Salicylate prevents hepatic insulin resistance caused by short-term elevation of free fatty acids *in vivo*

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Abstract

Recent evidence indicates that inflammatory pathways are causally involved in insulin resistance. In particular, IκBζ kinase β (IKKβ), which can impair insulin signaling directly via serine phosphorylation of insulin receptor substrates (IRS) and/or indirectly via induction of transcription of pro-inflammatory mediators, has been implicated in free fatty acid (FFA)-induced insulin resistance in skeletal muscle. However, it is unclear whether liver IKKβ activation plays a causal role in hepatic insulin resistance caused by acutely elevated FFA. In the present study, we wished to test the hypothesis that sodium salicylate, which inhibits IKKβ, prevents hepatic insulin resistance caused by short-term elevation of FFA. To do this, overnight-fasted Wistar rats were subject to 7-h i.v. infusion of either saline or Intralipid plus 20 U/ml heparin (IH; triglyceride emulsion that elevates FFA levels *in vivo*) with or without salicylate. Hyperinsulinemic-euglycemic clamp with tracer infusion was performed to assess insulin-induced stimulation of peripheral glucose utilization and suppression of endogenous glucose production (EGP). Infusion of IH markedly decreased (*P*<0.05) insulin-induced stimulation of peripheral glucose utilization and suppression of EGP, which were completely prevented by salicylate co-infusion. Furthermore, salicylate reversed IH-induced 1) decrease in IκBζ content; 2) increase in serine phosphorylation of IRS-1 (Ser 307) and IRS-2 (Ser 233); 3) decrease in tyrosine phosphorylation of IRS-1 and IRS-2; and 4) decrease in serine 473-phosphorylated Akt in the liver. These results demonstrate that inhibition of IKKβ prevents FFA-induced impairment of hepatic insulin signaling, thus implicating IKKβ as a causal mediator of hepatic insulin resistance caused by acutely elevated plasma FFA. *Journal of Endocrinology* (2007) 195, 323–331

Introduction

Numerous studies have established a close relationship between obesity, insulin resistance, and type 2 diabetes. This link is attributed to greater release of various adipocyte products, such as cytokines, resistin, and free fatty acids (FFAs), from the expanded adipose tissue in obese individuals. In particular, elevated circulating levels of FFA cause insulin resistance in both animals and humans (Roden *et al*. 1996, Boden *et al*. 2001, Yuan *et al*. 2001, Lam *et al*. 2002, Kim *et al*. 2004b). However, precise mechanisms by which FFAs impair insulin action in the liver and peripheral tissues are incompletely understood.

Recent studies have demonstrated that FFA cause insulin resistance in the skeletal muscle mainly via inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1 (Kim *et al*. 2001, 2004b, Yu *et al*. 2002), which is a critical step in insulin signal transduction. This process is likely mediated by the phosphorylation of serine residues on IRS-1 by certain serine kinases, such as protein kinase C (PKC), inhibitor of IκBζ kinase β (IKKβ), and c-Jun NH2-terminal kinase1 (JNK1). Shulman (Kim *et al*. 2001, 2004b, Yu *et al*. 2002) and others (Itani *et al*. 2000, 2002, Yuan *et al*. 2001, Boden *et al*. 2005) have implicated accumulation of intramyocellular lipid metabolites (e.g., diacylglycerol, long-chain fatty acyl CoA) and activation of serine kinases PKC (isoforms β and δ in humans, and ε and θ in rodents) and IKKβ as potentially causal events in the pathway of FFA-induced insulin resistance in skeletal muscle. In particular, it has been shown that mice lacking PKC-δ are protected from insulin resistance caused by a short-term lipid infusion (Kim *et al*. 2004b). Furthermore, treatment with high-dose sodium salicylate, an IKKβ inhibitor, or IKKβ-deficiency prevents fat-induced insulin resistance in rodent skeletal muscle (Kim *et al*. 2001).

