ± 3.8	$15.5 \pm 5.8^*$	16.5 ± 0.6	$11.2 \pm 1.2†$
Glucose utilization rate $(mg \cdot kg^{-1} \cdot min^{-1})$	32.5 ± 2.8	$21.5 \pm 1.8^*$	18.6 ± 0.4	$14.5 \pm 1.0^*$
Hepatic glucose production $(mg \cdot kg^{-1} \cdot min^{-1})$	3.1 ± 1.8	$6.1 \pm 2.0^*$	2.1 ± 0.4	$3.2 \pm 0.6^*$
Rg' (muscle) $(\mu mol \cdot 100 \ g^{-1} \cdot min^{-1})$	14.6 ± 1.2	$11.0 \pm 1.6^*$	12.4 ± 0.4	$8.7 \pm 0.6^*$

Data are means $\pm$ SE. $^*P < 0.05$. $†P < 0.01$ (control versus high-salt–fed rats.

uptake was attenuated by 33.6% in the high salt–fed rats as compared with the controls (Fig. 1*A*). In the 8-week salt-loading group, insulin-induced 2DG uptake was attenuated by 29.9% in the high salt–fed rats as compared with the controls (Fig. 1*B*). We assessed the soleus muscle GLUT4 content by Western blot analysis (Fig. 1*C*). In both in the 2-week and the 8-week salt-loading groups, GLUT4

protein expression levels in soleus muscle were similar in the controls and the high salt–fed rats.

Glycogen synthase activity in the liver. To investigate hepatic insulin action in high salt–fed rats, we evaluated insulin-stimulated glycogen synthase activation. Anesthetized rats were injected with normal saline or insulin; the livers were removed 15 min after the injection. Liver
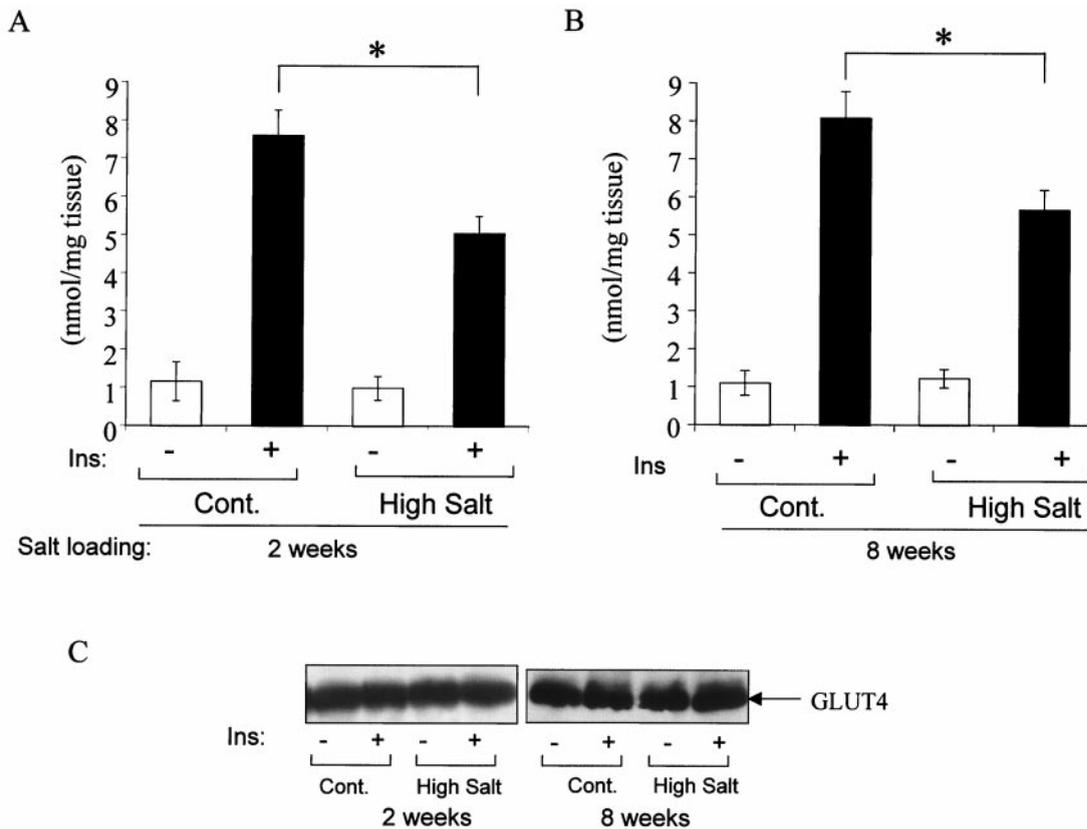

