

# Duration of rise in free fatty acids determines salicylate's effect on hepatic insulin sensitivity

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## Abstract

We have shown in rats that sodium salicylate (SS), which inhibits IκBα kinase B (IKKB), prevents hepatic and peripheral insulin resistance caused by short-term (7 h) i.v. administration of Intralipid and heparin (IH). We wished to further determine whether this beneficial effect of SS persisted after prolonged (48 h) IH infusion, which better mimics the chronic free fatty acid (FFA) elevation of obesity. Hence, we performed hyperinsulinemic euglycemic clamps with tritiated glucose methodology to determine hepatic and peripheral insulin sensitivity in rats infused with saline, IH, IH and SS, or SS alone. SS prevented peripheral insulin resistance ( $P < 0.05$ ) caused by prolonged plasma FFA elevation; however, it did not prevent hepatic insulin resistance. In skeletal muscle, protein levels of phospho-IκBα were augmented by prolonged IH administration and this was prevented by SS, suggesting that IH activates while SS prevents the activation of IKKB. Markers of IKKB activation, namely protein levels of phospho-IκBα and IκBα, indicated that IKKB is not activated in the liver after prolonged FFA elevation. Phosphorylation of serine 307 at insulin receptor substrate (IRS)-1, which is a marker of proximal insulin resistance, was not altered by IH administration in the liver, suggesting that this is not a site of hepatic insulin resistance in the prolonged lipid infusion model. Our results suggest that the role of IKKB in fat-induced insulin resistance is time and tissue dependent and that hepatic insulin resistance induced by prolonged lipid elevation is not due to an IRS-1 serine 307 kinase.

## Key Words

- ▶ free fatty acids
- ▶ insulin resistance
- ▶ liver
- ▶ glucose metabolism

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## Introduction

Obesity leads to type 2 diabetes mellitus (T2DM) because of insulin resistance, and insulin resistance of obesity is due to elevated circulating levels of free fatty acids (FFAs)

and cytokines (Boden 1997, Lewis *et al.* 2002, Galic *et al.* 2010). Inhibitor of κBα kinase B (IKKB), a serine/threonine kinase that is associated with insulin resistance, can be

activated by FFAs and cytokines (Boden *et al.* 2005, Park *et al.* 2007, Grund *et al.* 2008). The mechanisms through which IKKB induces insulin resistance are twofold: i) direct phosphorylation of insulin receptor substrate (IRS)-1 at serine 307 (Gao *et al.* 2002), which impairs tyrosine phosphorylation of IRS-1 and therefore dampens the insulin signal (Aguirre *et al.* 2000), and ii) activation of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B), which upregulates the expression of various cytokines (Barnes & Karin 1997). NF- $\kappa$ B is inhibited when bound to I $\kappa$ Ba in the cytoplasm, but upon phosphorylation of I $\kappa$ Ba by activated IKKB, I $\kappa$ Ba degradation is induced, culminating in the liberation and nuclear translocation of NF- $\kappa$ B (Ghosh & Baltimore 1990, Brown *et al.* 1995, Traenckner *et al.* 1995, Zandi *et al.* 1998, Karin 1999, Hacker & Karin 2006). IKKB is inhibited by salicylates, such as aspirin and sodium salicylate (SS; Yin *et al.* 1998).

Results obtained using rodent models show that IKKB mediates insulin resistance in multiple tissues. Hepatic overexpression of IKKB results in hepatic and peripheral insulin resistance (Cai *et al.* 2005). Hepatocyte-specific IKKB knockout (KO) mice show improved hepatic insulin sensitivity when challenged with high-fat feeding (Arkan *et al.* 2005). Salicylates ameliorate glucose tolerance as well as hepatic and skeletal muscle insulin signaling in Zucker *fa/fa* rats (Yuan *et al.* 2001). The role of IKKB in mediating insulin resistance is more controversial in humans than in rodents, as studies in humans have found salicylates to improve, decrease, or have no effect on insulin sensitivity (Newman & Brodows 1983, Hundal *et al.* 2002, Koska *et al.* 2009, Xiao *et al.* 2009). In patients with T2DM, aspirin has been found to alleviate peripheral insulin resistance, although no improvement was found in hepatic insulin sensitivity (Hundal *et al.* 2002).

I.v. lipid infusions have been used to determine the role of IKKB in insulin resistance induced by FFAs *per se*, as opposed to insulin resistance induced by other factors associated with obesity. Short-term lipid infusions represent the obesity-associated amplified acute increases in tissue FFA availability in the fasting–feeding cycle, while prolonged lipid infusions more closely approximate the chronically augmented plasma FFA concentrations found in obesity. Heterozygous IKKB KO mice are protected from whole-body and peripheral insulin resistance caused by short-term (5 h) lipid infusion (Kim *et al.* 2001). SS also prevents peripheral (Kim *et al.* 2001, Park *et al.* 2007) and hepatic (Park *et al.* 2007) FFA-induced insulin resistance caused by short-term lipid infusion in rats. In humans, salicylates have been reported to improve (Mohlig *et al.* 2006) or have no effect (Meex *et al.* 2011) on insulin

resistance caused by short-term lipid infusion. Using prolonged (48 h) lipid (Intralipid plus heparin, IH) infusion, our group has found that SS does not improve whole-body insulin resistance caused by prolonged plasma FFA elevation, although hepatic insulin sensitivity was not assessed (Xiao *et al.* 2009). The controversy surrounding the effectiveness of salicylates in improving insulin resistance in humans may be due to different dosages and types of salicylates used, and the often used oral route of administration, which limits the extent of bioavailability (Xiao *et al.* 2009). Studies on rodents facilitate the use of i.v. administration of salicylates and allow the collection of hepatic tissue for analysis of mediators and markers of insulin resistance. Therefore, in the current study, we sought to determine, in rodents, whether SS prevents hepatic and peripheral insulin resistance caused by prolonged (48 h) lipid (IH) infusion, as it did after short-term lipid infusion (Park *et al.* 2007).

## Materials and methods

### Animals

Female Wistar rats weighing 250–300 g were obtained from Charles River (Saint-Constant, QC, Canada) and housed in the Department of Comparative Medicine of the University of Toronto. Rats had free access to standard chow (Teklad Global Diet #2018; Harland Laboratories, Indianapolis, IN, USA) and water. All procedures were approved by the Animal Care Committee of the University of Toronto.

### Surgery

Surgery was performed under isoflurane anesthesia, following ~7–14 days of adaptation to the animal facility. Briefly, indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for blood sampling, as described previously (Lam *et al.* 2002, Park *et al.* 2007). Post-surgery recovery was at least 3 days.

