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Article

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Prevention of fat-induced insulin resistance by salicylate

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Insulin resistance is a major factor in the pathogenesis of type 2 diabetes and may involve fat-induced activation of a serine kinase cascade involving IKK-β. To test this hypothesis, we first examined insulin action and signaling in awake rats during hyperinsulinemic-euglycemic clamps after a lipid infusion with or without pretreatment with salicylate, a known inhibitor of IKK-β. Whole-body glucose uptake and metabolism were estimated using [3-³H]glucose infusion, and glucose uptake in individual tissues was estimated using [1-¹⁴C]2-deoxyglucose injection during the clamp. Here we show that lipid infusion decreased insulin-stimulated glucose uptake and activation of IRS-1–associated PI 3-kinase in skeletal muscle but that salicylate pretreatment prevented these lipid-induced effects. To examine the mechanism of salicylate action, we studied the effects of lipid infusion on insulin action and signaling during the clamp in awake mice lacking IKK-β. Unlike the response in wild-type mice, IKK-β knockout mice did not exhibit altered skeletal muscle insulin signaling and action following lipid infusion. In summary, high-dose salicylate and inactivation of IKK-β prevent fat-induced insulin resistance in skeletal muscle by blocking fat-induced defects in insulin signaling and action and represent a potentially novel class of therapeutic agents for type 2 diabetes.

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Introduction

Insulin resistance plays a primary role in the development of type 2 diabetes (1) and is a characteristic feature of other health disorders including obesity, dyslipidemias, hypertension, and cardiovascular disease (2). The mechanism by which insulin resistance occurs is unknown but may be related to alterations in fat metabolism (3). More than 30 years ago, Randle and colleagues (4) introduced the concept of substrate competition between glucose and fatty acid (i.e., glucose-fatty acid cycle) and postulated that fatty acids cause insulin resistance through an increase in the NADH/NAD and acetyl CoA/CoA ratios and citrate, leading to the inhibition of pyruvate dehydrogenase (5) and phosphofructokinase activity (6). Subsequently, glucose 6-phosphate concentration increases, which leads to the inhibition of hexokinase activity and glucose uptake (7). Recent studies by our group have challenged this substrate competition mechanism and found that acute elevations in plasma fatty acids in humans result in decreased glucose transport activity, as reflected by decreased concentrations of intracellular glucose 6-phosphate (8) and glucose (9). Furthermore, we have demonstrated that an acute elevation of plasma fatty acids resulted in activation of protein kinase C-θ (PKC-θ) (10), a serine kinase, and postulated that this leads to serine phosphorylation of insulin receptor substrate (IRS)-1, which reduces the ability of IRS-1 to activate phosphatidylinositol 3-kinase (PI 3-kinase), an important mediator of insulin signaling and action (11).

Almost 100 years ago, Williamson and colleagues (12) showed that high-dose salicylate treatment reduced the severity of glycosuria in diabetic patients, and in 1957, Reid and colleagues (13) further demonstrated that 10–14 days of aspirin treatment improved the results of oral glucose tolerance tests in diabetic patients. The mechanism by which salicylate may affect whole-body glucose homeostasis remained unknown until a recent finding by Yin and colleagues (14) that salicylate inhibits the activity of IκB kinase-β (IKK-β), a known serine kinase. In light of these findings, we hypothesized that salicylate might prevent fat-induced insulin resistance in skeletal muscle by inhibiting the activity of IKK-β and subsequent serine phosphorylation of IRS-1 by IKK-β and decreased activation of IRS-1–associated PI 3-kinase. To test this

hypothesis, we examined whether high-dose salicylate pretreatment would prevent fat-induced alterations in insulin activation of IRS-1–associated PI 3-kinase and skeletal muscle glucose uptake. In addition, we examined whether mice with inactivation of IKK-β are protected from fat-induced alterations in skeletal muscle insulin signaling and action.

Methods

Study 1: effects of salicylate pretreatment on fat-induced insulin resistance in rats

Animals. Male Wistar rats weighing 275–300 g were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA) and studied at least 5 days after arrival. Rats were housed under controlled temperature (23°C) and lighting (12 hours of light, 0600–1800 hours; 12 hours of dark, 1800–0600 hours) with free access to water and standard rat chow. All procedures were approved by the Yale University Animal Care and Use Committee.

Surgery and animal handling. At least 4 days before clamp experiments, rats were placed in individual cages with wire floors. The distal one third of each rat's tail was drawn through a hole placed low on the side of the cage and secured there with a rubber stopper. This arrangement was required to protect tail blood vessel catheters during experiments (15). Rats were free to move about and were allowed unrestricted access to food and water. Two tail vein infusion catheters were placed the day before the experiment, and one tail artery blood sampling catheter was placed 2–3 hours before the start of experiments (i.e., at 0700 hours). Catheters were placed percutaneously during local anesthesia with lidocaine while rats were restrained in a towel. Rats were returned to their cages after catheter placement with tails secured as described above and were free to move about during the experiments. Patency of the arterial catheter was maintained by a slow (0.016 ml/min) infusion of heparinized saline (10 U/ml).

Pretreatment of salicylate for 19 hours. Starting at 1200 hours on the day before the experiment, salicylate (for the salicylate and lipid-salicylate groups, 42 mg/kg; Sigma Chemical Co., St. Louis, Missouri, USA) or saline (for the control and lipid groups, matching volume) was fed using oral gavage at 6-hour internals (i.e., at 1200, 1800, and 2400 hours). The food was removed from the cage at 1700 hours for the overnight fasting of animals.

Infusion of lipid and/or salicylate for 5 hours. On the morning of experiment day (at 0700 hours), saline (control group, *n* = 7), salicylate (7 mg/kg/h; salicylate group, *n* = 6), lipid (5 ml/kg/h, Liposyn II, triglyceride emulsion, 20% wt/vol; Abbott Laboratories, North Chicago, Illinois, USA) and heparin (6 U/h; lipid group, *n* = 7), or lipid/heparin and salicylate (lipid-salicylate group, *n* = 7) was infused for 5 hours (preinfusion period). A blood sample (200 µl) was collected at the start of 5-hour lipid/salicylate infusion period for measurement of basal concentrations of glucose, insulin, free

fatty acid (FFA), and salicylate. To estimate basal whole-body glucose turnover, HPLC-purified [3-³H]glucose (NEN Life Science Products Inc., Boston, Massachusetts, USA) was infused at a rate of 0.05 µCi/min during the preinfusion period. Three blood samples (100 µl) were collected during the last 30 minutes of this period for measurements of plasma glucose, insulin, FFA, salicylate, and [³H]glucose concentrations.

