FFA-induced hepatic insulin resistance in vivo is mediated by PKCδ, NADPH oxidase, and oxidative stress

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Free fatty acids; insulin resistance; hyperinsulinemic-euglycemic clamp; antioxidant; antisense oligonucleotides

Free fatty acids (FFA), which are often elevated in obese individuals (3), play a causal role, together with adipokines, in the association between obesity and insulin resistance (34). Although the mechanisms of FFA-induced insulin resistance in muscle have been partially clarified, less is known about FFA-induced hepatic insulin resistance despite its key role in the pathogenesis of type 2 diabetes mellitus (T2DM). We (32) have previously demonstrated that FFA induce hepatic insulin resistance in parallel with PKCδ membrane translocation, which indicates its activation, and this link has been confirmed by others (7). PKC impairs tyrosine phosphorylation of IRS by phosphorylating its serine/threonine residues (28), at least in part via IKKβ and JNK (20). PKC is also a known activator of NADPH oxidase (53), which increases reactive oxygen species (ROS). ROS, in turn, can activate PKC (52) as well as IKKβ (27) and JNK (18). FFA-induced hepatic insulin resistance and PKCδ membrane translocation are associated with IKKβ activation (7), and we have found that FFA-induced hepatic insulin resistance is prevented by high-dose salicylate, an IKKβ inhibitor (44). JNK is upregulated in various animal models of obesity and insulin resistance (25) and, like IKKβ (19), is capable of inhibiting insulin signaling via serine phosphorylation of IRS (1).

Oxidative stress has been linked to multiple forms of insulin resistance (26). We (24) have demonstrated that the antioxidant N-acetyl-l-cysteine (NAC) or taurine prevents hyperglycemia-induced insulin resistance in rats. We have also shown that oral taurine partially prevents FFA-induced whole body insulin resistance in humans (56), and other authors obtained similar results using intravenous infusion of glutathione (43). However, whether oxidative stress is a causal factor in FFA-induced hepatic insulin resistance in vivo remains to be determined, and the signaling mechanisms upstream and downstream of oxidative stress have not yet been explored.

The objectives of the present study were to determine whether NAC prevents FFA-induced hepatic and peripheral insulin resistance (26). We (24) have demonstrated that the antioxidant N-acetyl-l-cysteine (NAC) or taurine prevents hyperglycemia-induced insulin resistance in rats. We have also shown that oral taurine partially prevents FFA-induced whole body insulin resistance in humans (56), and other authors obtained similar results using intravenous infusion of glutathione (43). However, whether oxidative stress is a causal factor in FFA-induced hepatic insulin resistance in vivo remains to be determined, and the signaling mechanisms upstream and downstream of oxidative stress have not yet been explored.

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insulin resistance; 2) FFA-induced hepatic insulin resistance is associated with PKCδ activation, oxidative stress, IKKβ/JNK activation, and serine phosphorylation of IRS-1 and -2; and 3) NAC prevents the FFA-induced changes in any of these parameters. To this end, rats were infused intravenously with saline (SAL), Intralipid plus heparin (IH) to elevate plasma FFA levels, IH along with NAC, or NAC alone for 7 h. During the last 2 h of the infusion, glucose tracer was infused to measure glucose production and utilization in the basal state or during hyperinsulinemic-euglycemic clamp (Fig. 1). In addition, to determine whether PKCδ activation contributes to FFA-induced hepatic insulin resistance and whether this involves NADPH oxidase activation, SAL/IH infusion was carried out with or without treatment with antisense oligonucleotide specific for PKCδ or a pharmacological inhibitor of NADPH oxidase. At the end of each experiment, liver tissue was collected for determination of signaling pathways potentially involved in FFA-induced hepatic insulin resistance in vivo.

MATERIALS AND METHODS

Animals and Surgery

The animal care committee of the University of Toronto approved all procedures. Female Wistar rats weighing 250–300 g were obtained from Charles River (Saint-Constant, QC, Canada) and fed standard rat chow (Purina 5001;Ralston Purina, St. Louis, MO) for the IH-NAC study and Teklad Global #2018 (Harland-Teklad Global Diets, Madison, WI) for the other studies until experiments, which occurred at least 3 days before experiments.