FIG. 1. Insulin-stimulated glucose uptake in skeletal muscle of high salt–fed rats. *A*: 2DG uptake in isolated soleus muscle after 2 weeks of salt loading. *B*: 2DG uptake in isolated soleus muscle after 8 weeks of salt loading. Soleus muscles of rats (*n* = 4–6) were isolated and incubated as described in RESEARCH DESIGN AND METHODS. The muscles were incubated in oxygenated KHB buffer in the presence or absence of human insulin (2 mU/ml) and then rinsed and incubated in KHB buffer containing 8 mmol/l 2-deoxy-D- [1,2-³H(N)]glucose and 32 mmol/l [¹⁴C]mannitol. After the incubation, muscles were solubilized and the uptake rate was counted using a scintillation counter. 2-deoxy-[³H]glucose uptake rates were corrected for extracellular trapping using [¹⁴C]mannitol counts. The bar represents quantitation of the results of triplicate samples. Data are means ± SE. *P < 0.05 high salt–fed rats versus controls treated with insulin. *C*: Expression level of GLUT4 in soleus muscle. Isolated soleus muscles were solubilized in homogenization buffer A, as described in RESEARCH DESIGN AND METHODS. The resultant lysates were subjected to SDS-PAGE and immunoblotted with anti-GLUT4 antibody. GLUT4 proteins were visualized with ECL. The data were representative of three independent experiments.

(G-6-P -/+ ratio)
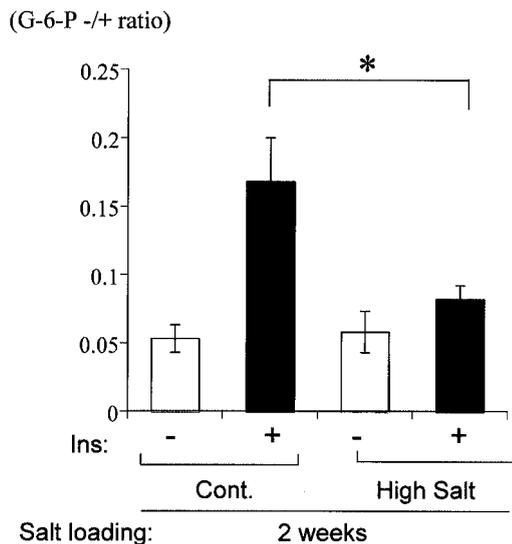
FIG. 2. Insulin-stimulated glycogen synthase activity in livers of high salt–fed rats. Normal saline (4 ml) with or without 10^{-5} mol/l insulin was injected into the portal veins of the anesthetized rats ($n = 3$) given a normal or high salt diet for 2 weeks. Livers were removed 15 min later, immediately homogenized in homogenization buffer B as described in RESEARCH DESIGN AND METHODS, and sonicated for 5 min. The insoluble matter was removed by centrifugation, and the supernatant was used for glycogen synthase assay. Insulin stimulation of glycogen synthase activity was determined as previously described (27), with modification. The liver glycogen synthase activity was determined in the absence or presence of 10 mmol/l G6P and given as the ratio of G6P-independent (in the absence) glycogen synthase activity divided by that of G6P-dependent (in the presence) glycogen synthase activity. The bar represents quantitation of the results of independent triplicate samples. Data are means ± SE. *$P < 0.05$, high salt–fed rats versus controls treated with insulin.

glycogen synthase activity was then determined in the absence or presence of 10 mmol/l G6P and given as the ratio of G6P-independent (in the absence) glycogen synthase divided by that of G6P-dependent (in the presence) glycogen synthase. As shown in Fig. 2, the insulin-stimulated activation of glycogen synthase in rats subjected to 2 weeks of salt loading was lower than that in controls (controls versus high salt–fed rats: 3.17- vs. 1.41-fold). This result suggests that salt loading impaired insulin action in terms of hepatic glycogen synthesis.

Insulin-induced tyrosine phosphorylation of the insulin receptor and IRS proteins. Next, we investigated the insulin-induced tyrosine phosphorylation of the insulin receptor (IR), IRS-1, IRS-2, and IRS-3 in liver and skeletal muscle. Rats were injected with insulin or saline into the portal vein, and then livers and hind limb muscles were removed. The homogenates of these tissues were immunoprecipitated with the anti-IRS or anti-phosphotyrosine (α-PY) antibodies. The tyrosine phosphorylation of IR was represented by an ~90-kDa band of the phosphotyrosine immunoblotting of the α-PY immunoprecipitated (Fig. 3A, upper panel). The hepatic tyrosine phosphorylation of IR was enhanced by 1.8-fold in high salt–fed rats as compared with controls (Fig. 3A, lower panel). The amounts of IRS-1, IRS-2, and IRS-3 protein were determined by immunoprecipitation and immunoblotting with antibodies against each protein. Hepatic expression levels of these proteins in high salt–fed rats were found to be similar to those of the controls (Fig. 3B–D, upper panels). Insulin-induced tyrosine phosphorylation was assessed by the immuno-

blotting with the α-PY antibody (Fig. 3B–D, middle panel). The insulin-induced tyrosine phosphorylations of IRS-1, IRS-2, and IRS-3 were enhanced by 1.8-fold, 2.0-fold, and 1.9-fold ($P < 0.05$), respectively, in the high salt–fed rats as compared with the controls (Fig. 3B–D, lower panels). In skeletal muscle, insulin-induced tyrosine phosphorylation of IR was increased by 1.5-fold (Fig. 3E). The amounts of IRS-1 and IRS-2 in high salt–fed rats were similar to those in the controls (Fig. 3F and G, upper panels). Insulin-induced tyrosine phosphorylations of IRS-1 and IRS-2 in the high salt–fed rats were enhanced by 1.8- and 1.4-fold, respectively, as compared with the controls (Fig. 3F and G, middle and lower panels). These results suggest that insulin-induced tyrosine phosphorylation of IR, IRS-1, IRS-2, and IRS-3 (liver only) in liver and skeletal muscle is enhanced by salt loading.