### Study design

On the first experiment day, the cannulated rats were placed in a circular cage and connected to the infusion apparatus, as described previously (Mason *et al.* 1999). After a basal blood sample was obtained (time=0 h), rats were given one of four i.v. treatments for 48 h: saline (5.5  $\mu$ l/min), IH (20% Intralipid plus 20 U/ml heparin at

5.5  $\mu\text{l}/\text{min}$ ), IH plus SS (Sigma–Aldrich) (7 mg/kg bolus plus 0.117 mg/kg per min), or SS alone. Heparinized saline (4 U/ml) was infused at 5  $\mu\text{l}/\text{min}$  into the carotid artery from 0 h to the 44 h mark to maintain patency. Blood samples were obtained at 18 h as well as 24 h and rats were fasted overnight. On clamp day, a blood sample was obtained at 44 h and immediately afterward, an 8  $\mu\text{Ci}$  bolus of [ $3\text{-}^3\text{H}$ ] glucose (Perkin-Elmer, Boston, MA, USA) was given, followed by a constant [ $3\text{-}^3\text{H}$ ] glucose infusion of 0.15  $\mu\text{Ci}/\text{min}$  that lasted until the end of treatment (time = 48 h). Insulin (5 mU/kg per min, Humulin R, Eli Lilly Canada) infusion was started at 46 h and plasma glucose was measured every 5 min using a glucose analyzer (Analox GM9D Analyser, Analox Instruments, Hammersmith, UK) and the infusion rate of a 50% glucose solution was altered accordingly to maintain euglycemia (Park *et al.* 2007). The glucose solution contained 48  $\mu\text{Ci}/\text{g}$  [ $3\text{-}^3\text{H}$ ] glucose to minimize decreases in plasma glucose specific activity, which are associated with underestimation of glucose production (Finegood *et al.* 1987). The 30 min before the start of insulin infusion is referred to as the basal period, while the last 30 min of the clamp are referred to as the clamp period. During the basal period and the clamp period, blood samples were obtained every 10 min to determine plasma glucose, FFA, insulin, and [ $3\text{-}^3\text{H}$ ] glucose specific activity. Once plasma was collected from centrifuged blood samples, red blood cells were mixed with heparinized saline (2 U/ml) in a 1:1 ratio and re-infused into animals. At the end of the clamp, rats were anesthetized, the liver was freeze-clamped with pre-cooled aluminum tongs, and the soleus muscle was collected. Tissues were stored at  $-80^\circ\text{C}$ . The tissue determinations have a lower  $n$  than the glucose kinetics determinations because we lost some tissue samples due to a freezer meltdown.

We have already shown that SS prevents IH-induced hepatic and peripheral insulin resistance in the 7-h model, which is analogous to the 48-h i.v. infusion model described above with the exception of treatments being shorter in time (Park *et al.* 2007). To avoid comparison with historical controls, 7-h studies were repeated using the same dosage of SS as in the 48-h studies, which is also the dosage of SS we previously found to prevent insulin resistance induced by 7-h IH infusion. The study design for these repeat 7-h studies was similar to that of the 48-h protocol, but all rats were infused with saline (5.5  $\mu\text{l}/\text{min}$ ) via the jugular vein from 0 to 41 h, and then on clamp day, at 41 h, rats were randomized into one of the four i.v. treatment groups that lasted 7 h: saline (5.5  $\mu\text{l}/\text{min}$ ), IH (5.5  $\mu\text{l}/\text{min}$ ), IH plus SS (7 mg/kg bolus plus

0.117 mg/kg per min), or SS alone. As for the 48-h protocol, [ $3\text{-}^3\text{H}$ ] glucose infusion was started 4 h before the end of treatment and a hyperinsulinemic (5 mU/kg per min) euglycemic clamp with concomitant [ $3\text{-}^3\text{H}$ ] glucose methodology was performed during the last 2 h of treatment.

### Plasma assays

To measure [ $3\text{-}^3\text{H}$ ] glucose in plasma and in glucose infusates, samples were deproteinized using  $\text{Ba}(\text{OH})_2$  and  $\text{ZnSO}_4$ , and after complete evaporation, radioactivity was counted (Park *et al.* 2007). Plasma insulin concentrations were determined by RIA (Millipore, Billerica, MA, USA) (Park *et al.* 2007). Plasma FFA concentrations were determined using a colorimetric assay (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (Park *et al.* 2007). Plasma adiponectin concentrations during the basal period were determined using a kit (Cat # EZRADP-62K, EMD Millipore) and 1:500 dilution of samples.

### Tissue preparation for western blot analysis

**Liver** Liver tissue (50 mg) was homogenized by sonication using a lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM  $\beta$ -glycerophosphate, 20 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 2 mM sodium pyrophosphate, 20  $\mu\text{M}$  leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 0.5  $\mu\text{M}$  okadaic acid. After centrifugation, the supernatant was stored at  $-80^\circ\text{C}$ . For western blot analysis of protein kinase C (PKC)- $\delta$ , liver cytosolic and membrane fractions were obtained as described previously (Park *et al.* 2007), except that 0.5  $\mu\text{M}$  okadaic acid was used instead of 1  $\mu\text{M}$  microcystin.

**Muscle** For phospho-IkBa and total IkBa, soleus muscle samples of 50 mg were homogenized using a glass homogenizer and a lysis buffer consisting of 128 mM NaCl, 24 mM  $\beta$ -glycerophosphate, 19 mM Tris–HCl (pH 7.5), 2 mM EDTA, 2 mM sodium pyrophosphate, 2 mM DL-dithiothreitol, 3 mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, 1% Triton X-100, 1.4  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  okadaic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 19 mM HEPES. Following centrifugation, the supernatant was stored at  $-80^\circ\text{C}$ . For soleus muscle phospho-IRS-1 and total IRS-1, the lysis buffer consisted of 50 mM Tris pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 2 mM EGTA, 10 mM sodium pyrophosphate,

100 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 50 nM okadaic acid, 1 mM PMSF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Following centrifugation, the supernatant was stored at -80 °C.