Experimental Design

NAC study. Rats were fasted overnight and randomly divided into either a basal protocol or a clamp protocol, which were performed in the conscious rat (Fig. 1). For each protocol, rats were subjected to 7-h intravenous infusion of 1) SAL control (5.5 μl/min); 2) IH (20% Intralipid + 20 U/ml heparin, 5.5 μl/min); 3) IH + NAC (Sigma-Aldrich, St. Louis, MO; 0.35 mg·kg⁻¹·min⁻¹); or 4) NAC alone. We (32) have previously shown that infusion of glycerol matching the plasma glycerol levels achieved with IH does not affect endogenous glucose production (EGP) or glucose utilization (GU) when compared with saline infusion.

For the basal protocol, during the last 2 h of the experiment, [6-3H]glucose (740 KBq bolus + 14.8 KBq/min infusion) was given to assess basal glucose production. The clamp protocol involved the same infusion groups (n = 6–9/group) as the basal protocol, with the additional infusion of insulin (5 mU·kg⁻¹·min⁻¹) during the last 2 h of the infusion period. Since we have previously reported that IH decreases insulin clearance in this model (32), in addition to the 5 mU·kg⁻¹·min⁻¹ insulin infusion group, we studied another IH group where we experimentally matched the insulin levels by lowering the insulin infusion rate to 2.5 mU·kg⁻¹·min⁻¹. In the IH + NAC and NAC-alone groups, we used the full dose of insulin. The details of the clamp and tissue collection have been reported previously (32). Tissue determinations were performed on the basal experiments, unless specified.

For measurement of insulin-stimulated tyrosine phosphorylation of IRS-1 and -2, and Ser⁴⁷³ phosphorylation of Akt in the liver, at the end of the basal experiment, under anesthesia, the rats received an insulin (Humulin R, Lilly; 2 U/kg over 1 min) or saline bolus through the portal vein.

To control for the caloric load of IH, we also performed a set of experiments where we elevated the FFA by direct oleate infusion (1.37 μmol/min) prepared in FFA-free bovine serum albumin (BSA) (5, 39, 42) with or without NAC. Oleate infusion was given for 7 h, and we performed a 2-h hyperinsulinemic clamp (5 mU·kg⁻¹·min⁻¹) in the last 2 h. The clamp protocol was the same as in our previous study with salicylate (44); i.e., basal and clamp EGP were assessed with [3-³H]glucose tracer at the same concentration. Similarly to our previous study (44), we did not perform a half-dose insulin infusion experiment, as the plasma insulin levels were matched with full-dose insulin infusion in all groups.

PKCδ antisense oligonucleotide study. Lead PKCδ antisense oligonucleotide (ASO) was selected based on dose-response characterization using several different types of PKCδ ASO provided by ISIS Pharmaceuticals (Carlsbad, CA). The control (CON) ASO contained a scrambled sequence and is not complementary to any known gene in public databases. The first five bases and last five bases of chimeric ASOs have a 2’-O-(2-methoxy)ethyl modification, and the ASOs also have a phosphorothioate backbone. This chimeric design has been shown to provide both increased nuclease resistance and mRNA affinity while maintaining the robust RNase H terminating mechanism used by these types of ASOs (22). Rats were injected with PKCδ ASO

![Fig. 1. Schema illustrating the protocols used in the main study (NAC study). SAL, saline; IH, Intralipid + heparin. NAC, N-acetyl-L-cysteine.](http://ajpendo.physiology.org/ by 10.220.33.1 on June 26, 2017)
Oxidative stress and fat-induced hepatic insulin resistance

For measurement of phosphorylated insulin receptor and carcino-embryonic antigen-related cell adhesion molecule 1 (CEACAM1), the liver samples were collected at the end of the clamp of the IH/NAC study (where changes in insulin clearance were observed). Livers of the full-dose IH experiments were used. Proteins (100 μg per bicinchoninic acid method, Pierce), were analyzed by 8% SDS-PAGE and sequential immunoblotting with polyclonal antibodies against phosphorylated CEACAM1 (Bethyl Laboratories) and rat CEACAM1 (α-Y513), raised against C-SSPTETVY113SVKKK peptide in the cytoplasmic tail, to assess the amount of the rat CEACAM1 protein. Other aliquots were analyzed by sequential immunoprobeing with a polyclonal antibody against insulin receptor β-subunit (C19, Santa Cruz Biotechnology), followed by a horseradish peroxidase-conjugated phosphotyrosine monoclonal antibody (4G10, Upstate), to assess insulin receptor phosphorylation. Reprobing with a monoclonal antibody against actin (Santa Cruz Biotechnology) was performed to normalize for the amount of proteins loaded.