Insulin-induced PI 3-kinase activation. Insulin-induced PI 3-kinase activation has been shown to be essential for glucose uptake in skeletal muscle (28) and glycogen synthesis in the liver (29). We measured PI 3-kinase activity in the immunoprecipitants with the respective antibodies against IRS-1, IRS-2, and IRS-3 (liver only). Insulin-stimulated PI 3-kinase activities associated with IRS-1, IRS-2, and IRS-3 in the livers of the high salt–fed rats were significantly increased to 4.1-fold, 2.3-fold, and 2.1-fold, respectively, compared with those in the livers of the controls ($P < 0.05$) (Fig. 4A–C). Insulin-stimulated PI 3-kinase activity associated with phosphotyrosine in the livers of the high salt–fed rats was significantly increased to 3.4 times that in livers of the controls ($P < 0.05$) (Fig. 4D). Insulin-stimulated PI 3-kinase activities associated with IRS-1, IRS-2, and phosphotyrosine in the skeletal muscle of high salt–fed rats was significantly increased to 3.1-fold, 3.8-fold, and 2.5-fold, respectively, of that in muscle of the controls ($P < 0.05$) (Fig. 4E–G). These results suggest that insulin-induced activations of PI 3-kinase associated with IRS-1, IRS-2, IRS-3 (liver only), and phosphotyrosine are enhanced by salt loading.

Insulin-induced Akt and GSK3 phosphorylation. Akt is reportedly one of the molecules downstream from PI 3-kinase. Akt kinase activity is regulated by PI 3-kinase products and by its serine/threonine phosphorylation (30,31). Ser-473 is one of the major phosphorylation sites of Akt (32), which has been linked to glucose transport, based on findings that overexpression of constitutively active Akt leads to enhanced glucose transport in 3T3-l1 adipocyte and L6 myotubes (33,34). We investigated the phosphorylation of Ser-473 of Akt in liver and muscle of high salt–fed rats. The amounts of Akt in liver were assessed by the immunoblotting of anti-Akt immunoprecipitants (Fig. 5A, upper panel). The amounts of Akt in livers of controls and high salt–fed rats were comparable. Insulin stimulation leads to a mobility shift in the band because of Akt phosphorylation (see insulin stimulation lanes in Fig. 5A). Insulin-stimulated phosphorylation of Ser-473 of Akt was demonstrated by immunoblotting with the phospho-Ser-473 specific antibody (Fig. 5A, middle panel). Phosphorylation of Ser-473 of Akt was enhanced 2.5-fold ($P < 0.001$) in the liver of the high salt–fed rats as compared with the controls. We also compared the phosphorylation of Ser-473 of Akt in skeletal muscle of high salt–fed rats and controls. In muscle as well, Akt expres-
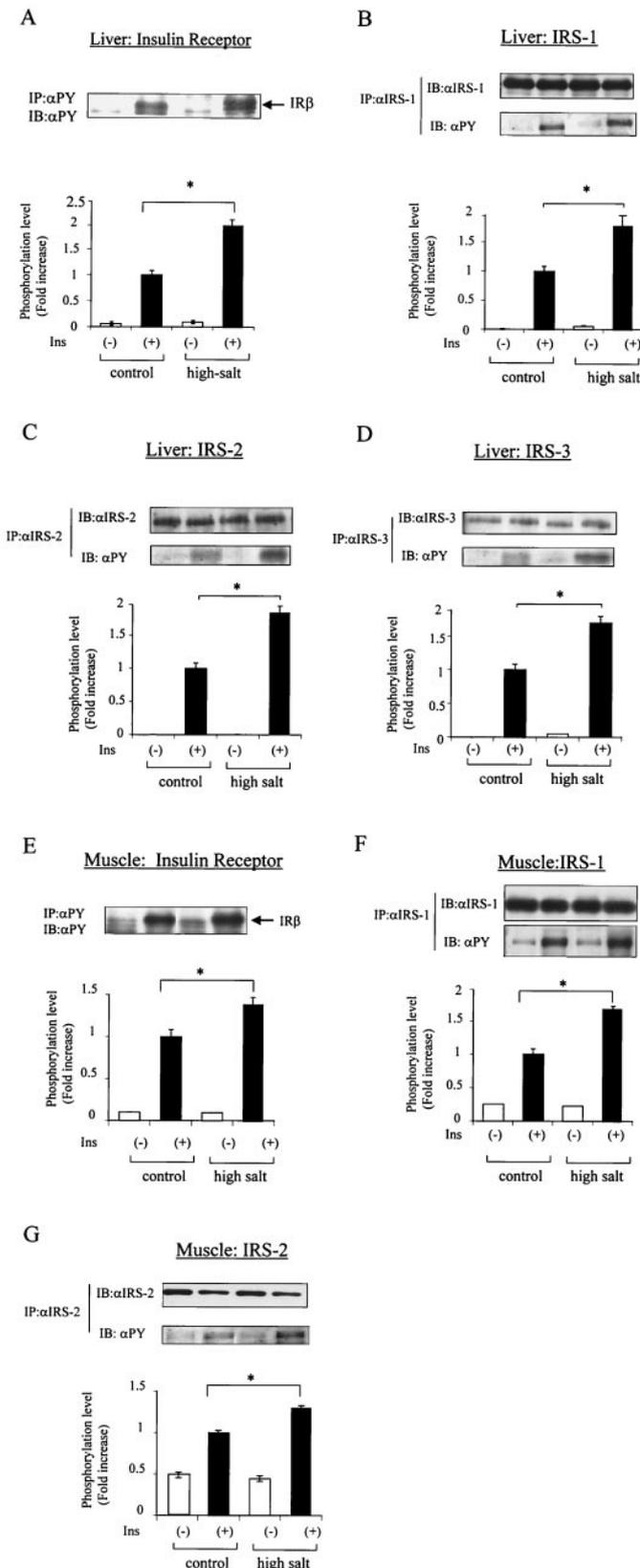
FIG. 3. Insulin-stimulated tyrosine phosphorylation of the IR and IRS proteins in liver and muscle of high salt–fed rats. Rats were anesthetized, and 4 ml normal saline with or without 10^{-5} mol/l insulin was injected ($n = 3$ in each group). Livers and hind limb muscles were removed 30 and 90 s later, respectively, and immediately homogenized in homogenization buffer A, as described in RESEARCH DESIGN AND METHODS. Lysates containing equal amounts of protein were incubated with anti-IRS-1, anti-IRS-2, anti-IRS-3 (liver lysate only), or α-PY antibody