Hyperinsulinemic-euglycemic clamp in awake rats. After the 5-hour lipid/salicylate infusion, a 70-minute hyperinsulinemic-euglycemic clamp was conducted with a prime-continuous infusion of human insulin (Humulin; Eli Lilly, Indianapolis, Indiana, USA) at a rate of 60 pmol/kg/min to raise plasma insulin concentration to approximately 3,000 pM. In the lipid and lipid-salicylate groups, lipid and heparin were infused as in the preinfusion period. Blood samples were collected at 10-minute interval for the immediate measurement of plasma glucose concentration, and 20% glucose was infused at variable rates to maintain plasma glucose at approximately 6 mM. Insulin-stimulated whole-body glucose flux were estimated using a prime-continuous infusion of [3-³H]glucose (20 µCi bolus, 0.2 µCi/min) throughout the clamps. To estimate rates of insulin-stimulated glucose uptake and metabolism in vivo in individual tissues, 2-deoxy-D-[1-¹⁴C]glucose (2-[¹⁴C]DG; NEN Life Science Products Inc.) was administered as a bolus (20 µCi) at 30 minutes before the end of clamps. Blood samples (60 µl) were taken at 42, 44, 47, 50, 55, 60, 65, and 70 minutes after the start of clamps for the determination of plasma [³H]glucose, ³H₂O, and 2-[¹⁴C]DG concentrations. Given that 2-deoxyglucose is a glucose analogue that is phosphorylated but not metabolized, insulin-stimulated glucose uptake in individual tissues can be estimated by determining the tissue content of 2-deoxyglucose-6-phosphate. Additional blood samples (100 µl) were obtained before the start and at the end of clamps for measurement of plasma insulin, FFA, and salicylate concentrations. All infusions were done using Razel syringe pumps (Razel Scientific Instrument, Stamford, Connecticut, USA). At the end of clamps, rats were anesthetized with sodium pentobarbital injection (2 mg/kg body weight). Within 5 minutes, four muscles (soleus, gastrocnemius, tibialis anterior, and quadriceps) from both hindlimbs, epididymal white adipose tissue, intrascapular brown adipose tissue, and liver were taken. Each tissue, once exposed, was dissected out within 2 seconds, frozen immediately using liquid N₂-cooled aluminum blocks, and stored at –70°C for later analysis.

Study 2: effects of lipid infusion in mice with inactivation of IKK-β

Animals and surgery. To determine whether IKK-β inactivation prevents fat-induced insulin resistance, the effects of lipid infusion on insulin action and signaling were examined in male whole-body heterozygous IKK-β knockout (IKK-β KO) and age-matched wild-

type littermates (16). At least 4 days before hyperinsulinemic-euglycemic clamp experiments, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and an indwelling catheter was inserted in the left internal jugular vein. The catheters were externalized through an incision in the skin flap behind the head, and the mice were returned to individual cages after the surgery (17). Mice fully recuperated from the surgery before in vivo experiments as reflected by their reaching preoperative weight. To conduct experiments in awake mice with minimal stress, a tail restraint method was used (17).

Hyperinsulinemic-euglycemic clamp in awake mice. After an overnight fast, saline or lipid (5 ml/kg/h, Liposyn II, triglyceride emulsion, 20% wt/vol; Abbott Laboratories) and heparin (6 U/h) were infused for 5 hours (basal period) in the wild-type mice (control group, *n* = 4; control-lipid group, *n* = 3) and IKK-β KO mice (IKK-β KO group, *n* = 4; IKK-β KO-lipid group, *n* = 5). To estimate basal whole-body glucose turnover, [3-^{3}H]glucose was infused at a rate of 0.05 μCi/min during the basal period. A blood sample (80 μl) was collected at the end of basal period for measurement of basal concentrations of glucose, insulin, FFA, and [^{3}H]glucose. After the basal period, a 120-minute hyperinsulinemic-euglycemic clamp was conducted with a prime-continuous infusion of human insulin at a rate of 15 pmol/kg/min to raise plasma insulin within a physiological range (~730 pM). Blood samples (20 μl) were collected at 20- to 30-minute intervals for the immediate measurement of plasma glucose concentration, and 20% glucose was infused at variable rates to maintain plasma glucose at basal concentrations. Insulin-stimulated whole-body glucose flux were estimated using a prime-continuous infusion of [3-^{3}H]glucose (10 μCi bolus, 0.1 μCi/min; NEN Life Science Products Inc.) throughout the clamps. All infusions were done using microdialysis pumps (CMA/Microdialysis, North Chelmsford, Massachusetts, USA). To estimate insulin-stimulated glucose uptake and metabolism in individual tissues, 2-[^{14}C]DG (NEN Life Science Products Inc.) was administered as a bolus (10 μCi) at 75 minutes after the start of clamps. Blood samples (20 μl) were taken at 80, 85, 90, 100, 110, and 120 minutes after the start of clamps for the determination of plasma [^{3}H]glucose, ^{3}H$_2$O, and 2-[^{14}C]DG concentrations. At the end of clamps, mice were anesthetized, and tissues were taken for later analysis as described in Study 1.

Insulin signaling analysis. Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase activity in skeletal muscle were measured by immunoprecipitating IRS-1 using antibody to IRS-1 (kindly provided by M. White, Joslin Diabetes Center, Boston, Massachusetts, USA) and assessing the incorporation of ^{32}P into PI to yield phosphatidylinositol-3-monophosphate (17). Insulin-stimulated tyrosine phosphorylation of insulin receptor in skeletal muscle were measured using antibodies to phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, New York, USA).