### Western blot analysis

Protein concentration was determined using the Bradford method (Bio-Rad Laboratories). Dried liver and muscle samples were mixed with Laemmli buffer boiled, loaded onto an electrophoretic apparatus, and subjected to SDS-PAGE. Subsequently, proteins were transferred to polyvinylidene fluoride membranes. Membranes were then blocked by incubating with 5% non-fat milk in Tris-buffered saline-Tween (TBST). For membranes that were probed for phospho-proteins, 5% BSA in TBST was used for blocking. Next, membranes were incubated with primary antibodies. The following primary antibodies were used: rabbit polyclonal antibody against serine 32- and serine 36-phosphorylated IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-IκBα antibody (Santa Cruz), mouse monoclonal anti-α-tubulin antibody (Santa Cruz), goat polyclonal anti-actin antibody (Santa Cruz), rabbit polyclonal antibody against serine 307-phosphorylated IRS-1 (Millipore, Temecula, CA, USA), rabbit polyclonal anti-IRS-1 antibody (Millipore), rabbit polyclonal antibody against serine 473-phosphorylated Akt (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-Akt antibody (Cell Signaling Technology), and rabbit polyclonal anti-PKC-δ antibody (Santa Cruz). The following day, membranes were washed with TBST and incubated with secondary antibodies (all from Santa Cruz and conjugated with HRP). Afterward, membranes were washed with TBST. ECL substrate (ThermoScientific, Rockford, IL, USA) was used to develop the membranes. Membranes that were probed for phospho-IκBα, IκBα, phospho-IRS-1, and phospho-Akt were subsequently stripped and reprobed for IκBα, α-tubulin (for muscle samples) or actin (for liver samples), IRS-1, or Akt respectively. Developed membranes were rinsed with TBST, stripped with stripping buffer (ThermoScientific), and washed with TBST. Blocking and subsequent steps were done as described above.

Quantification of the bands obtained on film was done using ImageJ (National Institutes of Health, Bethesda, MD, USA).

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Mini extraction kit (Qiagen) and subjected to RT before PCR amplification

with the Two-Step Master Mix (Applied Biosystems). TaqMan fluorogenic probes and oligonucleotide primers were designed by Applied Biosystems. TaqMan PCR assays were carried out in triplicate in 96-well optical plates with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). For every PCR sample, an amplification plot was generated from the collected data, and a threshold cycle (CT) value was calculated with the software suite. CT values were used to calculate the initial quantity of cDNA by subtracting the CT value of β-actin (*Actb*), which was used as a housekeeping gene. The real-time quantitative PCR data were analyzed using the  $2^{-\Delta\Delta CT}$  method of Livak & Schmittgen (2001). TaqMan assays for interleukin6 (*Il6*) (Rn01410330\_m1), phosphoenolpyruvate carboxykinase (*Pck1*) (Rn01529014\_m1), tumor necrosis factor-α (*Tnf*) (Rn01525859\_g1), and *Actb* (Rn00667869\_m1) were obtained from Applied Biosystems.

### Calculations of glucose kinetics

Calculations of the rate of glucose disappearance ( $R_d$ ) and of endogenous glucose production (EGP) were done based on [<sup>3</sup>H] glucose data and using modified Steele's equations that take into account the tracer included in the glucose infusion during the clamp (Steele *et al.* 1956, Finegood *et al.* 1987). During the basal period,  $R_d$  equals EGP. During the clamp period,  $R_d$  equals the sum of the rate of exogenous glucose infusion and EGP. During euglycemia,  $R_d$  represents glucose utilization by tissues because renal glucose clearance equals zero (Park *et al.* 2007). Averages of EGP and  $R_d$  for the basal and clamp periods (steady state) as well as the average of the glucose infusion rate during the clamp period were calculated and presented herein.

### Statistical analysis

Data are presented as means ± S.E.M. To compare parameters between more than two treatment groups, one-way ANOVA was used and *post hoc* analysis was done using Tukey's test. When comparing parameters between two treatment groups, the independent samples *t*-test was used. Significance was set at 0.05. Statistical analysis was carried out using IBM SPSS Statistics, Version 20 (IBM Corporation, Armonk, NY, USA).

### Results

Prolonged lipid administration increased plasma FFA concentrations by ~1.5-fold during the basal period (last 30 min before the start of the hyperinsulinemic

euglycemic clamp) in both the IH-treated group and the group co-infused with IH and SS compared with controls ( $P < 0.05$ , Table 1). During the last 30 min of the hyperinsulinemic euglycemic clamp (clamp period), plasma FFA concentrations were also higher in the IH and co-infusion groups ( $P < 0.05$ , Table 1). SS did not appear to affect insulin-induced suppression of FFA, as previously found after 7 h of lipid infusion and consistent with a lack of effect of IH on markers of IKKB activation in fat (Park *et al.* 2007). Insulin concentrations were higher during the basal period in the IH group compared with the co-infusion and SS alone groups ( $P < 0.05$ , Table 1), likely secondary to insulin resistance. There were no differences in insulin concentrations between groups during the clamp (Table 1). Basal EGP corresponds to basal  $R_d$  and it was higher in the IH group vs the other groups ( $P < 0.05$ , Fig. 1A). Co-infusion with SS appeared to normalize basal EGP- $R_d$  as well as basal hyperinsulinemia. During the hyperinsulinemic clamp, EGP was suppressed as expected, but it was higher in the IH and co-infusion groups (Fig. 1A). Percentage suppression of EGP from basal indicates that SS did not prevent hepatic insulin resistance caused by prolonged IH administration (Fig. 1B). However, the decrease in insulin-stimulated glucose utilization caused by prolonged IH administration was completely prevented by SS co-administration (Fig. 2A). As the glucose infusion rate, which is indicative of whole-body insulin sensitivity, is the difference between glucose utilization and EGP during the clamp period, we found that SS only partially prevents whole-body insulin resistance caused by prolonged IH treatment (Fig. 2B). In contrast with these results, we have previously shown that SS completely prevents whole-body, hepatic, and peripheral insulin resistance caused by short-term (7 h) IH infusion in rats (Park *et al.* 2007). To avoid historical controls, the studies were repeated and we found the same results (Figs 3 and 4).