sions were similar in high salt–fed rats and controls. In contrast to the liver, levels of phosphorylation of Akt in muscle were similar in controls and high salt–fed rats (Fig. 5*B*).

GSK-3 is a substrate of Akt, and the phosphorylation of GSK-3 by Akt is reportedly the same as that of phosphorylation induced by insulin stimulation (35). Insulin activates glycogen synthase in part by decreasing the activity of GSK-3 (36). Akt has been shown to phosphorylate Ser-21 of GSK-3 and to inactivate it (35). We investigated the phosphorylation of Ser-21 of the GSK-3α isoform in the livers of high salt–fed rats. The amounts of GSK-3α were assessed by immunoblotting of liver and muscle cell lysates (Fig. 5*C*, upper panel). The amounts of GSK-3α in livers of controls and high salt–fed rats were comparable. Insulin-stimulated phosphorylation of Ser-21 of GSK-3α was demonstrated by immunoblotting with phospho-specific GSK-3α Ser-21 antibody (Fig. 5*C*, middle and lower panels). Phosphorylation of Ser-21 of hepatic GSK-3α in high salt–fed rats was double ($P < 0.001$) that in the controls. These results suggest that salt loading enhances phosphorylation of Akt and GSK-3 in liver but not phosphorylation of Akt in muscle.

The effect of administration of thiazolidinedione on decreased insulin sensitivity induced by a high salt diet. Next, we investigated the reversibility of the decreased insulin sensitivity associated with high salt loading. Rats were fed a normal or a high salt diet, with or without 0.02% pioglitazone, for 2 weeks ($\sim$20 mg/kg body wt per day). This amount of pioglitazone is reportedly sufficient to improve insulin resistance in Zucker *fa/fa* rats (37). In high salt–fed rats, a slight increase in blood pressure was unaffected by administration of pioglitazone (Table 4). Hyperinsulinemic-euglycemic clamp study showed that administration of pioglitazone did not normalize the decreased glucose infusion rate, glucose utilization rate, or glucose metabolic index in the soleus muscle or increase the hepatic glucose production (Table 5). These results suggest that administration of pioglitazone has no effect on decreased insulin sensitivity associated with high salt loading.

DISCUSSION

Multiple lines of evidence have shown an association between insulin resistance and hypertension, as well as a relationship between dietary salt intake and hypertension. In addition, several clinical reports have shown that patients with insulin resistance or diabetes tend to have the salt-sensitive type of hypertension (12–14). Furthermore, normal subjects given a high-salt diet were reported to be more insulin-resistant than those on a low-salt diet, based on a hyperinsulinemic-euglycemic clamp study (16).