In vivo glucose flux analysis. Plasma glucose concentrations during clamps were analyzed using 10 μl plasma by a glucose oxidase method on a Beckman glucose analyzer II (Beckman Instruments Inc., Fullerton, California, USA), and plasma insulin concentrations were measured by RIA using kits from Linco Research Inc. (St. Charles, Missouri, USA). Plasma FFA concentrations were determined using an acyl-CoA oxidase-based colorimetric kit (Wako Chemicals USA Inc., Richmond, Virginia, USA), and plasma salicylate concentration (Study 1) was determined using salicylate assay kit (Sigma Chemical Co.). For the determination of plasma [3-^{3}H]glucose and 2-[^{14}C]DG concentrations, plasma was deproteinized with ZnSO$_4$ and Ba(OH)$_2$, dried to remove ^{3}H$_2$O, resuspended in water, and counted in scintillation fluid (Ultima Gold; Packard Instrument Co., Meriden, Connecticut) on dual channels for separation of ^{3}H and ^{14}C. The plasma concentration of ^{3}H$_2$O was determined by the difference between ^{3}H counts without and with drying. For the determination of tissue 2-[^{14}C]DG-6-phosphate (2-DG-6-P) content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG, as described previously (15). The radioactivity of ^{3}H in muscle glycogen was determined by digesting muscle samples in KOH and precipitating glycogen with ethanol as described previously (15). Skeletal muscle and liver triglyceride concentrations (Study 1) were determined using triglyceride assay kit (Sigma Chemical Co.) and a method adapted from Storlien et al. (18).

Calculations. Rates of basal endogenous glucose production (EGP) and insulin-stimulated whole-body glucose uptake were determined as the ratio of the [^{3}H]glucose infusion rate (disintegrations per minute [dpm]/min) to the specific activity of plasma glucose (dpm/μmol) during the final 30 minutes of basal and clamp periods, respectively. Endogenous glucose production during clamps was determined by subtracting the glucose infusion rate from the whole-body glucose uptake. Whole-body glycolysis was calculated from the rate of increase in plasma ^{3}H$_2$O concentration, determined by linear regression of the measurements at 50, 55, 60, 65, and 70 minutes for Study 1 and at 80, 85, 90, 100, 110, and 120 minutes for the Study 2. Whole-body glycogen and lipid synthesis were estimated by subtracting whole-body glycolysis from whole-body glucose uptake, assuming that glycolysis and glycogen/lipid synthesis account for the majority of insulin-stimulated glucose uptake (19). Glucose uptake in individual tissues was calculated from plasma 2-[^{14}C]DG profile, which was fitted with a double exponential curve using MLAB (Civilized Software, Bethesda, Maryland, USA) and tissue 2-DG-6-P content as described previously (15). Skeletal muscle glycogen synthesis was calculated from ^{3}H incorporation to muscle glycogen as described elsewhere (15). Skeletal muscle glycolysis was then estimated as the difference between muscle glucose uptake and muscle glycogen synthesis.

Figure 1

Metabolic parameters and insulin-stimulated whole-body and skeletal muscle (soleus) glucose uptake in awake control (black bars), salicylate (light gray bars), lipid (open bars), and lipid-salicylate (dark gray bars) rats. (**a**) Plasma salicylate concentrations during basal and hyperinsulinemic-euglycemic clamp. (**b**) Plasma FFA concentrations during basal and hyperinsulinemic-euglycemic clamp. (**c**) Insulin-stimulated whole-body glucose uptake in vivo. (**d**) Insulin-stimulated skeletal muscle glucose uptake in vivo. Values are means ± SE for six to seven experiments. *$P < 0.05$ versus control group.
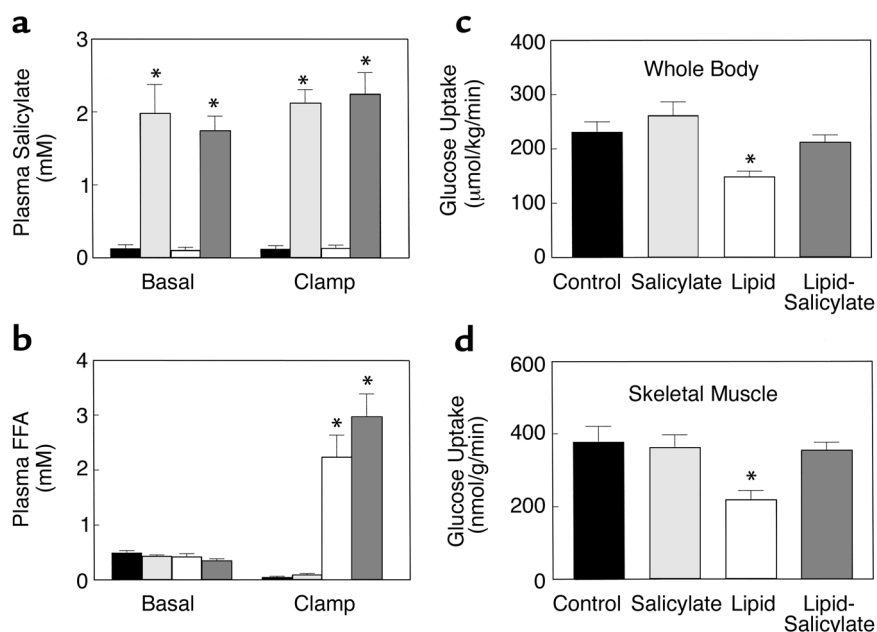
Statistical analysis. Data are expressed as means ± SE. The significance of the difference in mean values among control, salicylate, lipid, and lipid-salicylate groups in Study 1 and among control, IKK-β KO, control-lipid, and IKK-β KO-lipid groups in Study 2 was evaluated using the Duncan's multiple range test.