Recently, treatment of primary mouse hepatocytes with saturated fatty acids was found to activate JNK in association with increased IRS-1 serine 307 phosphorylation and decreased insulin-stimulated Akt activation, which were absent in hepatocytes isolated from JNK1−/− mice (Solinas *et al*. 2006). However, potentially causal roles of serine kinases PKC, IKKβ, and JNK1 in FFA-induced hepatic insulin resistance have not been extensively investigated *in vivo* models. Our laboratory has previously established an association between PKC-δ membrane translocation, a
marker of its activation, and FFA-induced hepatic insulin resistance (Lam et al. 2002). This association has recently been confirmed by Boden et al. (2005), who, in the same study, also linked hepatic IKKβ activation with FFA-induced hepatic insulin resistance. However, it has not been clearly shown whether IKKβ causes hepatic insulin resistance induced by short-term elevation of circulating FFA. While Arkan et al. (2005) have demonstrated that hepatocyte-specific IKKβ knockout mice are protected from hepatic insulin resistance caused by high-fat feeding or genetically induced obesity, both of these models exhibit chronically elevated FFA levels, which may cause insulin resistance via a different mechanism than short-term FFA elevation. Thus, in the present study, we wished to test the hypothesis that IKKβ activation is causally involved in the mechanism of hepatic insulin resistance induced by acute FFA elevation. To do this, we used a rat model of short-term (7 h) i.v. lipid infusion with or without co-infusion of sodium salicylate to examine whether inhibition of IKKβ results in restoration of hepatic insulin action via prevention of FFA-induced impairment of hepatic insulin signaling.

Materials and Methods

Animal models

Female Wistar rats (Charles River, Quebec, Canada) weighing 250–300 g were used for experiments. The rats were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12 h light:12 h darkness cycle and were fed rat chow (Teklad Global # 2018; Harland Teklad Global Diets, Madison, WI, USA) and water ad libitum. The Animal Care Committee of the University of Toronto approved all procedures.

Surgery

After 3–5 days of adaptation to the facility, rats were anesthetized with isoflurane, and indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for blood sampling, as previously described (Lam et al. 2002). Briefly, polyethylene (PE) catheters (PE-50; Cay Adams, Boston, MA, USA), each extended with a segment of silastic tubing (length 3 cm and 0.58 mm in internal diameter; Dow Corning, Midland, MI, USA), were used. The venous catheter was inserted to the level of the right atrium and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1000 U/ml) to maintain patency and were closed with a metal pin. The rats were allowed for a minimum of 3 days to recover from the surgery before experiments.

Experimental design

After overnight fasting (10–12 h), the rats (n=6–8/group) were subject to a 7-h i.v. infusion of either saline (SAL), Intralipid plus heparin (IH; 20% Intralipid plus 20 U/ml heparin at 5.5 µl/min), IH plus sodium salicylate (SS; 7 mg/kg bolus plus 0.117 mg/kg per min), or sodium salicylate alone. The dose of sodium salicylate (Sigma–Aldrich) used in the study was derived from a previous in vivo study by Kim et al. (2001) in which SS treatment was shown to prevent FFA-induced insulin resistance in skeletal muscle. After 3 h of infusion, [3-3H] glucose (Perkin–Elmer, Boston, MA, USA) was initiated (8 µCi bolus followed by constant infusion at 0.15 µCi/min) to assess peripheral glucose utilization and endogenous glucose production (EGP). Hyperinsulinemic–euglycemic clamp was performed with tracer infusion during the last 2 h of the 7-h infusion period to assess hepatic and peripheral insulin sensitivity. During 30 min preceding the clamp (‘basal period’), measurements were taken at 10-min interval for plasma glucose, insulin, FFA, and [3-3H] glucose-specific activity. At the onset of the clamp, an infusion of porcine insulin at 5 mU/kg per min, resulting in plasma insulin levels in the postprandial range, was initiated. To maintain euglycemia during insulin infusion, a variable infusion of 20% glucose was given through the jugular catheter and adjusted according to glycemic determinations every 5 min. The glucose infusion was radiolabeled with 48 µCi/g [3-3H] glucose to maintain plasma glucose-specific activity constant. The total blood volume withdrawn was ~3-8 ml. After plasma separation, red blood cells were diluted at 1:1 ratio in heparinized saline (4 U/ml) and re-infused into the rats. At the end of the experiments, the rats were anesthetized with i.v. administration of ketamine:xylazine:acepromazine cocktail (87:1.7:0.4 mg/ml), immediately after which liver, soleus muscle, and white adipose tissue samples were freeze clamped with pre-cooled aluminum tongs, while infusions were maintained through the jugular vein.