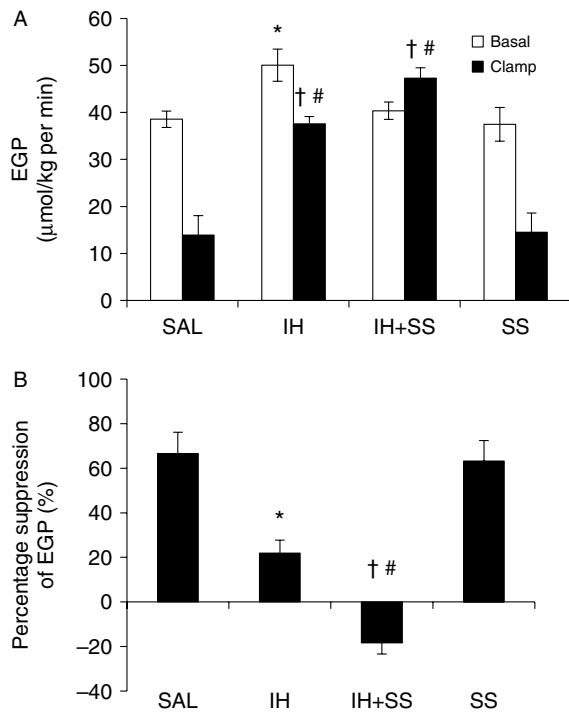
We also examined liver and skeletal muscle tissues collected after the hyperinsulinemic euglycemic clamp in the prolonged infusion model for markers of IKKB activation and insulin sensitivity. IKKB activation causes the degradation of I $\kappa$ B $\alpha$  protein, and, hence, a decrease in I $\kappa$ B $\alpha$  protein content is often used as a marker of IKKB activation. We found that prolonged IH administration does not affect I $\kappa$ B $\alpha$  protein levels in the liver (Fig. 5A), suggesting that IKKB is not activated after prolonged plasma FFA elevation. We also determined the extent of serine 32 and serine 36 phosphorylation of I $\kappa$ B $\alpha$  as a more direct indicator of IKKB activation, compared to I $\kappa$ B $\alpha$  protein levels (DiDonato *et al.* 1995) because IKKB directly phosphorylates I $\kappa$ B $\alpha$  at serine 32 and 36, and it is this phosphorylation that results in the degradation of I $\kappa$ B $\alpha$  (Brown *et al.* 1995, Zandi *et al.* 1998, Hacker & Karin 2006). In liver tissue, we found that the content of phosphorylated I $\kappa$ B $\alpha$  at serine 32 and 36 divided by the content of total I $\kappa$ B $\alpha$  did not differ between treatments (Fig. 5B). This further suggests that hepatic IKKB is not activated after prolonged IH administration. We found that hepatic mRNA levels of *I $\kappa$ B* (IH = 0.97  $\pm$  0.55-fold of saline group (normalized control)) or of *Tnf* (IH = 1.05  $\pm$  0.39-fold of saline group) were not altered after prolonged IH administration.

We also determined serine 307 phosphorylation of IRS-1, which is an established *ex vivo* marker of insulin resistance. We did not find any differences in serine 307-phosphorylated IRS-1 divided by total IRS-1 (Fig. 6). Total IRS-1 was also not affected. This suggests that this is not a primary site of hepatic insulin resistance caused by prolonged FFA elevation. Next, we assessed serine 473 Akt phosphorylation in the liver. IH decreased serine 473 phosphorylation of Akt, thereby indicating IH-induced impairment of insulin signaling. However, SS co-infusion completely prevented the IH-induced reduction in phosphorylation of Akt (Fig. 7A), and, moreover, SS alone increased phosphorylation of Akt

**Table 1** Plasma glucose, FFA, and insulin concentrations during the basal and clamp periods after prolonged (48 h) treatment in Wistar rats

	Basal period				Clamp period			
	SAL	IH	IH+SS	SS	SAL	IH	IH+SS	SS
Glucose (mmol/l)	6.42 $\pm$ 0.19	6.09 $\pm$ 0.22	6.28 $\pm$ 0.13	6.37 $\pm$ 0.07	6.49 $\pm$ 0.17	6.28 $\pm$ 0.15	6.29 $\pm$ 0.14	6.41 $\pm$ 0.13
FFA ( $\mu$ Eq/l)	545 $\pm$ 30	876 $\pm$ 55 <sup>*,†</sup>	909 $\pm$ 99 <sup>*,†</sup>	525 $\pm$ 52	157 $\pm$ 48	491 $\pm$ 43 <sup>*,†</sup>	538 $\pm$ 50 <sup>*,†</sup>	158 $\pm$ 26
Insulin (pM)	68 $\pm$ 11	111 $\pm$ 19 <sup>*,†</sup>	43 $\pm$ 5	31 $\pm$ 6	579 $\pm$ 20	608 $\pm$ 49	563 $\pm$ 12	594 $\pm$ 41

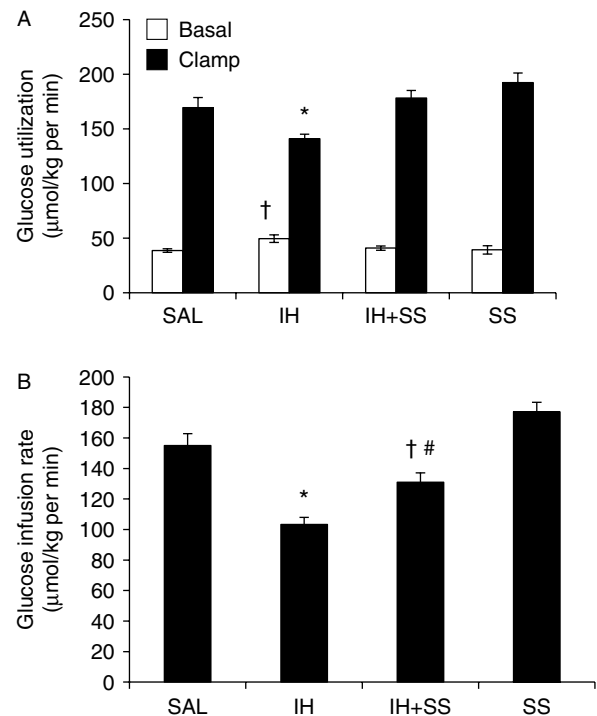
Treatments: SAL, saline (5.5  $\mu$ l/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5  $\mu$ l/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n = 9-14$ /group. \* $P < 0.05$  vs SAL.  $^\dagger P < 0.05$  vs SS.  $^\ddagger P < 0.05$  vs IH+SS.