Results

The effect of high-dose salicylate pretreatment on fat-induced insulin resistance was examined in awake rats. Plasma salicylate concentrations were raised to approximately 2 mM after a 19-hour salicylate pretreatment and remained elevated during clamps in the salicylate and lipid-salicylate groups (Figure 1a). Plasma fatty acid concentrations were increased to approximately 2.5 mM after a 5-hour lipid infusion and remained elevated during clamps in the lipid and lipid-salicylate groups (Figure 1b). In contrast, plasma fatty acid concentrations were suppressed by approximately 80% during clamps in the control and salicylate groups. A 19-hour salicylate pretreatment or 5-hour lipid infusion did not alter plasma glucose and insulin concentrations (Table 1). During the clamps, plasma insulin concentrations were raised to approximately 3,000 pM, whereas plasma glucose was clamped at approximately 5.9 mM in all groups (Table 1). The rate of glucose infusion needed to maintain euglycemia increased rapidly in the control and salicylate groups and reached a

Figure 2

Insulin-stimulated whole-body and skeletal muscle (soleus) glucose metabolic flux in awake control (black bars), salicylate (light gray bars), lipid (open bars), and lipid-salicylate (dark gray bars) rats. (**a**) Insulin-stimulated whole-body glycolysis in vivo. (**b**) Insulin-stimulated whole-body glycogen/lipid synthesis in vivo. (**c**) Insulin-stimulated skeletal muscle glycolysis in vivo. (**d**) Insulin-stimulated skeletal muscle glycogen synthesis in vivo. Values are means ± SE for six to seven experiments. *$P < 0.05$ versus control group.
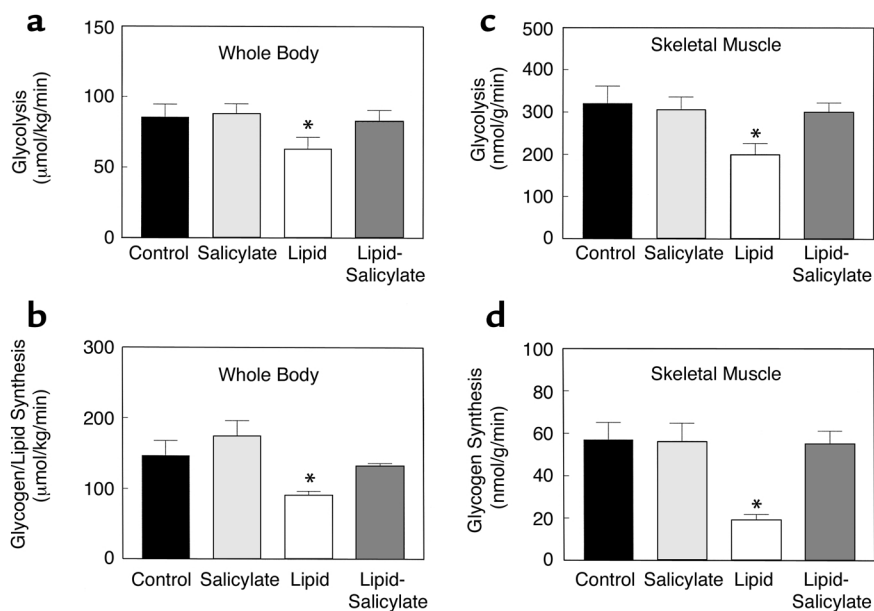
Table 1

Metabolic parameters during basal and hyperinsulinemic-euglycemic clamp periods and steady-state glucose infusion rate (GINF)[A]

		Basal period					Clamp period				
	n	Body Weight (g)	Plasma Glucose (mM)	Plasma Insulin (pM)	Plasma FFA (mM)	Plasma Salicylate (mM)	Plasma Glucose (mM)	Plasma Insulin (pM)	Plasma FFA (mM)	Plasma Salicylate (mM)	GINF (μmol/ kg/min)
Control	7	314 ± 11	5.7 ± 0.2	39 ± 8	0.49 ± 0.04	0.13 ± 0.05	6.0 ± 0.2	3,330 ± 182	0.05 ± 0.02	0.12 ± 0.05	231 ± 19
Salicylate	6	322 ± 11	5.4 ± 0.3	41 ± 6	0.44 ± 0.02	1.98 ± 0.40[B]	5.5 ± 0.2	2,515 ± 459	0.09 ± 0.02	2.11 ± 0.20[B]	262 ± 25
Lipid	7	301 ± 14	6.1 ± 0.3	63 ± 18	0.42 ± 0.06	0.11 ± 0.04	5.8 ± 0.2	3,172 ± 313	2.24 ± 0.40[B]	0.13 ± 0.05	148 ± 11[B]
Lipid-salicylate	7	310 ± 9	6.0 ± 0.3	51 ± 14	0.35 ± 0.03	1.74 ± 0.20[B]	6.2 ± 0.1	2,832 ± 319	2.97 ± 0.41[B]	2.24 ± 0.30[B]	213 ± 13

[A]Obtained from averaged rates of 40–70 minutes of clamps in the control, salicylate, lipid, and lipid-salicylate groups; [B]$P < 0.001$ vs. control group by the Duncan's multiple range test.

steady state within 30 minutes. In contrast, the glucose infusion rate required to maintain euglycemia in the lipid group was markedly reduced (Table 1), indicating that lipid infusion caused insulin resistance in these animals. Salicylate pretreatment prevented the lipid-induced decrease in steady-state glucose infusion rate.

Insulin-stimulated whole-body glucose uptake was decreased by 37% with the lipid infusion (Figure 1c). Salicylate pretreatment prevented decreases in insulin-stimulated whole-body glucose uptake caused by lipid infusion. Decreases in insulin-stimulated whole-body glucose uptake were due to a 41% decrease in insulin-stimulated glucose uptake in skeletal muscle (soleus) with lipid infusion (Figure 1d). Salicylate pretreatment also prevented the lipid-induced decrease in insulin-stimulated skeletal muscle glucose uptake. Furthermore, insulin-stimulated whole-body glycolysis and glycogen/lipid synthesis were significantly decreased by 27% and 43%, respectively, with the lipid infusion (Figure 2, a and b). Salicylate pretreatment prevented these decreases in insulin-stimulated whole-body glucose metabolic flux caused by lipid infusion. Similar to the decreases in insulin-stimulated whole-body glucose metabolic flux with lipid infusion, insulin-stimulated skeletal muscle glycolysis was decreased by 36% with lipid infusion (Figure 2c), but the most profound change was a 66% decrease in insulin-stimulated skeletal muscle glycogen synthesis with lipid infusion (Figure 2d). Salicylate pretreatment also prevented the lipid-induced decreases in insulin-stimulated skeletal muscle glucose metabolism.