Plasma assays

Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman, Fullerton, CA, USA). Plasma radioactivity from [3-3H] glucose was determined after deproteinization with Ba(OH)2 and ZnSO4, and subsequent evaporation to dryness. Aliquots of [3-3H] glucose and of the tritiated glucose infusate were assayed together with the plasma samples (Lam et al. 2002). Insulin levels in plasma were determined by RIAs using kits specific for rat insulin (but with 100% cross-reactivity with porcine insulin used for infusion), as previously described (Lam et al. 2002). Plasma FFA levels were measured using a colorimetric kit from Wako Industrials (Richmond, VA, USA), as previously described (Lam et al. 2002).

Immunoprecipitation and Western blot analysis

Liver samples (150 mg) were homogenized by hand-held glass homogenizer in buffer A (50 mM Tris–HCl (pH 7.5): 10 mM


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were mixed with equal volumes of 3 modified Lowry microassay, using a standardized assay kit. Performed as described previously. The protein concentration (6.86 M urea, 4.29% SDS, 300 mM dithiothreitol, and purity of the cytosolic and membrane fractions was previously supernatant provided the solubilized membrane fraction. The described (Zinker liver, proteins were extracted from rat liver tissue as previously dilutions of 1/500, 1/1000, 1/500, and 1/500 respectively. Temecula, CA, USA), and Serine 233 IRS-2 (Labvision, Antibodies against IRS-1, Serine 307 IRS-1, IRS-2 (Upstate, phosphorylated IRS-1 in the soleus muscle and fat. and IRS-2 in the cytosolic fraction of liver and serine- was used to determine serine-phosphorylated and total IRS-1 was measured in the cytosolic fraction). The same procedure was measured in the IH and IH plus SS groups compared with the SAL post hoc test in order to compare results between treatment groups for the following parameters: whole body insulin sensitivity, I\textsubscript{B} and IRS-2, total IRS-1 and IRS-2, and serine 473-phosphorylated Akt. For plasma glucose, FFAs, and insulin as well as EGP and peripheral glucose utilization, we used two-way ANOVA with Tukey's test in order to compare results between treatment groups.

EGTA; 2 mM EDTA; 1 mM NaHCO\textsubscript{3}; 5 mM MgCl\textsubscript{2}; 1 mM Na\textsubscript{2}VO\textsubscript{4}; 1 mM NaF; 1 \mu g/ml aprotinin, leupeptin, and pepstatin; 0.1 mM phenylmethylsulfonyl fluoride; and 1 \mu M microcystin). The homogenates were centrifuged at 100 000 \textit{g} for 1 h at 4°C, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in buffer B (buffer A + 1% Triton X-100), homogenized by passing through a 23-gauge needle thrice, incubated for 15 min on ice, and centrifuged at 100 000 \textit{g} for 1 h at 4°C. The supernatant provided the solubilized membrane fraction. The purity of the cytosolic and membrane fractions was previously assessed by assaying glucose-6-phosphate dehydrogenase (Sigma) and 5'-nucleotidase activities (Sigma) respectively (Lam et al. 2002). The results showed that the index of purity of both fractions were >90%. Homogenization of muscle (Wong et al. 2006) and fat (Beard et al. 2006) samples was performed as described previously. The protein concentration in all samples was determined by the detergent-compatible modified Lowry microassay, using a standardized assay kit.