**Immunoprecipitation**

The same procedure was used as in our previous study (44).

**Carbonyl Protein Assay**

Liver tissue was homogenized at 4°C in a solution containing HEPES (10 mM, pH 7.4), NaCl (137 mM), KCl (4.6 mM), KH₂PO₄ (1.1 mM), MgSO₄ (0.6 mM), EDTA (1.1 mM), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), PMSF (40 μg/ml), and aprotinin (0.5 μg/ml) to solubilize protein, and cell debris was removed by centrifugation. Protein concentrations were determined as described previously (32). Oxidative protein damage, assessed by the formation of carbonyl groups, was measured as described by Levine et al. (33). To remove nucleic acids, samples were incubated with 10% streptomycin sulfate until a final concentration of 1% streptomycin sulfate was reached. Carboxyl content was calculated from the absorbance of samples at 365 nm compared with their complementary HCl-treated blanks, using a molar absorption coefficient of 22,000 M⁻¹ cm⁻¹.

**mRNA for Gluconeogenic Enzymes**

Total RNA was extracted from rat livers with the RNeasy Mini Kit from Qiagen (Valencia, CA) according to the manufacturer’s instructions. Liver samples (20 mg) were disrupted and homogenized using Buffer RLT (β-mercaptoethanol added). The RNA was quantified spectrophotometrically and then stored at −80°C. cDNAs were generated by reverse transcriptase reactions using TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR analysis was

**Liver Fractionation and Western Blot Analysis**

Liver samples were separated into cytosolic and membrane fractions, and Western blot was performed as previously described (32). Antibody for PKCβ [Sigma for the NAC study and Santa Cruz Biotechnology (Santa Cruz, CA) for the PKCβ ASO study] was used at a concentration of 1:2,000. Tyr⁸¹⁷-phosphorylated PKCβ and Ser⁵⁴⁵-phosphorylated p47phox were measured in the membrane fraction with antibodies from Biosource (Life Technologies, Carlsbad, CA; 1:2,500) and from the laboratory of Dr. J. El-Benna (1:2,500) (12), respectively. Hepatic content of IκBα, and serine-phosphorylated and total IRS-1 and -2 were determined in the cytosolic fraction as described previously (44). Cytosolic Thr³⁸⁸/Tyr¹⁴⁵-phosphorylated JNK (1:250) and total JNK (1:500) were assessed with antibodies from Santa Cruz Biotechnology. The antibody for actin was from Santa Cruz Biotechnology.

**Table 1. Plasma levels of metabolic parameters and GIR in the basal state and during 2-h hyperinsulinemic-euglycemic clamp**

<table>
<thead>
<tr>
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<th>Basal</th>
<th>Clompa</th>
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<tr>
<td></td>
<td>SAL</td>
<td>IH</td>
</tr>
<tr>
<td></td>
<td>SAL</td>
<td>IH</td>
</tr>
<tr>
<td>FFA, mM</td>
<td>0.48 ± 0.04</td>
<td>1.63 ± 0.23*</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.7 ± 0.3</td>
<td>7.3 ± 0.28</td>
</tr>
<tr>
<td>Insulin, pm</td>
<td>89 ± 8</td>
<td>147 ± 16*</td>
</tr>
<tr>
<td>C-peptide, nM</td>
<td>0.28 ± 0.05</td>
<td>0.85 ± 0.18*</td>
</tr>
<tr>
<td>GIR, μmol·kg⁻¹·min⁻¹</td>
<td>0.03 ± 0.03</td>
<td>0.07 ± 0.02</td>
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</table>