Decreases in insulin-stimulated glucose uptake and metabolism in skeletal muscle were associated with a 59% decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 in skeletal muscle (gastrocnemius) with lipid infusion (Figure 3b). Also, insulin-stimulated IRS-1–associated PI 3-kinase activity in skeletal muscle was decreased by 38% with lipid infusion (Figure 3c). These findings suggest that skeletal muscle insulin resistance with lipid infusion may be secondary to the observed defects in skeletal muscle insulin signaling. Lipid-induced decreases in both insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase activity in skeletal muscle were prevented with salicylate pretreatment. In contrast, insulin-stimulated tyrosine phosphorylation of insulin receptor was not altered with lipid infusion or salicylate pretreatment (Figure 3a). These findings further suggest that lipid-induced defect and the effect of salicylate on skeletal muscle insulin signaling lie downstream to the insulin receptor and likely reside at the level of insulin receptor substrate.

In contrast to the defects in skeletal muscle insulin signaling and action with lipid infusion, basal EGP and insulin's ability to suppress basal EGP were not altered
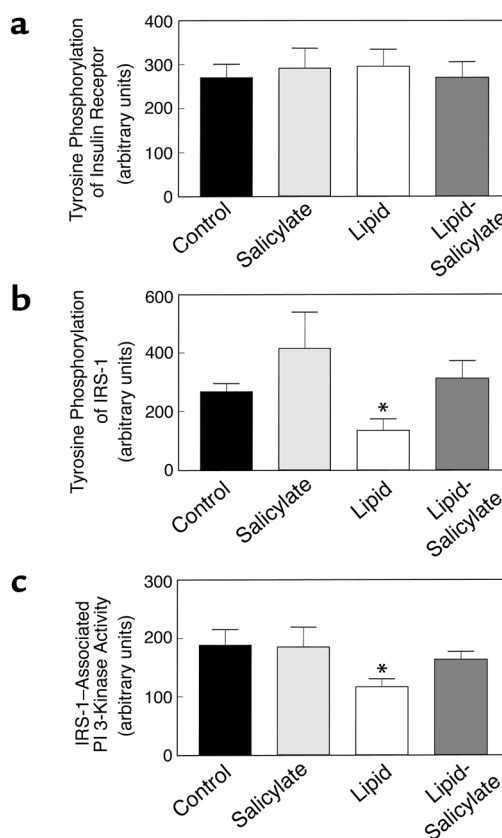
Figure 3

Insulin signaling in skeletal muscle (gastrocnemius) of the control (black bars), salicylate (light gray bars), lipid (open bars), and lipid-salicylate (dark gray bars) rats. (**a**) Insulin-stimulated tyrosine phosphorylation of insulin receptor. (**b**) Insulin-stimulated tyrosine phosphorylation of IRS-1. (**c**) Insulin-stimulated IRS-1–associated PI 3-kinase activity. Values are means ± SE for six to seven experiments. *$P < 0.05$ versus control group.
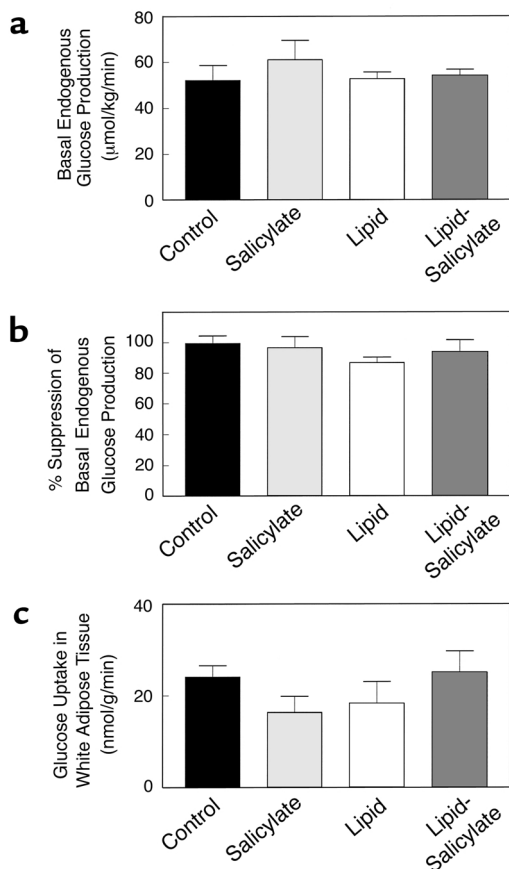
Figure 4
Insulin action in liver and epididymal white adipose tissue in the control (black bars), salicylate (light gray bars), lipid (open bars), and lipid-salicylate rats (dark gray bars). (**a**) Basal rates of endogenous glucose production. (**b**) Percent suppression of basal endogenous glucose production during insulin-stimulated state. (**c**) Insulin-stimulated glucose uptake in epididymal white adipose tissue. Values are means ± SE for six to seven experiments.

with lipid infusion or salicylate pretreatment (Figure 4, a and b). Moreover, insulin-stimulated glucose uptake in epididymal white adipose tissue was not altered with lipid infusion or salicylate pretreatment (Figure 4c). These findings indicate that increased plasma fatty acid concentrations caused a selective decrease in insulin responsiveness in skeletal muscle, which may be

attributed to decreases in skeletal muscle insulin signaling and action, and that these defects were prevented with salicylate pretreatment.