Fifty micrograms of protein in liver, muscle, or fat samples were mixed with equal volumes of 3X sample-loading buffer (6-86 M urea, 4-29% SDS, 300 mM dithiothreitol, and 43 mM Tris–HCl (pH 6.8)) and left at room temperature for 30 min. The mixture was then vortexed and subjected to SDS-PAGE (10% polyacrylamide). Following electrophoretic separation, protein was transferred to polyvinylidene fluoride membranes. The membranes were then incubated for 1 h at room temperature in Tris-buffered saline–Tween (TBST) containing 5% nonfat dried milk (pH 7.4). After the blocking step, membranes were incubated overnight in TBST plus 5% milk containing affinity-purified polyclonal antibody specific for IkB\textalpha (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 1:2000 (note that hepatic IkB\textalpha content was measured in the cytosolic fraction). The same procedure was used to determine serine-phosphorylated and total IRS-1 and IRS-2 in the cytosolic fraction of liver and serine-phosphorylated IRS-1 in the soleus muscle and fat. Antibodies against IRS-1, Serine 307 IRS-1, IRS-2 (Upstate, Temecula, CA, USA), and Serine 233 IRS-2 (Labvision, Fremont, CA, USA) were added to the western membranes at dilutions of 1/500, 1/1000, 1/500, and 1/500 respectively.

For detection of tyrosine phosphorylation of IRS in the liver, proteins were extracted from rat liver tissue as previously described (Zinker et al. 2002). Liver lysates containing equal amounts of protein (1 mg) were subject to immunoprecipitation overnight at 4°C with agarose-conjugated anti-IRS-1 antibody (Upstate) or with anti-IRS-2 antibody (Upstate) followed by 2-h incubation with Protein A/G PLUS–Agarose immunoprecipitation reagent (Santa Cruz) at room temperature. Immune complexes were collected by brief centrifugation (12 000 \textit{g}) and washed four times with ice-cold PBS. The equivalent amount of protein samples was then resuspended in 1X Laemmlli sample buffer (2% SDS, 10% glycerol, 62:5 mM Tris (pH6-8), 0:1% bromophenol blue, and 5% \beta-mercaptoethanol), boiled for 5 min, and separated by SDS-PAGE (10% polyacrylamide) under reducing conditions, as described above. This was followed by immunoblotting with anti-phosphotyrosine antibody (Santa Cruz; 1/1000 dilution). Note that serine 473-phosphorylated Akt (antibody purchased from Cell Signaling (Beverly, MA, USA); 1/1000 dilution) was measured by western blotting using the same liver lysates that were used for immunoprecipitation of IRS-1 and IRS-2.

After washing thrice with TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Santa Cruz). The membranes were then washed several times with double-distilled water and developed using ECL reagent (Amersham Biosciences). The bands obtained from immunoblotting were quantified by scanning laser densitometry.

Calculations

Glucose turnover (rate of appearance of glucose determined with [3-3H] glucose) was calculated using Steele's non-steady-state equation (Altszuler et al. 1956), taking into account the extra tracer infused with the glucose infusate (Finegood et al. 1987). In the basal state, the total rate of glucose appearance corresponds to the EGP. During the clamp, EGP was calculated by subtracting the exogenous glucose infusion rate from the total rate of glucose appearance. At steady state, glucose disappearance corresponds to the rate of glucose appearance and at euglycemia glucose disappearance corresponds to tissue glucose utilization as renal glucose clearance is zero. Data are presented as average values of the samples that were taken during the 30 min preceding the clamp and during the last 30 min of the clamp.

Statistical analysis

One-way ANOVA followed by Tukey's post hoc test was used to compare differences between treatment groups for the following parameters: whole body insulin sensitivity, IkB\textalpha content, serine-phosphorylated IRS-1 and IRS-2, tyrosine-phosphorylated IRS-1 and IRS-2, total IRS-1 and IRS-2, and serine 473-phosphorylated Akt. For plasma glucose, FFAs, and insulin as well as EGP and peripheral glucose utilization, we used two-way ANOVA with Tukey's post hoc test in order to compare results between treatment groups and, if necessary, to compare basal and clamp data within each group. Statistical calculations were performed using Statistica software (Statistical Analysis System, Cary, NC, USA). Significance was accepted at a \(P<0.05\).

Results

Table 1 shows plasma glucose, FFA, and insulin levels during the basal period and hyperinsulinemic–euglycemic clamp. Plasma glucose levels were not different between treatments during the basal period or the clamp. Plasma FFA levels that were measured during the basal period increased by \(\sim 100\%\) in the IH and IH plus SS groups compared with the SAL
As expected, plasma FFA levels were lower during the hyperinsulinemic clamp than during the basal period in all treatment groups due to the anti-lipolytic and fat esterification effects of insulin. Plasma insulin levels were not different between groups during the basal period and were markedly elevated during the clamp compared with the basal period in all treatment groups due to insulin infusion.