**Figure 1**

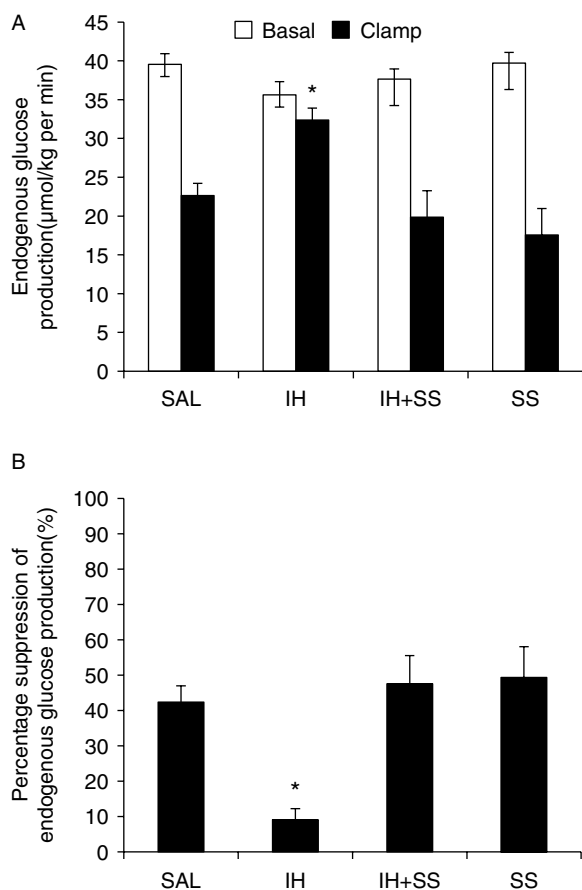
EGP in the prolonged (48 h) infusion model in Wistar rats. (A) EGP during the basal period (30 min immediately before the hyperinsulinemic euglycemic clamp) and the clamp period (last 30 min of the hyperinsulinemic euglycemic clamp). (B) Percent suppression in EGP in the clamp period compared with the basal period. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=9-14$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test. \* $P<0.05$  vs other groups. † $P<0.05$  vs SAL. # $P<0.05$  vs SS.

( $P<0.05$  vs saline, Fig. 7A). These results suggest that i) after 48 h of IH infusion, there is a disconnect in hepatic insulin resistance assessed *in vivo* vs *ex vivo* (liver) and ii) after 48 h of SS treatment, SS itself increases hepatic Akt activation. To further explore the mechanism of the latter observation, we determined plasma adiponectin because in high-fat-diet-fed mice SS administration augments adiponectin gene expression in adipose tissue (Nixon *et al.* 2012) and adiponectin can stimulate Akt activation (Ouchi *et al.* 2004, Yamauchi *et al.* 2007). Indeed, we found that plasma adiponectin levels were higher in the co-infusion group compared with the saline and IH groups ( $P<0.05$ , Fig. 7B); plasma adiponectin levels in the SS alone group were intermediate, between the levels in the co-infusion group and the saline or IH groups (Fig. 7B).

PKC-δ impairs insulin signaling, but it does not phosphorylate IRS-1 at serine 307 directly (Greene *et al.* 2004), and by activating p38 MAPK, PKC-δ can increase gluconeogenic enzyme mRNA independent of Akt (Collins *et al.* 2006). We have previously reported that short-term (7 h) IH infusion activates PKC-δ in the liver, based on its translocation from cytosol to membrane (Lam *et al.* 2002). Here, we show that prolonged (48 h) IH infusion also activates PKC-δ in the liver and addition of SS did not prevent this effect (Fig. 8). Furthermore, the mRNA levels of the gluconeogenic enzyme *Pck1* were over twofold greater in both the IH and IH plus SS groups compared with the saline group, but statistical significance was not reached across treatments ( $2.99 \pm 1.14$ -fold of saline in IH group vs  $2.39 \pm 0.87$  in IH plus SS group vs  $1.14 \pm 0.22$  in SS alone group).

**Figure 2**

Glucose utilization and glucose infusion rate in the prolonged (48 h) infusion model in Wistar rats. (A) Glucose utilization during basal period (30 min immediately before the hyperinsulinemic euglycemic clamp) and clamp period (last 30 min of the hyperinsulinemic euglycemic clamp). (B) Glucose infusion rate during the clamp period. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=9-14$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test. \* $P<0.05$  vs other groups. † $P<0.05$  vs SAL. # $P<0.05$  vs SS.

**Figure 3**

EGP in the short-term (7 h) infusion model in Wistar rats. (A) EGP during the basal period (30 min immediately before the hyperinsulinemic euglycemic clamp) and the clamp period (last 30 min of the hyperinsulinemic euglycemic clamp). (B) Percent suppression in EGP in the clamp period compared with the basal period. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=3-6$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test.  $*P<0.05$  vs other groups.

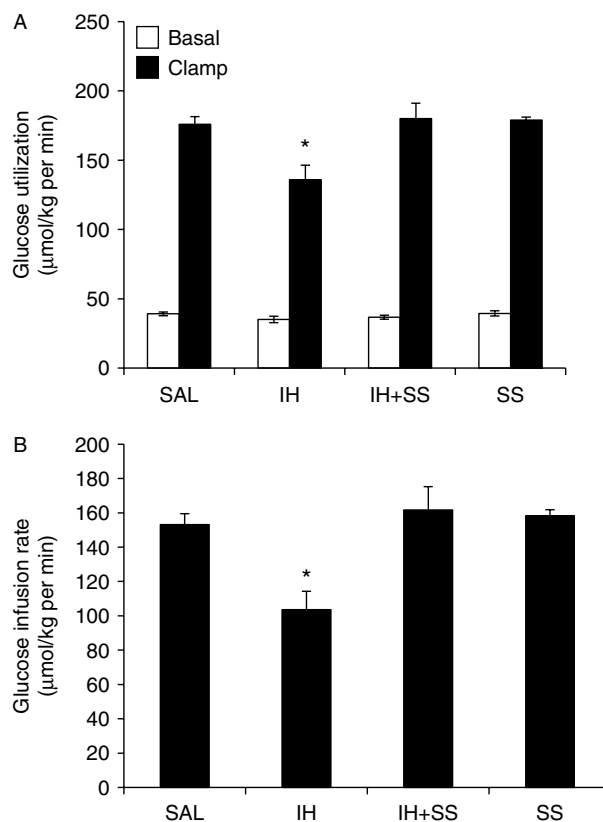
In skeletal muscle, although IκBα protein levels were not altered after prolonged IH administration (Fig. 9A), IκBα phosphorylation was augmented and the effect of IH was prevented by SS ( $P<0.05$  vs other groups, Fig. 9B). Although phosphorylation of serine 307 IRS-1 in skeletal muscle tended to be increased after prolonged IH administration, the difference was not significant (Fig. 10) and addition of SS did not reverse this tendency.