To further determine the mechanism of salicylate action, we examined whether mice with inactivation of IKK-β are protected from fat-induced insulin resistance in skeletal muscle. A 5-hour lipid infusion increased plasma fatty acid concentrations threefold during the basal period and more than 15-fold during the clamps compared with the control group (Table 2 and Figure 5a). During the clamps, plasma insulin concentrations were raised to approximately 730 pM, whereas plasma glucose was clamped at approximately 6.5 mM in all groups (Table 2). Lipid infusion markedly decreased the glucose infusion rate required to maintain euglycemia in the control mice, indicating that lipid infusion caused insulin resistance in these mice. In contrast, lipid infusion did not alter steady-state glucose infusion rate in the IKK-β KO mice compared with the control and IKK-β KO mice without lipid infusion (Table 2). Basal EGP and insulin's ability to suppress basal EGP were unaltered with lipid infusion in the control and IKK-β KO mice (Figure 5b).
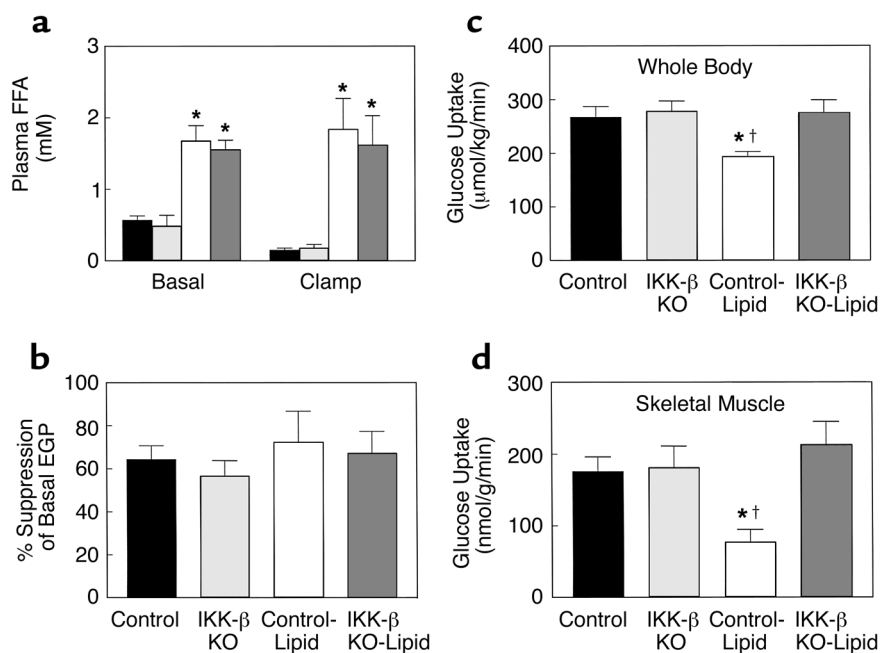
Insulin-stimulated whole-body glucose uptake was decreased by 28% with lipid infusion, and this change was due to a 56% decrease in insulin-stimulated skeletal muscle glucose uptake with lipid infusion in the control mice (Figure 5, c and d). In contrast, lipid infusion did not alter insulin-stimulated whole-body and skeletal muscle glucose uptake in the IKK-β KO mice compared with the control mice (Figure 5, c and d). Furthermore, insulin-stimulated whole-body glycolysis was unaltered, whereas whole-body glycogen/lipid synthesis was significantly decreased by 62% with lipid infusion in the control mice (Figure 6, a and b). Insulin-stimulated skeletal muscle glycolysis and glycogen synthesis were significantly decreased by 56% and 58%, respectively, with lipid infusion in the control mice (Figure 6, c and d). These defects in insulin-stimulated whole-body and skeletal muscle glucose metabolism with lipid infusion were prevented in the IKK-β KO mice (Figure 6).

Decreases in insulin-stimulated glucose uptake and metabolism in skeletal muscle of control mice were associated with a 43% decrease in insulin-stimulated

Table 2
Metabolic parameters during basal and hyperinsulinemic-euglycemic clamp periods in the control, IKK-β KO, lipid-infused control, and lipid-infused IKK-β KO mice at ~20 weeks of age

			Basal period			Clamp period			
	n	Body Weight (g)	Plasma Glucose (mM)	Plasma Insulin (pM)	Plasma FFA (mM)	Plasma Glucose (mM)	Plasma Insulin (pM)	Plasma FFA (mM)	GINF (μmol/kg/min)
Control	4	28 ± 2	6.8 ± 0.6	60 ± 14	0.57 ± 0.06	7.0 ± 0.2	678 ± 97	0.15 ± 0.03	231 ± 20
IKK-β KO	4	25 ± 2	7.9 ± 0.4	52 ± 12	0.49 ± 0.15	6.5 ± 1.0	804 ± 130	0.18 ± 0.05	230 ± 17
Control-Lipid	3	31 ± 2	7.9 ± 1.2	44 ± 23	1.68 ± 0.21[A]	6.1 ± 0.6	764 ± 120	1.84 ± 0.43[A]	143 ± 17[A]
IKK-β KO-Lipid	5	28 ± 3	7.6 ± 0.5	40 ± 5	1.55 ± 0.14[A]	6.5 ± 1.0	770 ± 80	1.62 ± 0.41[A]	240 ± 36

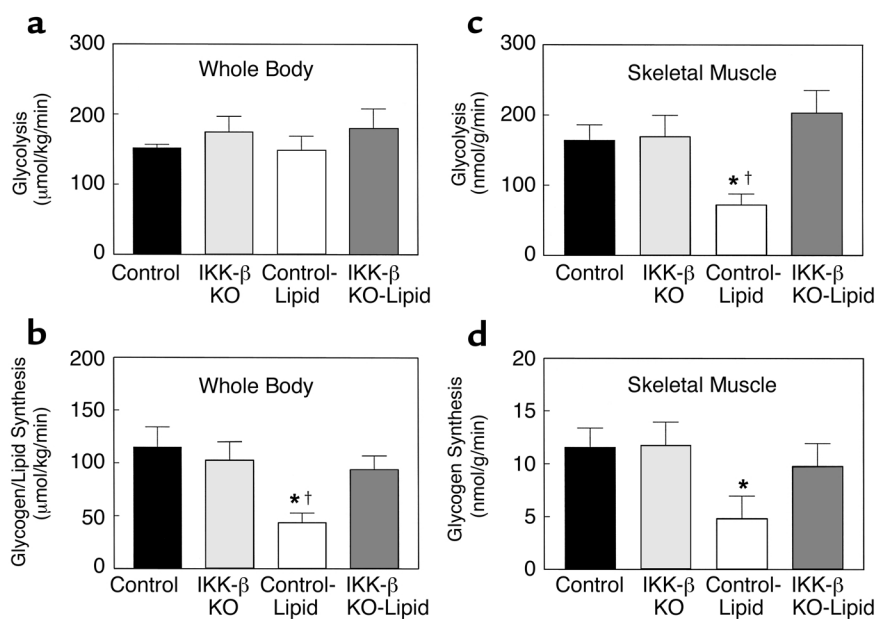[A]$P < 0.05$ versus control group by the Duncan's multiple range test.