(10 μg/ml each) and then incubated with 10 ml protein A-Sepharose. The samples were washed and boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol. Immunoprecipitant (10 μl) was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with each of the antibodies. Proteins were visualized with ECL, and band intensities were quantified with a Molecular Imager GS-525 using Imaging Screen-CH. *A*: Liver insulin receptor. *B*: Liver IRS-1. *C*: Liver IRS-2. *D*: Liver IRS-3. *E*: Muscle insulin receptor. *F*: Muscle IRS-1. *G*: Muscle IRS-2. Upper and middle panels represent immunoblots. The bar represents quantitation of the results of independently obtained, in triplicate, tyrosine phosphorylation bands. Data are means ± SE. *$P < 0.05$, high salt–fed rats versus controls treated with insulin.
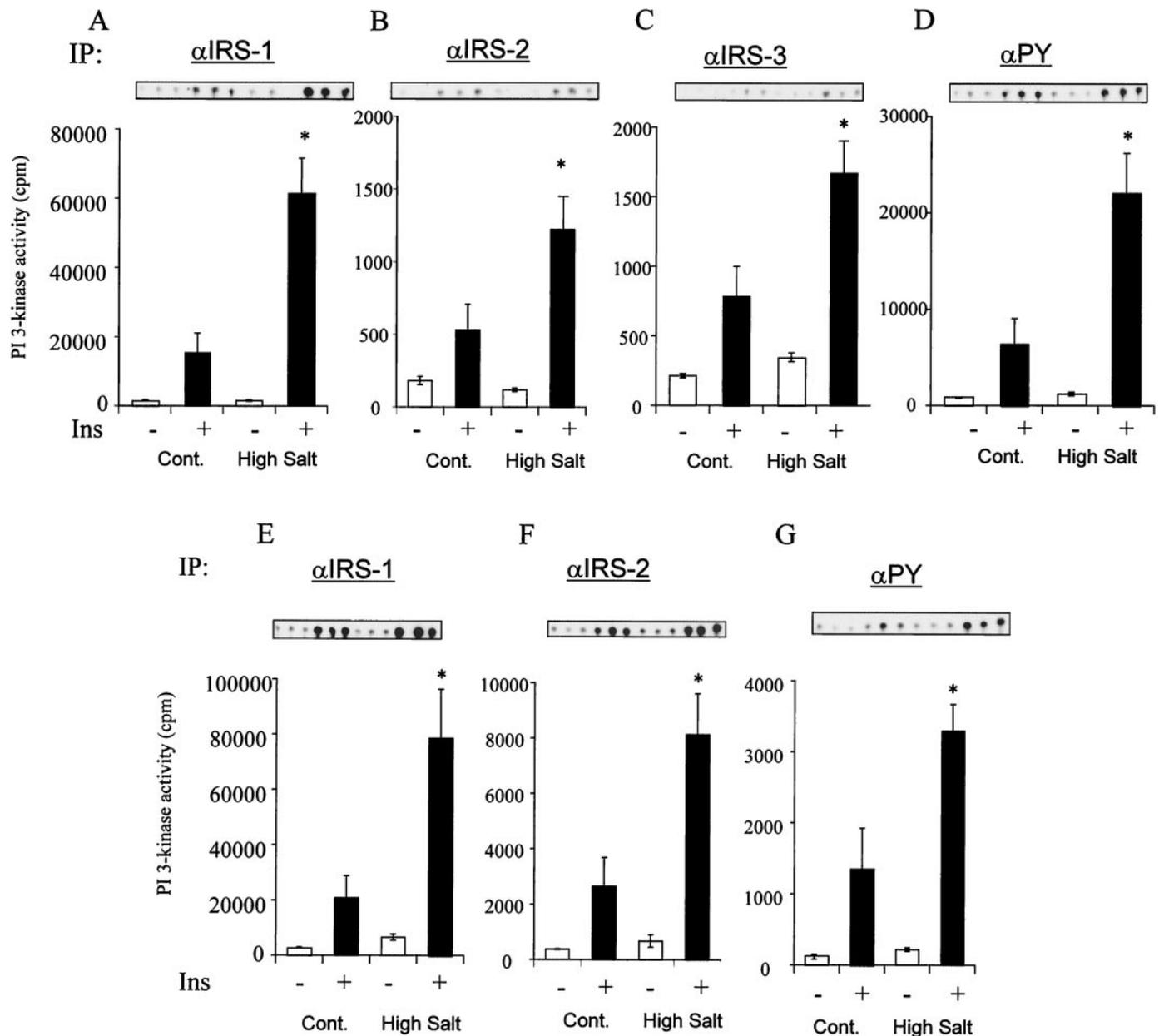
FIG. 4. Insulin-stimulated PI 3-kinase activation in liver and muscle of high salt–fed rats. Rats were anesthetized, and 4 ml normal saline with or without 10^{-5} mol/l insulin was injected. Livers and hind limb muscles were removed and homogenized ($n = 3$ in each group). The lysates were subjected to immunoprecipitation with anti-IRS-1, anti-IRS-2, anti-IRS-3, or α-PY antibodies followed by protein A-Sepharose 6MB. The assays of PI 3-kinase activity in the immunoprecipitant were performed as previously described (18). *A*: Liver IRS-1. *B*: Liver IRS-2. *C*: Liver IRS-3. *D*: Liver phosphotyrosine. *E*: Muscle IRS-1. *F*: Muscle IRS-2. *G*: Muscle phosphotyrosine. Upper panels show spots of PI(3)P from independent triplicate assay samples. The bar represents PI 3-kinase activity of independent triplicate experiments. Data are means ± SE. *$P < 0.05$, high salt–fed rats versus controls treated with insulin.