## Discussion

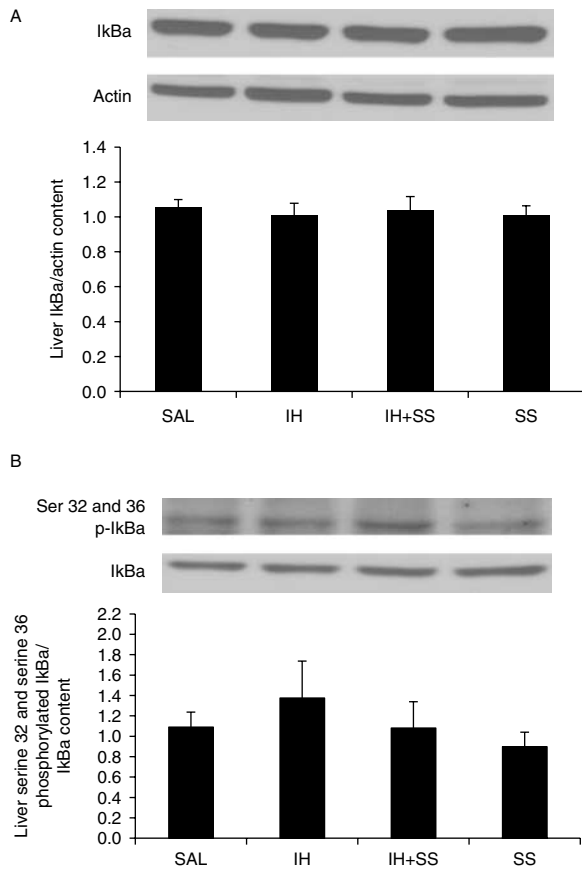
In the current study, we show that hepatic insulin resistance caused by prolonged FFA elevation is not

associated with IKKB activation in the liver and, accordingly, it is not prevented by SS. We also found that hepatic serine 307 of IRS-1 is not a target for phosphorylation by IKKB or other serine/threonine kinases in the liver in response to prolonged lipid infusion. Lastly, we found that prolonged FFA elevation causes peripheral insulin resistance and activates IKKB in skeletal muscle, both of which are prevented by SS.

Our findings are in contrast to the findings that liver-specific IKKB KO mice on a high-fat diet show improved, albeit not normalized, *in vivo* hepatic insulin sensitivity relative to controls fed a high-fat diet (Arkan *et al.* 2005). It is possible that in the high-fat diet model, cytokines rather than fat are the main inducers of IKKB activation,

**Figure 4**

Glucose utilization and glucose infusion rate in the short-term (7 h) infusion model in Wistar rats. (A) Glucose utilization during basal period (30 min immediately before the hyperinsulinemic euglycemic clamp) and clamp period (last 30 min of the hyperinsulinemic euglycemic clamp). (B) Glucose infusion rate during the clamp period. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=3-6$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test.  $*P<0.05$  vs other groups.

**Figure 5**

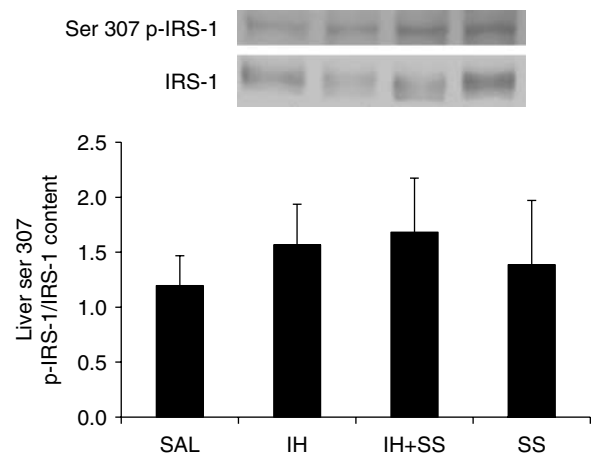
Hepatic phosphorylated IκBα and IκBα protein levels after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats. (A) Hepatic IκBα protein content divided by actin protein content. (B) Hepatic serine 32 and serine 36-phosphorylated IκBα protein content divided by IκBα protein content. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone. *n* = 5–6/group. Data were analyzed by one-way ANOVA followed by Tukey's test.

unlike our model where we found that prolonged IH administration did not alter hepatic expression of *Il6* and *Tnf*. Interestingly, in a model of cytokine-induced glucose intolerance, liver-specific IKKB KO mice showed better glucose tolerance than controls (Arkan *et al.* 2005). Our results, however, do agree with studies on T2DM subjects, where treatment with high-dose aspirin improved peripheral insulin sensitivity but failed to improve insulin action on EGP (Hundal *et al.* 2002). Indeed, we have also found that prolonged infusion of olive oil (48 h) in rats induces hepatic insulin resistance that is not prevented by SS co-infusion (Park *et al.* 2005).

Our current results, together with our other previously published results (Park *et al.* 2007), suggest that IKKB

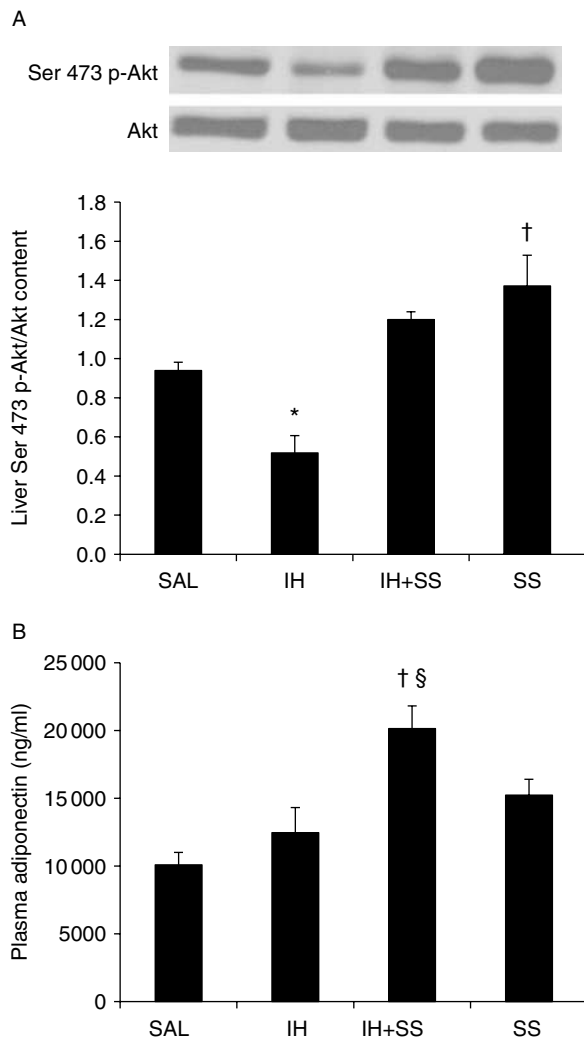
is causal in fat-induced hepatic insulin resistance in the short term, but IKKB does not mediate hepatic insulin resistance due to prolonged FFA elevation. In terms of markers of hepatic IKKB activation, we did not find that either total IκBα or phosphorylated IκBα normalized by total IκBα was altered by prolonged lipid infusion. By contrast, we previously found that short-term lipid infusion decreases total IκBα and this is prevented by SS co-infusion (Park *et al.* 2007). It is possible that as the length of lipid infusion increases from 7 to 48 h, the negative feedback loop whereby NF-κB increases IκBα gene expression (Sun *et al.* 1993) takes effect. This feedback loop, however, does not explain the lack of effect of prolonged lipid infusion on IκBα phosphorylation, suggesting mechanisms of IKKB inactivation with prolonged duration of lipid infusion.