Figure 5
Metabolic parameters and insulin-stimulated whole-body and skeletal muscle (gastrocnemius) glucose uptake in awake control (black bars), IKK-β KO (light gray bars), control-lipid (open bars), and IKK-β KO-lipid (dark gray bars) mice. (**a**) Plasma FFA concentrations during basal and hyperinsulinemic-euglycemic clamp. (**b**) Insulin-stimulated percent suppression of basal EGP. (**c**) Insulin-stimulated whole-body glucose uptake in vivo. (**d**) Insulin-stimulated skeletal muscle glucose uptake in vivo. Values are means ± SE for three to five experiments. *P < 0.05 versus control group; †P < 0.05 versus IKK-β KO-lipid group.

tyrosine phosphorylation of IRS-1 in skeletal muscle (gastrocnemius) with lipid infusion (Figure 7a). Also, insulin-stimulated IRS-1–associated PI 3-kinase activity in skeletal muscle was decreased by 37% with lipid infusion in the control mice (Figure 7b). Lipid-induced decreases in both insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase activity in skeletal muscle were prevented in the IKK-β KO mice (Figure 7, a and b). These findings suggest that the lipid-induced decreases in insulin-stimulated skeletal muscle glucose uptake in the control mice were secondary to the defects in muscle insulin signaling and that the IKK-β inactivation prevented these lipid-induced defects in muscle insulin signaling and action.

In addition to lipid-induced defects in skeletal muscle insulin signaling and action, insulin-stimulated glucose uptake in brown adipose tissue was decreased by 54% with lipid infusion in the control mice, and this lipid-induced defect was also prevented in the IKK-β KO mice (Figure 7c). Interestingly, insulin-stimulated glucose uptake in epididymal white adipose tissue showed a tendency to increase with lipid infusion in the IKK-β KO mice but did not reach statistical significance (Figure 7d).

Discussion
In this study, we found that a 5-hour lipid infusion decreased insulin-stimulated glucose uptake and

Figure 6
Insulin-stimulated whole-body and skeletal muscle (gastrocnemius) glucose metabolic flux in awake control (black bars), IKK-β KO (light gray bars), control-lipid (open bars), and IKK-β KO-lipid (dark gray bars) mice. (**a**) Insulin-stimulated whole-body glycolysis in vivo. (**b**) Insulin-stimulated whole-body glycogen/lipid synthesis in vivo. (**c**) Insulin-stimulated skeletal muscle glycolysis in vivo. (**d**) Insulin-stimulated skeletal muscle glycogen synthesis in vivo. Values are means ± SE for three to five experiments. *P < 0.05 versus control group; †P < 0.05 versus IKK-β KO-lipid group.

Figure 7

Insulin signaling in skeletal muscle (gastrocnemius) and insulin action in fat of the control (black bars), IKK-β KO (light gray bars), control-lipid (open bars), and IKK-β KO-lipid (dark gray bars) mice. (**a**) Insulin-stimulated tyrosine phosphorylation of IRS-1 in skeletal muscle. (**b**) Insulin-stimulated IRS-1–associated PI 3-kinase activity in skeletal muscle. (**c**) Insulin-stimulated glucose uptake in intrascapular brown adipose tissue. (**d**) Insulin-stimulated glucose uptake in epididymal white adipose tissue. Values are means ± SE for three to five experiments. *$P < 0.05$ versus control group; †$P < 0.05$ versus IKK-β KO-lipid group.
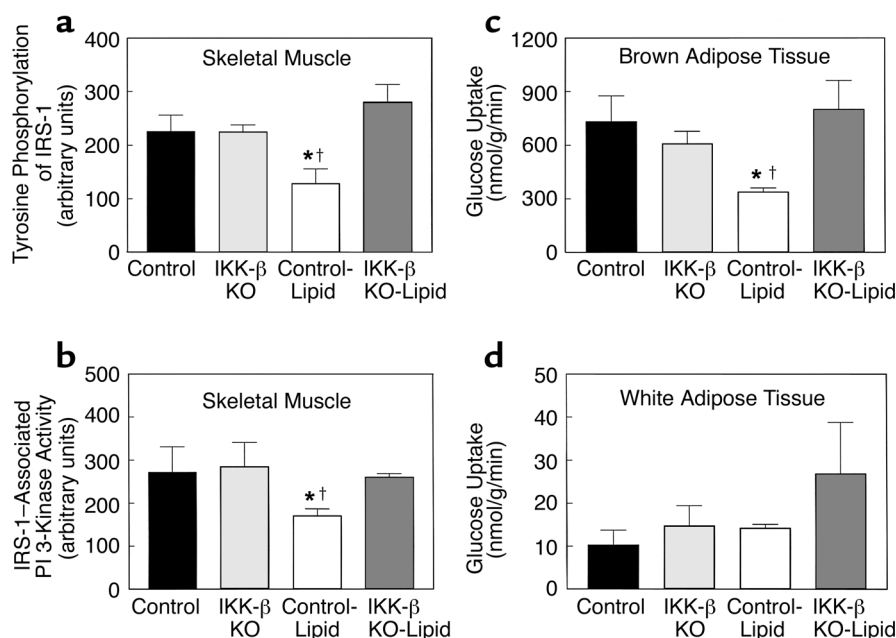
metabolism in rat skeletal muscle. Given that glucose transport is rate-controlling for glucose utilization in skeletal muscle (20, 21), decreases in glucose transport may account for the parallel decreases in glycolysis and glycogen synthesis with lipid infusion. These abnormalities were associated with defects in insulin activation of tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase in skeletal muscle. Recent studies have shown that IRS-1–associated PI 3-kinase is an important intracellular mediator of insulin signaling in skeletal muscle (22), and insulin stimulation of both glucose transport and glycogen synthase activity has been associated with activation of IRS-1–associated PI 3-kinase in skeletal muscle (23). These findings suggest that defects in skeletal muscle insulin action may be secondary to the observed defects in skeletal muscle insulin signaling with lipid infusion. Interestingly, the decrease in insulin-stimulated muscle glycogen synthesis was more profound than the decrease in muscle glucose uptake. This may be due to additional downstream defects in insulin activation of PI 3-kinase that in turn affect insulin-stimulated glycogen synthase activity in skeletal muscle with lipid infusion. In contrast to the defects in insulin activation of tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase in skeletal muscle, insulin-stimulated tyrosine phosphorylation of the insulin receptor in the muscle was unaltered with lipid infusion. This finding suggests that the defect in insulin signaling with lipid infusion was not due to an alteration in plasma membrane or membrane-associated insulin receptors (24). It further suggests that the mechanism of blunted insulin signaling with lipid infusion occurs downstream of the insulin receptor and at the level of the IRS-1. Furthermore, a 5-hour lipid infusion did not alter insulin's ability to suppress EGP or stimulate glucose uptake in white adipose tissue.