Glucose infusion rate (GIR) during the last 30 min of the 2-h hyperinsulinemic–euglycemic clamp is an indication of whole body insulin sensitivity. IH infusion significantly decreased GIR (P < 0.01) compared with saline infusion (Fig. 1). Salicylate co-infusion with IH prevented the IH-induced decrease in GIR (P < 0.01 versus IH). Salicylate alone did not have any effect.

During the basal period, EGP is equal to glucose utilization and there was no significant difference between treatment groups (Figs 2A and 3A). During the clamp, we were able to maintain fluctuations in plasma glucose-specific activity from basal at low levels (SAL, 8%; IH, 7%; IH + SS, 2%; SS, 4%; average percentage difference between basal and clamp), which prevents significant errors in calculation of glucose production (Fisher et al. 1996). IH infusion decreased insulin-stimulated increase in peripheral glucose utilization during the clamp when compared with SAL infusion (P < 0.05; Fig. 2B). This IH-induced decrease was completely abolished by salicylate co-infusion (P < 0.05 versus IH). The insulin-stimulated increase in peripheral glucose utilization in the SS group did not differ from the SAL group.

Hepatic insulin sensitivity is measured as the capacity of insulin to suppress the EGP from the basal state. In the SAL group, EGP decreased by 37% from basal during the last 30 min of hyperinsulinemic clamp. However, in IH group, suppression of EGP during clamp was only 7%, which is significantly less than suppression of EGP observed in the SAL group (P < 0.05; Fig. 3B). Co-infusion of salicylate prevented IH-induced decrease in suppression of EGP during the clamp (P < 0.05 versus IH). Salicylate alone did not have any effect.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose (mM/l)</th>
<th>FFA (mEq/l)</th>
<th>Insulin (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>6.77 ± 0.36</td>
<td>0.64 ± 0.052</td>
<td>152 ± 25</td>
</tr>
<tr>
<td>IH</td>
<td>7.07 ± 0.29</td>
<td>1.199 ± 0.080</td>
<td>153 ± 33</td>
</tr>
<tr>
<td>IH + SS</td>
<td>6.79 ± 0.34</td>
<td>0.795 ± 0.044</td>
<td>179 ± 36</td>
</tr>
<tr>
<td>SS</td>
<td>7.25 ± 0.80</td>
<td>0.509 ± 0.030</td>
<td>214 ± 34</td>
</tr>
<tr>
<td>IH + SS</td>
<td>6.97 ± 0.39</td>
<td>0.754 ± 0.091</td>
<td>7.0 ± 106</td>
</tr>
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<td>0.509 ± 0.030</td>
<td>214 ± 34</td>
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SAL, saline; IH, Intralipid plus heparin; IH + SS, Intralipid plus heparin co-infused with salicylate (0.117 mg/kg per min); SS, salicylate alone. *P < 0.01 versus SAL and SS groups. †P < 0.01 versus basal period.
In order to assess the effect of salicylate on IKKβ activity in the liver, we measured hepatic IκBα content (Fig. 4A), which is a marker of IKKβ activation as IκBα upon phosphorylation by IKKβ is targeted for degradation. IH infusion decreased hepatic IκBα levels (P < 0.05), which were restored to control levels by salicylate co-infusion. Salicylate alone did not have any effect on hepatic IκBα levels. IH infusion also decreased IκBα content in the soleus muscle (P < 0.05) and salicylate co-infusion reversed the IH-induced decrease in IκBα content (P < 0.05 versus IH; Fig. 4B). Again, salicylate alone did not have any effect on soleus muscle IκBα levels. In adipose tissue, however, IH infusion did not change IκBα content (Fig. 4C).