Data are means ± SE (n = 6–9/group) and refer to the last 30 min of each experiment. SAL, saline; IH, Intralipid + heparin; NAC, N-acetyl-L-cysteine; FFA, free fatty acids; GIR, glucose infusion rate; *Insulin infusion rate 5 μU·kg⁻¹·min⁻¹ in SAL, IH, IH+NAC, NAC. Insulin infusion rate 2.5 μU·kg⁻¹·min⁻¹ in IH (half insulin) to match clamp insulin levels. †P < 0.01 and ‡P < 0.05 vs. SAL; #P < 0.05 and &&P < 0.001 vs. SAL and NAC; **P < 0.01 vs. other treatment groups; ††P < 0.01 and ‡‡P < 0.001 vs. SAL, IH+NAC and NAC.
Glucose turnover was calculated using Steele’s equation, taking into account the extra tracer infused with the glucose infusate (15). Data are presented as averages during the last 30 min of experiments.

Statistics

One-way ANOVA for repeated measures followed by Tukey’s post hoc test was used to compare differences between treatments in both the basal and clamp groups. Two-way ANOVA with interaction was used to compare differences induced by treatment between basal and clamp. In the oleate, PKCδ ASO, and apocynin studies, in which basal and clamp evaluations of glucose turnover were performed in the same rat, percentage suppression of EGP from basal was calculated. Statistics were performed using SAS (Statistical Analysis System, Cary, NC).

RESULTS

NAC Prevents IH-Induced Hepatic and Peripheral Insulin Resistance

IH infusion for 7 h increased plasma FFA approximately three- to fourfold, and FFA were lower during the hyperinsulinemic clamp than during the basal state (Table 1). Coinfusion of the antioxidant NAC did not affect plasma FFA. Basal plasma glucose was higher in IH than in the other groups, but during the hyperinsulinemic clamp there was no significant difference in plasma glucose between groups. IH increased basal plasma insulin and C-peptide, suggesting that FFA inhibited insulin secretion; this was not prevented by NAC coinfusion. During the clamp, plasma C-peptide levels were lower than those in the basal state in all groups, indicating that insulin secretion was almost completely suppressed by insulin. During the clamp, the IH group receiving 5 mU·kg⁻¹·min⁻¹ had higher insulin levels (Table 1), indicating a decrease in insulin clearance, as previously observed (32). Clamp insulin levels were matched in the SAL and IH groups by infusing insulin at higher insulin levels (Table 1), indicating a decrease in insulin clearance, as previously observed (32). Clamp insulin levels were matched in the SAL and IH groups by infusing insulin at higher insulin levels (Table 1), indicating a decrease in insulin clearance. 

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IH decreased EGP both in the basal state and during the clamp (Fig. 2A). During the clamp with both insulin doses, IH infusion also decreased GU (Fig. 2B), which was prevented by coinfusing NAC. NAC alone had no effect on EGP or GU. These data show that NAC prevents FFA-induced hepatic and peripheral insulin resistance.

To control for the caloric load of IH, we also performed experiments where we elevated FFA by direct infusion of oleate with or without NAC. FFA were elevated to ~1 mM, which was less than in the IH + NAC study, and the plasma insulin concentrations were matched with the same rate of insulin infusion in all groups (Fig. 3). Basal EGP was also not significantly elevated by oleate (Table 3). Similar to IH, oleate induced hepatic and peripheral insulin resistance, and both were prevented by NAC (Fig. 3 and Table 3).

**PKCδ Plays a Causal Role in IH-Induced Hepatic Insulin Resistance, Likely Downstream of Oxidative Stress**

To confirm that IH infusion induced oxidative stress specifically in the liver and that this was prevented by NAC, hepatic protein carbonyl content was assessed as a marker of oxidative stress (51). IH elevated hepatic protein carbonyl content two-fold, and coinfusion of NAC completely prevented this effect (Fig. 4A). NAC alone had no effect.