Consistent with the lack of increased IκBα phosphorylation, serine 307 of IRS-1 does not show changes in phosphorylation in the liver across treatments, suggesting that IKKB is not activated and that this is not a source of hepatic insulin resistance caused by prolonged lipid infusion. Our results also suggest that other kinases that target serine 307 in IRS-1, such as c-jun NH<sub>2</sub> terminal kinase (JNK; Aguirre *et al.* 2000), are likely not involved in hepatic insulin resistance after prolonged lipid infusion. Nevertheless, impairments at the level of IRS proteins may still exist because of phosphorylation of other

**Figure 6**

Hepatic serine 307-phosphorylated IRS-1 (Ser 307 p-IRS-1) protein content divided by IRS-1 protein content after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone. *n* = 6/group. Data were analyzed by one-way ANOVA followed by Tukey's test.



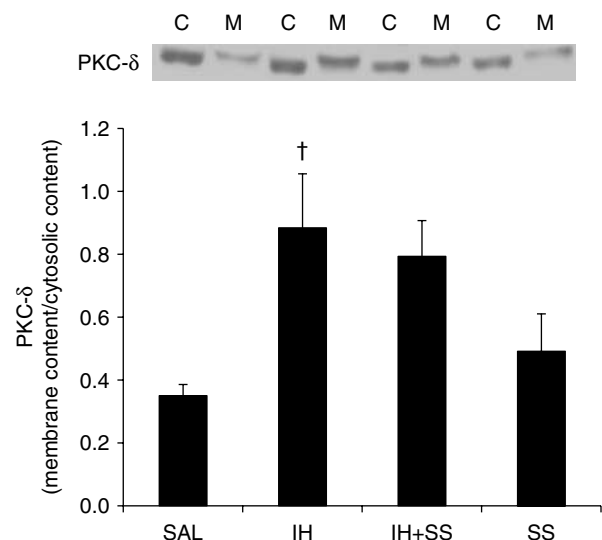
**Figure 7**

(A) Hepatic serine 473-phosphorylated Akt (Ser 473 p-Akt) protein content divided by Akt protein content, after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats.  $n=4-6$ /group. (B) Plasma adiponectin concentrations during the basal period (30 min period immediately before the hyperinsulinemic euglycemic clamp) in the prolonged (48 h) infusion model in Wistar rats.  $n=8-14$ /group. Treatments: SAL, saline (5.5  $\mu$ l/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5  $\mu$ l/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone. Data were analyzed by one-way ANOVA followed by Tukey's test. \* $P<0.05$  vs other groups. † $P<0.05$  vs SAL. § $P<0.05$  vs IH.

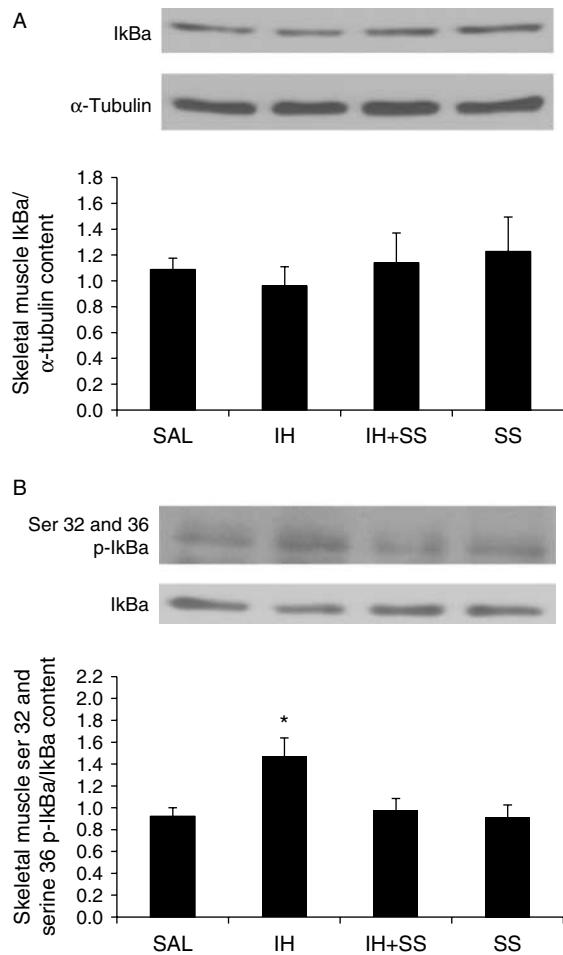
serine/threonine residues and/or impairment of tyrosine phosphorylation. A novel PKC isoform, PKC- $\epsilon$ , binds to the insulin receptor in the liver *in vivo* and it can diminish the receptor's kinase activity, thereby inducing hepatic insulin resistance after a 3-day high-fat diet (Samuel *et al.* 2007). We (Lam *et al.* 2002) and others (Boden *et al.* 2005) have reported that hepatic insulin

resistance due to short-term lipid infusion is associated with membrane translocation (marker of activation) of another novel PKC isoform, PKC- $\delta$ , in the liver, and, interestingly, PKC- $\delta$  does not directly phosphorylate IRS-1 at serine 307 (Greene *et al.* 2004). We herein found that PKC- $\delta$  membrane translocation is increased by IH and not affected by SS, which raises the possibility that hepatic insulin resistance in the prolonged IH infusion model may be mediated by PKC- $\delta$ , although this should be tested in future inhibition studies. PKC- $\delta$  could act by reducing tyrosine phosphorylation of IRS-1 (Greene *et al.* 2004), which should still affect Akt, or via p38 MAPK, which would directly affect gluconeogenic enzyme gene expression (Collins *et al.* 2006). P38 MAPK can also be activated by insulin (Fujishiro *et al.* 2001), and, therefore, our liver samples obtained after a hyperinsulinemic euglycemic clamp are not adequate for assessment of its activation. Our preliminary data suggest that short-term IH infusion in the absence of hyperinsulinemic clamp increases phosphorylation of p38 MAPK in the liver (data not shown).

Unexpectedly, salicylate, which did not affect IH-induced hepatic insulin resistance, normalized hepatic phosphorylation of Akt. The reduced Akt phosphorylation

**Figure 8**

Membrane PKC- $\delta$  protein content (M) divided by cytosolic PKC- $\delta$  protein content (C), after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats. Treatments: SAL, saline (5.5  $\mu$ l/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5  $\mu$ l/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min).  $n=4-6$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test. † $P<0.05$  vs SAL.