IKKβ is known to phosphorylate IRS on serine residues (Gao et al. 2002), thereby decreasing the insulin-induced tyrosine phosphorylation. IH infusion markedly increased serine 307 phosphorylation of IRS-1 (Fig. 5A; P < 0.05) and serine 233 phosphorylation of IRS-2 (Fig. 5B; P < 0.05) in the liver. Co-infusion of salicylate, however, completely reversed these effects of IH (P < 0.05 versus IH). IH infusion, however, did not change total IRS-1 and IRS-2 levels (data not shown) and salicylate alone did not have any effect. Furthermore, IH infusion dramatically increased serine 307 phosphorylation of IRS-1 in the soleus muscle (P < 0.01), which was completely reversed by salicylate co-infusion (P < 0.01 versus IH; Fig. 5C). IH did not alter serine 307 phosphorylation of IRS-1 in the adipose tissue (Fig. 5D).

Tyrosine phosphorylation of IRS by the insulin receptor is a critical step in insulin signaling. Shulman’s laboratory has previously shown that short-term lipid infusion impairs tyrosine phosphorylation of IRS-1 in rat skeletal muscle (Kim et al. 2001, 2004b, Yu et al. 2002). In liver, we found that IH infusion decreased tyrosine phosphorylation of IRS-1 (Fig. 6A) and IRS-2 (Fig. 6B; both P < 0.05) and that salicylate co-infusion prevented the IH-induced decreases (both P < 0.05 versus IH).

When activated, Akt is phosphorylated at serine 473. IH infusion decreased serine 473-phosphorylated Akt (P < 0.05) in the liver. This inhibitory effect of IH on Akt was prevented by salicylate co-infusion (Fig. 7; P < 0.05 versus IH).

Discussion

Recent studies have implicated inflammatory pathways, particularly IKKβ/nuclear factor κB (NFκB) system, in various animal models of insulin resistance (Kim et al. 2001, Yuan et al. 2001, Itani et al. 2002, Arkan et al. 2005, Boden et al. 2005, Cai et al. 2005). However, it is unclear whether...
IKKβ activation is causally involved in the impairment of hepatic insulin action caused by short-term elevation of FFA. Therefore, the present study in rats was performed to investigate whether inhibition of IKKβ activity provides protection from FFA-induced hepatic insulin resistance. We demonstrate that treatment with high-dose sodium salicylate completely prevents hepatic insulin resistance caused by short-term lipid infusion in association with prevention of impairment of hepatic insulin signaling.

As expected, i.v. infusion of IH markedly elevated plasma FFA levels. IH is a triglyceride emulsion containing heparin that is broken down to non-esterified fatty acids and glycerol in vivo by lipoprotein lipase. It is thus possible that glycerol derived from triglycerides by itself affects EGP measured in the present study; however, we have shown in a previous study (Lam et al. 2002) that glycerol infusion (5 mg/kg per min) resulting in plasma glycerol levels similar to that achieved by 7 h of IH infusion has no effect on EGP when compared with saline infusion either in the basal fasting state or during the hyperinsulinemic–euglycemic clamp.

Infusion rate of exogenous glucose during the clamp is an indication of whole body insulin sensitivity. Numerous studies (Boden 1996, Boden et al. 2001, 2005, Kim et al. 2001, 2004b, Lam et al. 2002, 2003, Yu et al. 2002) have shown that lipid infusion causes whole body insulin resistance and our results are consistent with these studies. The whole body insulin resistance caused by IH infusion was completely reversed with salicylate co-infusion, suggesting that the site of salicylate’s effect includes both liver and peripheral tissues. Using infusion of [3-3H] tracer, which enabled us to separately assess hepatic and peripheral insulin sensitivity, we show that IH infusion causes both hepatic and peripheral insulin resistance, as indicated by decreases in insulin-induced suppression of EGP from basal and in insulin-stimulated peripheral glucose utilization respectively. These results are in agreement with our previous findings (Lam et al. 2002). With salicylate co-infusion, IH-induced insulin resistance in both liver and periphery was completely prevented.