In this study, we first investigated whether a high-salt diet induces insulin resistance in normal SD rats. After salt loading for 2 weeks, neither body weight nor food intake was altered, whereas blood pressure was slightly but significantly elevated. Then, we examined insulin sensitivity in high salt–fed rats using a hyperinsulinemic-euglycemic clamp method, which revealed that high salt–fed rats were significantly insulin-resistant after both 2 weeks and 8 weeks of salt loading as indicated by the decreased glucose infusion rate (Table 3). We analyzed insulin action using skeletal muscle and liver tissues and found that insulin-stimulated glucose uptake in the isolated muscle and glycogen synthase activation in the liver were attenu-

ated in high salt–fed rats. These results indicate that salt loading induces insulin resistance in both muscle and liver. The decreased triglyceride level in high salt–fed rats suggests that a decreased insulin action in liver promotes triglyceride synthesis.

Next, we investigated the molecular mechanism underlying insulin resistance induced by salt loading. Recently, rigorous studies have been published on the intracellular signaling pathways leading to various insulin actions, although much still remains unknown (38,39). In skeletal muscle cells and adipocytes, the rate-limiting step of glucose uptake is determined by the activity of glucose transporters on the cell surface. In translocation of the
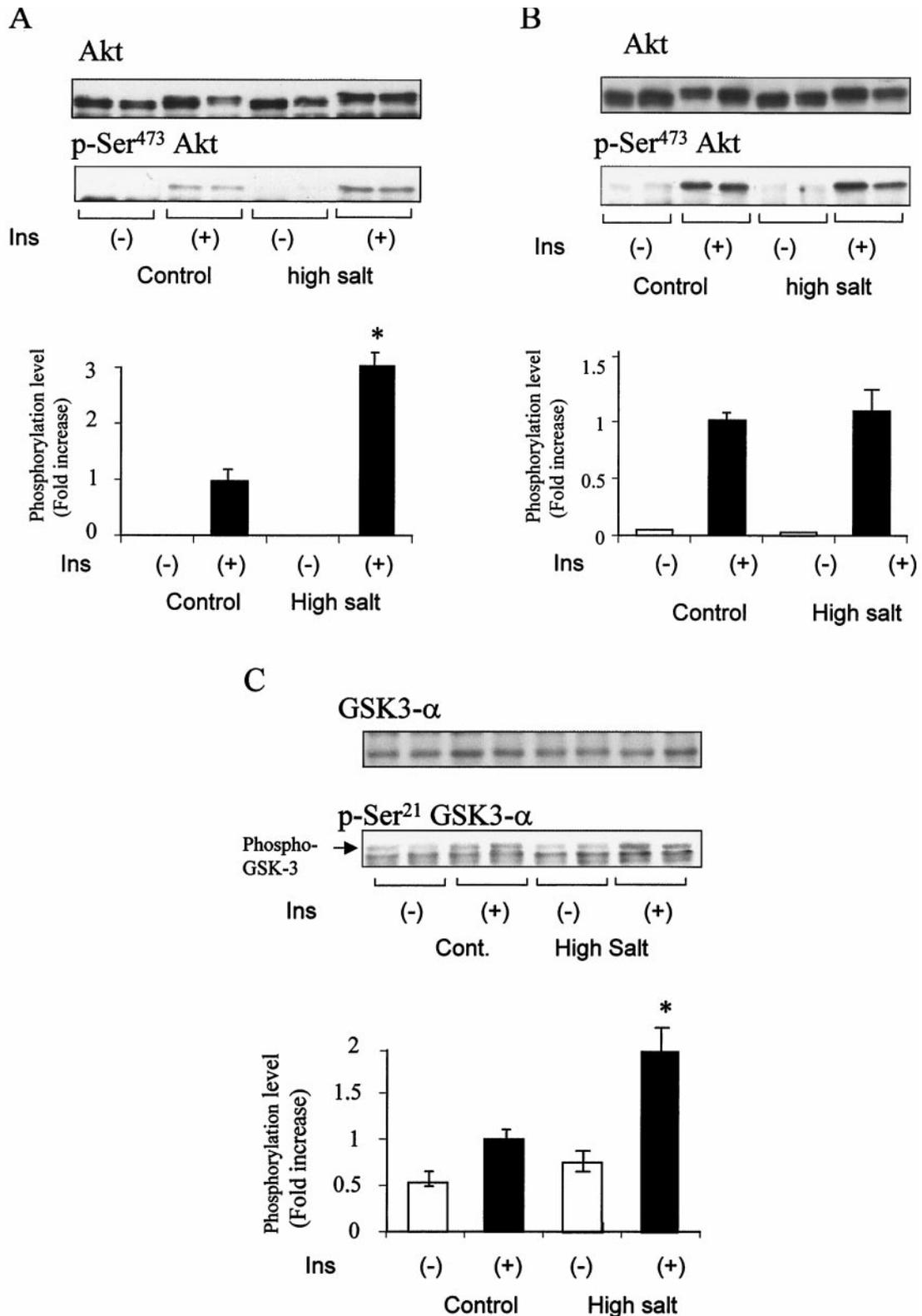
FIG. 5. Insulin-stimulated phosphorylation of Akt in liver and muscle and of GSK-3 in liver and muscle of high salt–fed rats. Rats were anesthetized, and 4 ml normal saline with or without 10^{-5} mol/l insulin was injected ($n = 3$ in each group). Livers and hind limb muscles were removed and homogenized. The lysates were subjected to immunoprecipitation with anti-Akt antibody and then incubated with 10 μl protein A-Sepharose. The immunoprecipitants were washed and then boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol. Immunopre-cipitant (10 μg) was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against Akt or phosphorylated Ser-473 of Akt. The total liver lysates were subjected to immunoblotting with antibody against GSK-3a and phosphorylated Ser-21 of GSK-3a. Proteins were visualized with ECL and band intensities were quantified with a Molecular Imager GS-525 using Imaging Screen-CH. *A*: The amount of Akt protein and phosphorylation of Ser-473 of Akt in liver. *B*: The amount of Akt protein and phosphorylation of Ser-473 of Akt in muscle. *C*: The amount of GSK-3α protein and phosphorylation of Ser-21 of GSK-3α in liver. Upper and middle panels are representative immunoblots. The bar represents quantitation of the results of independently obtained, in triplicate, tyrosine phosphorylation bands. Data are means ± SE. *$P < 0.05$, high salt–fed rats versus controls treated with insulin.