IH infusion decreased cytosolic PKCδ and increased membrane PKCδ content (Fig. 4B) in the liver, indicating PKCδ membrane translocation and, therefore, PKCδ activation. However, NAC did not prevent IH-induced PKCδ membrane translocation, indicating that oxidative stress was likely not involved in this process. PKCδ activation in response to oxidative stress is associated with Tyr311 phosphorylation (30); however, we found no change in PKCδ Tyr311 phosphorylation in response to IH or NAC infusion (SAL = 100 ± 10.4 vs. IH = 95.7 ± 9.7 vs. IH + NAC = 97.0 ± 12.1 vs. NAC = 108.5 ± 17.1%, means ± SE). Together, these findings argue against the involvement of oxidative stress in IH-induced PKCδ activation.

Other than oxidative stress, lipid metabolites such as LCFA-CoA and DAG can activate PKC during lipid infusion. Under our experimental conditions, IH did not significantly increase the hepatic content of ceramide or LCFA-CoA (ceramide: SAL = 14 ± 1.1 vs. IH = 15.4 ± 2.0 pmol/nmol total phospholipids; LCFA-CoA: SAL = 31 ± 5.0 vs. IH = 27 ± 3.0 pmol/mg tissue; means ± SE). In contrast, the hepatic levels of DAG and TG were increased by IH and were not normalized by NAC (Table 2).

### Table 3. Plasma levels of metabolic parameters, GIR, EGP, and GU in the basal period and during 2-h hyperinsulinemic-euglycemic clamp

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BSA</th>
<th>OLE</th>
<th>OLE+NAC</th>
<th>Clamp</th>
<th>BSA</th>
<th>OLE</th>
<th>OLE+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mM</td>
<td>6.9 ± 0.3</td>
<td>6.6 ± 0.5</td>
<td>6.5 ± 0.2</td>
<td>6.6 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>6.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>GIR, μmol·kg⁻¹·min⁻¹</td>
<td>46.7 ± 2.4</td>
<td>50.0 ± 3.2</td>
<td>57.3 ± 2.8</td>
<td>137 ± 7</td>
<td>101 ± 8*</td>
<td>138 ± 5</td>
<td></td>
</tr>
<tr>
<td>EGP, μmol·kg⁻¹·min⁻¹</td>
<td>47.7 ± 2.5</td>
<td>52.0 ± 3.5</td>
<td>57.6 ± 2.7</td>
<td>36.7 ± 2.8</td>
<td>54.4 ± 5.1*</td>
<td>45.0 ± 4.7</td>
<td></td>
</tr>
</tbody>
</table>
| GU, μmol·kg⁻¹·min⁻¹ | 174.7 ± 7.8 | 154.8 ± 10.6* | 181.5 ± 3.8 | *P < 0.05 vs. other treatment groups.

Data are means ± SE (n = 5–6/group) and for the basal period refer to the 30 min before the clamp, while for the clamp, data refer to the last 30 min of each experiment. BSA, bovine serum albumin (vehicle control for oleate); OLE, oleate; EGP, endogenous glucose production; GU, glucose utilization.

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**Figure 3:** Effects of oleate and NAC on plasma insulin levels and suppression of EGP during the clamp (n = 5–6/group). A: plasma insulin levels were matched with full insulin dose insulin infusion in all groups; therefore, a half-dose insulin infusion was not performed. B: oleate infusion decreased insulin-induced suppression of EGP, which was prevented by NAC coinfusion. Data are means ± SE. BSA, bovine serum albumin (vehicle for oleate); OLE, oleate. Basal, 30 min before 2-h hyperinsulinemic euglycemic clamp; Clamp, last 30 min of 2-h hyperinsulinemic euglycemic clamp.

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**Figure 4:** Effect of coinfusion of NAC on hepatic protein carbonyl content and PKCδ membrane translocation. Error bars indicate ± SE. BSA, bovine serum albumin (vehicle control for oleate); OLE, oleate; OLE+NAC, oleate+NAC; IH, Insulin; NAC, N-acetyl-L-cysteine; *P < 0.05 vs. other treatment groups.
PKCδ levels in the liver (Fig. 5A). Hepatic PKCe protein expression was not affected by PKCδ ASO (Fig. 5B).

PKCδ membrane-to-cytosolic ratio in the CON ASO + SAL group was less than that of the SAL ASO group in the NAC study (Fig. 4); thus, we cannot exclude a nonspecific effect of CON ASO. Nevertheless, only PKCδ ASO, and not CON ASO, prevented IH-induced PKCδ membrane translocation. This was indicated by the increase in membrane PKCδ in response to IH in the rats that received CON ASO, whereas in the rats treated with PKCδ ASO we observed no increase in membrane PKCδ (Fig. 5A). PKCe did not translocate from the cytosol to the membrane in response to IH in either group (Fig. 5B).