Consistent with the effect of salicylate to prevent IH-induced decrease in peripheral glucose utilization during the clamp, salicylate co-infusion prevented IH-induced decrease in IkBz content in parallel with reversal of IH-induced increase in serine 307 phosphorylation of IRS-1 in the skeletal muscle. In the adipose tissue, IH infusion did not alter IkBz content or serine 307 phosphorylation of IRS-1. Although FFA have been shown to decrease glucose uptake in association with the activation of IKKβ and increased serine 307 phosphorylation of IRS-1 in 3T3-L1 adipocytes (Gao et al. 2004), the differences in the experimental models used likely explains the discrepancies in the results. It is noteworthy that, in Kim’s study (Kim et al. 2001), short-term lipid infusion did not alter insulin-stimulated glucose uptake in white adipose tissue, which is in keeping with our findings. These results indicate that liver and skeletal muscle were selectively targeted by salicylate and that prevention of FFA-induced peripheral insulin resistance by salicylate is associated with the reversal of serine phosphorylation of IRS-1 in skeletal muscle.

As far as we know, this study is the first to show that salicylate is effective in restoring hepatic insulin sensitivity in a model of acute FFA elevation. While recent studies (Yuan et al. 2001, Arkan et al. 2005) have demonstrated that IKKβ deficiency is protective against insulin resistance caused by high-fat feeding or genetically induced obesity in rodents, there is an important distinction in the experimental model of insulin resistance used between these studies and our present study. High-fat feeding and genetically induced obesity usually result in chronically elevated FFA levels, which may induce insulin resistance via a
different mechanism than acute FFA elevation. For instance, in high-fat-fed animals, inhibition of NFκB appears to be sufficient to restore insulin sensitivity without inhibition of IKKβ activity (Cai et al. 2005), suggesting that the insulin resistance is secondary to transcriptional effects of NFκB, which may not be true after short-term lipid infusion. Furthermore, these models are associated with increased release of adipocyte-derived factors other than FFA, such as pro-inflammatory cytokines, tumor necrosis factor α (TNFα), interleukin-1β, and interleukin-6 (Hotamisligil & Spiegelman 1994, Rotter et al. 2003, Kim et al. 2004a, He et al. 2006, Jager et al. 2007) which are known to induce insulin resistance. Accordingly, in these studies, insulin resistance on which IKKβ inhibition was shown to have a protective effect cannot be attributed only to FFA. Instead, the model of insulin resistance we used is similar to that used by Kim et al. (2001) in their investigation of salicylate’s effect on fat-induced insulin resistance in rat skeletal muscle. In this study, however, a high rate of insulin infusion (60 pmol/kg per min) used during the clamp completely suppressed EGP in all groups, potentially masking the effect of salicylate to prevent FFA-induced hepatic insulin resistance. Using a much lower rate of insulin infusion (30 pmol/kg per min) and maintaining plasma glucose-specific activity constant to avoid underestimation of glucose production during the clamp (Finegood et al. 1988), we were able to reveal the ability of salicylate to prevent hepatic insulin resistance caused by FFA.

Numerous recent studies (Kim et al. 2001, Arkan et al. 2005, Boden et al. 2005, Cai et al. 2005) have implicated activation of intracellular inflammatory pathway in fat-induced insulin resistance, although it is not clear whether this defect is due to the direct inhibitory effect of IKKβ on insulin signaling or its indirect effect to promote NFκB-mediated production of pro-inflammatory cytokines. Since short-term fat infusion can activate IKKβ (Kim et al. 2001, Boden et al. 2005) and IKKβ can directly phosphorylate serine residues of IRS-1 (Gao et al. 2002), which prevents tyrosine phosphorylation of IRS and thus insulin signaling (Peraldi & Spiegelman 1998), it is likely that salicylate prevents fat-induced hepatic insulin resistance directly through prevention of IKKβ activity. In support of this notion, we found that salicylate completely prevents IH-induced decrease in hepatic IκBα content, which indicates increased IKKβ activity as IκBα upon phosphorylation by IKKβ is targeted for degradation. Interestingly, salicylate alone had no effect on hepatic IκBα content in the absence of elevated FFA, presumably because the dose of salicylate we used was not sufficiently high to suppress IKKβ activity beyond its baseline levels.

The preventive effect of salicylate on IH-induced IKKβ activation occurred in association with prevention of IH-induced increase in serine 307 phosphorylation and decrease in tyrosine phosphorylation of IRS-1 in muscle and fat, and this prevention was not associated with changes in serine 233 phosphorylation of IRS-2. These results suggest that salicylate prevents IKKβ activation downstream of IRS-1 and that this effect leads to prevention of hepatic insulin resistance.