TABLE 4
Characterization of controls and high salt–fed rats with or without administration of pioglitazone

Administration of pioglitazone	Controls		High salt–fed rats	
	(−)	(+)	(−)	(+)
n	5	5	5	5
Body weight (g)	314.0 ± 6.7	310.7 ± 3.8	305.6 ± 7.1	295.0 ± 3.1
Systolic pressure (mmHg)	102.8 ± 2.5	102.5 ± 3.5	122.1 ± 5.5*	128.1 ± 7.1
Diastolic pressure (mmHg)	69.9 ± 5.3	72.1 ± 2.8	89.4 ± 2.9*	85.4 ± 8.1
Food intake per rat (g/day)	30.6 ± 0.8	30.2 ± 0.7	29.5 ± 0.7	30.4 ± 0.9

Data are means ± SE. *$P < 0.05$ (control versus high-salt group without pioglitazone.)

highly insulin-sensitive glucose transporter GLUT4 to the cell surface, insulin-stimulated phosphorylation of the insulin receptor and its substrates, IRS-1 and IRS-2, and their associated PI 3-kinase activation have been shown to play an important role, whereas the contribution of Akt is controversial (39). In the liver, insulin-stimulated PI 3-kinase activation has been shown to be essential for glycogen synthesis (29) and suppression of gluconeogenesis (40). Insulin-induced PI 3-kinase activation leads to Akt activation and subsequent GSK-3 phosphorylation, resulting in glycogen synthase activation (41).

Thus, we investigated how the insulin-induced activation of PI 3-kinase and phosphorylation of Akt and GSK-3 were affected in muscle and liver by the high salt–diet feeding. Surprisingly, this insulin signaling was apparently enhanced in both muscle and liver of high salt–fed rats, despite the presence of insulin resistance. In the liver and muscle of high salt–fed rats, insulin-induced phosphorylation of IR, IRS-1, IRS-2, and IRS-3 (liver only) was increased 1.4- to 2-fold, as compared with controls (Fig. 3). In addition, PI 3-kinase activities with anti-IRS-1, anti-IRS-2, or anti-IRS-3 were increased, more markedly than the tyrosine phosphorylation levels, to 2.1–4.1 times those in controls (Fig. 4). In the livers of high salt–fed rats, insulin-induced phosphorylations of Akt and GSK-3 were enhanced 2.9- and 2-fold, respectively, as compared with the controls, although this was not the case in the muscle of high salt–fed rats (Fig. 5). Based on these findings, it is very likely that insulin signal transduction leading to GLUT4 translocation is impaired at the step located downstream from PI 3-kinase activation in muscle. On the other hand, in the livers of high salt–fed rats, insulin-induced

phosphorylation of Akt and GSK-3 were markedly enhanced, despite the decreased activation of glycogen synthase. The molecular mechanism responsible for this discrepancy between enhanced GSK-3 phosphorylation and decreased glycogen synthase activation cannot be pinpointed based on the data presented in this study. Glycogen synthase activity is reportedly regulated by the balance between dephosphorylation by protein phosphatase-1 and phosphorylation by GSK-3 (38). Therefore, we speculate that some modification of glycogen synthase activation by molecules other than GSK-3 or some change in the intracellular distribution of GSK-3 and glycogen synthase accounts for this discrepancy. Further study is needed to clarify this issue.