The mechanism by which lipid infusion may affect muscle insulin signaling may involve increases in intracellular fatty acid derived metabolites (i.e., fatty acyl CoA, diacylglycerol, ceramides) and subsequent activation of a serine/threonine kinase (e.g., PKC-θ, IKK-β). Chalkley et al. (25) have reported that a 5-hour lipid infusion increased muscle triglyceride and long-chain fatty acyl CoA contents, and this increase in fatty acyl CoA might lead to an increase in diacylglycerol, a known potent activator of PKC-θ (26). Moreover, recent studies by our group have shown that an acute elevation of plasma fatty acids for 5 hours resulted in activation of PKC-θ, which was associated with decreased tyrosine phosphorylation of IRS-1 (10). Mechanisms responsible for decreased insulin-stimulated tyrosine phosphorylation of IRS-1 may involve serine phosphorylation of IRS-1 (27–29), proteasome-mediated degradation of IRS-1 (30, 31), or phosphatase-mediated dephosphorylation of IRS-1 (32, 33). In this regard, serine phosphorylation of IRS-1 impairs the ability of IRS-1 to associate with the insulin receptor, which inhibits subsequent insulin-stimulated tyrosine phosphorylation of IRS-1 (10, 24, 34) and activation of IRS-1–associated PI 3-kinase (27, 28). Moreover, 5-hour lipid infusion did not alter IRS-1 protein content in rat skeletal muscle (222 ± 21 vs. 246 ± 26 arbitrary units in controls). Thus, accumulation of diacylglycerol or fatty acyl CoA's due to lipid infusion (i.e., increased delivery of fatty acid into skeletal muscle) may be responsible for defects in insulin's ability to activate IRS-1–associated PI 3-kinase in skeletal muscle and subsequent insulin action. Furthermore, it is also possible that increased concentrations of other intracellular fatty acid derived metabolites, such as ceramide, may play a role in the lipid-induced insulin resistance in skeletal muscle. Summers and

colleagues have shown that ceramide suppressed insulin-stimulated glucose transport in 3T3-L1 adipocytes by inhibiting phosphorylation and activation of Akt/protein kinase B, a serine/threonine protein kinase that is activated by insulin in a PI 3-kinase-dependent manner (35) and involved in the translocation of GLUT4 to the cell membrane (36).

The defects in skeletal muscle insulin signaling and action with lipid infusion were completely prevented with salicylate pretreatment, and the mechanism by which salicylate prevents insulin resistance may involve inhibition of IKK-β. Recently, Yin et al. (14) have shown that salicylate may prevent activation by NF-κB of genes involved in the pathogenesis of the inflammatory response by inhibiting the activity of IKK-β and phosphorylation of IκB (at serines S32 and S36 in IκBα and S19 and S23 in IκBβ). DiDonato et al. (37) and Zandi et al. (38) have recently shown that serine phosphorylation of IκB triggers ubiquitination and degradation of the IκB and eventually activation of NF-κB, a transcription factor involved in immune and inflammatory response (39). Furthermore, TNF-α, a well-known proinflammatory cytokine, activates NF-κB by stimulating IKK activity (38), and salicylate has been demonstrated to inhibit TNF-α–induced stimulation of IKK activity (14). Interestingly, TNF-α has also been shown to promote insulin resistance in adipocytes (40) and skeletal muscle (41) by decreasing insulin activation of IRS-1 tyrosine phosphorylation (42) and IRS-1–associated PI 3-kinase (43). From these findings, we hypothesized that lipid infusion may cause skeletal muscle insulin resistance by activating IKK-β, which subsequently leads to increased serine phosphorylation of IRS-1. Consistent with this hypothesis, we found that salicylate pretreatment prevented lipid-induced insulin resistance in skeletal muscle by possibly inhibiting the IKK-β activity and preventing the lipid-induced decreases in tyrosine phosphorylation of IRS-1 and IRS-1–associated PI 3-kinase activity.

To further examine the hypothesis that salicylate prevents lipid-induced insulin resistance in skeletal muscle by inhibiting IKK-β, we determined the effects of 5-hour lipid infusion on skeletal muscle insulin signaling and action in mice with IKK-β inactivation (16). A 5-hour lipid infusion significantly decreased insulin-stimulated glucose uptake and metabolic flux (i.e., glycogen synthesis, glycolysis) in skeletal muscle of the control mice. These lipid-induced defects in skeletal muscle insulin action were secondary to defects in muscle insulin signaling, as observed in rats. In contrast, lipid infusion failed to alter insulin-stimulated glucose uptake, tyrosine phosphorylation of IRS-1, and IRS-1–associated PI 3-kinase activity in skeletal muscle of IKK-β knockout mice. These findings implicate the protective role of IKK-β inactivation on fat-induced development of skeletal muscle insulin resistance and further support the effects of salicylate on insulin action via inhibition of IKK-β activity in skeletal muscle. However, we cannot rule out that salicylate may have additional roles on

ribosomal S6 kinase 2 activity (44), kinase Erk activity (45), NF-κB activity (37, 44, 45), or other potential targets of anti-inflammatory action (46) that in turn affect insulin action in skeletal muscle.

In summary, salicylate pretreatment prevents lipid-induced skeletal muscle insulin resistance by inhibiting lipid-induced decreases in insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1–associated PI 3-kinase activation. The effect of salicylate, a known inhibitor of IKK-β, on insulin action is further supported by our findings that mice with inactivation of IKK-β are protected from lipid-induced skeletal muscle insulin resistance. Overall, these results provide important new insights into the mechanism of fat-induced insulin resistance in skeletal muscle and suggest a potentially novel class of therapeutic agents for type 2 diabetes.

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