Figure 5 Serine phosphorylation of the insulin receptor substrate (IRS) in the liver, skeletal muscle, and fat following 2-h hyperinsulinemic-euglycemic clamp (n=6–8/group). (A) Effect of IH and salicylate on serine 307 phosphorylation of IRS-1 in the liver. (B) Effect of IH and salicylate on serine 233 phosphorylation of IRS-2 in the liver. (C) Effect of IH and salicylate on serine 307 phosphorylation of IRS-1 in the soleus muscle. (D) Effect of IH and salicylate on serine 307 phosphorylation of IRS-1 in fat. A representative image of immunoblots is shown at the top of each graph. Data are means ± S.E.M. SAL, saline; IH, Intralipid plus heparin; IH+SS, Intralipid plus heparin co-infused with salicylate (0.117 mg/kg per min); SS, salicylate alone. **P<0.01 versus other groups. **P<0.01 versus other groups.
phosphorylation of IRS-1 in the liver (Figs 5A and 6A, respectively). While there are numerous serine residues on IRS that are associated with impaired insulin signaling, serine 307 appears to be a critical site (Birnbaum 2001). For instance, TNFα, by triggering activation of JNK1, has been shown to increase serine phosphorylation of IRS-1 at 307 site, which decreases tyrosine phosphorylation of IRS-1 and thus impedes insulin signaling (Aguirre et al. 2000). It was recently shown that FFA cause insulin resistance through IKKβ- and JNK1-mediated serine (307) phosphorylation of IRS-1 in 3T3-L1 adipocytes (Gao et al. 2004). Furthermore, serine 307 phosphorylation of IRS-1 caused by short-term fat infusion was associated with decreased tyrosine phosphorylation of IRS-1 and impairment of insulin signaling in rat skeletal muscle, although the serine kinase responsible was not identified (Yu et al. 2002). Our results show that salicylate also prevents IH-induced increase in serine 233 phosphorylation of IRS-2 (Fig. 5B), which has been linked to insulin resistance (Scioscia et al. 2006). Although it is still unknown whether serine 233 phosphorylation interferes with tyrosine phosphorylation of IRS-2, our finding of concomitant decrease in tyrosine phosphorylation of IRS-2 indicates that these processes may be linked.

In addition to defects in tyrosine phosphorylation of IRS, IH infusion was found to reduce serine 473 phosphorylation of Akt, which was prevented with salicylate co-infusion. Taken together, our results suggest that the effect of salicylate to restore hepatic insulin sensitivity is closely associated with its reversal of fat-induced impairment of hepatic insulin signaling.

Although our findings confirm the ability of high-dose salicylate to inhibit IKKβ, we cannot exclude the possibility that salicylate prevents IKKβ-induced transcription of various pro-inflammatory cytokines, thereby inhibiting their autocrine effect to impair hepatic insulin signaling. However, relatively short exposure to elevated FFA may preclude IKKβ-induced transcription of pro-inflammatory genes. Furthermore, while salicylate is a relatively weak inhibitor of cyclooxygenase (COX) due to a lack of acetyl group that is required for deactivation of the enzyme, it is nonetheless possible that salicylate had some effect in inhibiting COX-mediated prostaglandin production. However, given that neither a treatment with non-steroidal anti-inflammatory drugs, which are robust inhibitors of COX, nor COX deficiency prevented insulin resistance in Fao hepatoma cells and in obese mice (Yuan et al. 2001) respectively, it is unlikely that potentially inhibitory effect of salicylate on prostaglandin played a role in the present study.

In summary, the present study demonstrates that 1) a high-dose treatment with sodium salicylate prevents hepatic insulin resistance caused by short-term elevation of plasma FFA and 2) the effect of salicylate to restore hepatic insulin sensitivity occurs in association with prevention of FFA-induced hepatic IKKβ activation and corresponding impairment of hepatic insulin signaling. The findings of this study suggest that IKKβ is a causal mediator of hepatic insulin resistance induced by acutely elevated plasma FFA. Therefore, hepatic IKKβ represents a potential therapeutic target to prevent or treat fat-induced hepatic insulin resistance and associated diseases.

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