To date, almost all insulin-resistant animal models as well as human subjects have been shown to exhibit an impairment of insulin-induced PI 3-kinase activation. For example, PI 3-kinase activation was shown to be impaired in several tissues from insulin-resistant animal models such as ob/ob mice (17), Zucker fatty rats (18,42), dexamethasone-treated rats (21), and aged rats (20). Impaired insulin-induced PI 3-kinase activation was also reported in muscles from obese or nonobese patients with type 2 diabetes (43,44). The only exception is the livers of high-fat–fed rats, which we recently reported to show marked enhancement of insulin-induced PI 3-kinase activation despite insulin resistance (19). In muscle and fat from high-fat–fed rats, insulin-induced PI 3-kinase activation was shown to be decreased.

Based on data from this study and the aforementioned previous reports, we conclude that a high-salt diet induces insulin resistance by a mechanism completely different

TABLE 5
Hyperinsulinemic euglycemic clamp data and effects of administration of pioglitazone on hepatic glucose production and glucose uptake into skeletal muscle

Administration of pioglitazone	Controls		High salt–fed rats	
	(−)	(+)	(−)	(+)
n	5	5	5	5
Plasma insulin (ng/dl)	321 ± 19	316 ± 21	337 ± 22	314 ± 37
Blood glucose (mmol/l)	5.52 ± 0.28	5.60 ± 0.30	5.45 ± 0.24	5.49 ± 0.33
Glucose infusion rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	23.5 ± 1.2	24.2 ± 1.0	17.1 ± 0.6*	17.6 ± 1.2
Glucose utilization rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	25.7 ± 0.9	26.3 ± 1.4	22.1 ± 0.6†	22.3 ± 1.2
Hepatic glucose production ($mg \cdot kg^{-1} \cdot min^{-1}$)	2.2 ± 0.7	2.2 ± 0.4	5.0 ± 0.7*	4.7 ± 0.6
Rg′ (muscle) ($\mu mol \cdot 100\ g^{-1} \cdot min^{-1}$)	14.1 ± 1.8	13.8 ± 1.2	10.7 ± 1.2*	11.3 ± 1.8

Data are means ± SE. *$P < 0.05$. †$P < 0.01$ (control versus high-salt group without pioglitazone)

from those observed in other insulin-resistant animal models. Recently, thiazolidinediones and their derivatives have come to be used for improving insulin resistance, and their main mechanism of action is reportedly enhancement of an early step in the insulin-signaling pathway leading to PI 3-kinase activation (24,45,46). Thus, we believe that it is important to understand whether PI 3-kinase activation is impaired or enhanced in the insulin-resistant state. In fact, thiazolidinediones and their derivatives have been shown to be highly effective in insulin-resistant animal models with impairment of PI 3-kinase activation, such as Zucker fatty rats (24). However, troglitazone, one of the thiazolidinediones, was not effective in rats with insulin resistance due to high-fat feeding (47). Such rats show enhancement of hepatic PI 3-kinase activity. A major finding of this study is that pioglitazone failed to improve the insulin resistance in high salt–fed rats. Although a previous report showed pioglitazone to be effective in reversing the insulin resistance in high salt–fed Dahl salt-sensitive rats (48), our results using normal SD rats were not consistent with their observation, probably because different experimental conditions were used.

Recently, large-scale studies such as the U.K. Prospective Diabetes Study have shown that treatment of hypertension improves the prognosis of patients with type 2 diabetes (2). Thus, rigorous studies on the mechanism and the treatment of insulin resistance with hypertension are needed. Nevertheless, to our knowledge, no study in the field of insulin signal transduction research has focused on how insulin signaling is modified in the hypertensive state. A previous report demonstrated that genetically hypertensive Dahl salt-sensitive rats become insulin resistant in accordance with the development of severe hypertension induced by a high salt diet (49). However, our SD rats, which are not an animal model of hypertension, also became insulin-resistant despite minimal elevation of blood pressure. Our results seem to be consistent with observations made in the human study reported by Donovan et al. (16). In our study, the impairment of insulin action both in vivo and in isolated muscle was at a similar level at 2 and 8 weeks of salt loading, despite the blood pressure elevation being greater at 8 weeks than at 2 weeks. Thus, we can speculate that a high-salt diet induces insulin resistance independently of the development of hypertension, but further study is necessary to clarify this issue.

This report is the first to clearly demonstrate that a high-salt diet can induce insulin resistance via a unique mechanism and is anticipated to open the way to future treatment strategies for patients with diabetes and hypertension. In conclusion, *1*) a high-salt diet may be a factor promoting insulin resistance, *2*) the insulin-signaling step impaired by high-salt intake is likely to be downstream from PI 3-kinase or Akt activation, and *3*) this unique insulin resistance mechanism may be present in patients with diabetes and hypertension.

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