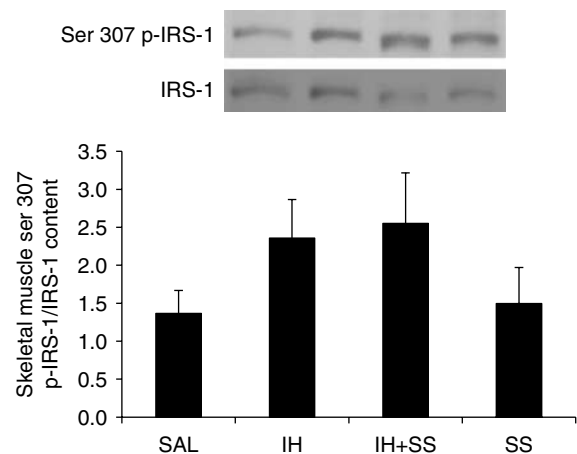
**Figure 9**

Skeletal muscle phosphorylated IκBα (p-IκBα) and IκBα protein levels after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats. (A) Skeletal muscle IκBα protein content divided by α-tubulin protein content. (B) Skeletal muscle serine (Ser) 32- and Ser 36-phosphorylated IκBα protein content divided by IκBα protein content. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=5-6$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test. \* $P<0.05$  vs other groups.

caused by prolonged IH infusion could be mediated by ceramides (Schmitz-Peiffer *et al.* 1999) or the protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is stabilized by p38 MAPK (Liu *et al.* 2007). The IH-induced Akt inhibition was prevented by SS co-infusion in association with increased plasma concentrations of adiponectin. It has been documented that SS increases mRNA levels of adiponectin in adipose tissue by a mechanism that may not be IKKB related (Nixon *et al.* 2012) (an IKKB-independent mechanism would be in

accordance with our data in the 7-h IH infusion model, where we could not find an increase in markers of IKKB activation in adipose tissue (Park *et al.* 2007)). In obese subjects, treatment with salicylate, a salicylate dimer, elevated circulating adiponectin concentrations (Fleischman *et al.* 2008). Moreover, adiponectin increases Akt activation, for instance via increased ceramidase activity (Schmitz-Peiffer *et al.* 1999, Holland *et al.* 2011). Hepatic levels of Akt phosphorylation did not correlate with *in vivo*-determined hepatic insulin sensitivity, which raises the possibility that hepatic insulin resistance caused by prolonged IH infusion may be due to the counteraction of insulin's effects on gluconeogenic enzyme gene expression rather than impairment in insulin signaling. In fact, the mRNA levels of *Pck1* were in accordance with *in vivo*-determined hepatic glucose production. The effect of IH on *Pck1* mRNA could be via counteraction of insulin's effects on gluconeogenic enzyme gene expression, via the PKC-δ/p38 MAPK axis.

While it is likely that prolonged IH infusion induced insulin resistance directly on the liver, an indirect effect mediated in the CNS is another possibility. Thus, insulin-stimulated suppression of EGP can be impaired in the context of normal insulin signaling in the liver when insulin signaling in the hypothalamus is reduced (Okamoto *et al.* 2005). Fat at the level of the brain induces central insulin resistance (Posey *et al.* 2009) as

**Figure 10**

Skeletal muscle serine 307-phosphorylated IRS-1 (Ser 307 p-IRS-1) protein content divided by IRS-1 protein content after 48 h of treatment, at the end of the hyperinsulinemic euglycemic clamp, in Wistar rats. Treatments: SAL, saline (5.5 μl/min); IH, Intralipid plus heparin (20% Intralipid plus 20 U/ml heparin at 5.5 μl/min); IH+SS, Intralipid plus heparin co-infused with sodium salicylate (7 mg/kg bolus plus 0.117 mg/kg per min); SS, sodium salicylate alone.  $n=6$ /group. Data were analyzed by one-way ANOVA followed by Tukey's test.

well as hepatic insulin resistance evaluated with a hyperinsulinemic euglycemic clamp (Clement *et al.* 2002, Benoit *et al.* 2009). Insulin acting in the brain suppresses EGP, and this brain–liver circuit appears to be independent of hepatic Akt activation (Inoue *et al.* 2006, Myers 2006). At the level of the liver, central insulin suppresses hepatic glucose production through hepatic IL6 production, which in turn activates STAT3, a transcription factor that decreases the expression of gluconeogenic genes (Inoue *et al.* 2006, Myers 2006). Hepatic *Il6* mRNA levels were not affected by IH in our model, which makes the importance of this brain–liver circuit less likely. Furthermore, although i.c.v. palmitate administration can result in hypothalamic IKKB activation (Posey *et al.* 2009) and adenoviral delivery of constitutively active IKKB into the hypothalamus induces hypothalamic insulin resistance (Zhang *et al.* 2008), it is unlikely that IKKB activation in the brain causes hepatic insulin resistance in our prolonged lipid infusion model because SS, which can cross the blood–brain barrier (Su *et al.* 2009), was ineffective in preventing hepatic insulin resistance.

Prolonged plasma FFA elevation stimulated I $\kappa$ B $\alpha$  phosphorylation, a marker of IKKB activation, in skeletal muscle and this was prevented by SS co-infusion. However, prolonged plasma FFA elevation did not significantly increase phosphorylation of IRS-1 at serine 307. As previously mentioned, serine 307 of IRS-1 is a target of IKKB, but addition of SS did not abolish the tendency of IRS-1 serine 307 phosphorylation to increase. The improvement observed in IH-induced peripheral insulin resistance when SS was co-infused may have been due to transcriptional effects of NF- $\kappa$ B activation rather than the effect of SS on IKKB. Another possibility to explain the SS effect in muscle is a beneficial effect of adiponectin on glucose uptake. This could be mediated via AMP-activated protein kinase (AMPK; Bruce *et al.* 2005), and interestingly, salicylate has been reported to directly activate AMPK (Hawley *et al.* 2012).

Our results suggest that as the duration of FFA elevation increases, the IKKB pathway in the liver becomes inactivated. This study also suggests that normalization of Akt phosphorylation is not sufficient to normalize hepatic insulin sensitivity after prolonged plasma FFA elevation, raising the possibility that prevention of FFA effect on gluconeogenic enzyme expression may also be required, perhaps by inhibiting the PKC- $\delta$ /p38 MAPK axis. We conclude that hepatic insulin resistance caused by prolonged FFA elevation is mediated by factors that do not

target serine 307 of IRS-1 and remain to be identified, but PKC- $\delta$  is a plausible candidate.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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