IH-induced hepatic insulin resistance was prevented by PKCδ ASO (Fig. 5C). Peripheral insulin sensitivity was not affected by PKCδ ASO, as expected, likely due to preferential uptake of ASO by the liver (49), as shown by similar GU during the clamp in both IH-infused CON ASO and PKCδ ASO groups (CON ASO: SAL = 184.3 ± 5.2, IH = 118.1 ± 19.1; PKCδ ASO: SAL = 181.6 ± 6.1, IH = 109.4 ± 26.3 μmol·kg⁻¹·min⁻¹, means ± SE; P < 0.01 IH vs. SAL in both CON ASO and PKCδ ASO groups).

PKCδ ASO also prevented the IH-induced increase in hepatic membrane Ser³⁴⁵-phosphorylated p47phox (Fig. 5D), which is a marker of NADPH oxidase activation (12). Furthermore, apocynin (APO), an inhibitor of NADPH oxidase, prevented IH-induced impairment of suppression of EGP (Fig. 5E). APO also prevented IH from decreasing peripheral GU (GU during the clamp: SAL = 184.6 ± 14.0, IH = 125.2 ± 7.1, IH + APO = 172.4 ± 9.8, APO = 189.5 ± 4.8 μmol·kg⁻¹·min⁻¹, means ± SE; P < 0.01 for both SAL vs. IH and IH vs. IH + APO). This suggests that NADPH oxidase-derived superoxide plays a causal role in hepatic and peripheral insulin resistance.

Interestingly, and for unclear reasons that may relate to lower FFA-releasing property of the new heparins, in both the PKCδ ASO and the apocynin studies that were performed after the NAC study, FFA levels achieved with IH (~1.2 mM) were lower than in the NAC study and comparable to those achieved with olate. Similar to the results obtained in the oleate study, insulin levels were not significantly different, with the same rate of insulin infusion in all groups, and basal EGP was not significantly elevated by IH.

NAC Prevents IH-Induced Impairments of Hepatic Insulin Sensitivity and Insulin Clearance

IH significantly decreased IkBα content, which reflects phosphorylation and degradation of IkBα by activated IKKβ (7). The IH-induced decrease in hepatic IkBα content was prevented by NAC (Fig. 6A). Additionally, IH increased JNK phosphorylation (a marker of its activation), which was also prevented by NAC (Fig. 6B).

IKKβ and/or JNK activation cause insulin resistance via serine phosphorylation of IRS-1 and IRS-2 (1, 19). Phosphorylation of Ser³⁴⁷ and Ser³⁷³ on IRS-1 and IRS-2, respectively, was increased by IH and this was prevented by coinfusion with NAC (Fig. 6, C and D). There was no change in total protein content of hepatic IRS-1 or IRS-2 in this model. Furthermore, NAC coinfusion protected against the decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 (Fig. 7, A and B), as well as Ser⁴⁷³ phosphorylation of IRS-2.

**Fig. 4. Effects of IH and NAC on hepatic protein carbonyl content and hepatic PKCδ (n = 6–9/group). A: NAC coinfusion prevented IH-induced increase in hepatic protein carbonyl levels. NAC alone did not have any effect. B: Both IH and IH plus NAC treatments decreased cytosolic and increased membrane levels of PKCδ protein. IH infusion increased the membrane to cytosolic ratio of hepatic PKCδ, indicating increased membrane translocation. IH-induced PKCδ membrane translocation was not affected by NAC coinfusion. Liver samples assayed were collected at the end of the basal infusions. Data are mean ± SE. C: Cytosol; M: Membrane. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *P < 0.01 vs. other treatment groups. &P < 0.05 Membrane vs. Cytosol. #P < 0.05 vs. SAL and NAC. **P < 0.01 vs. SAL and NAC.**
phosphorylation of Akt (Fig. 7C), in the liver of rats undergoing IH infusion.

Although tyrosine phosphorylation of IRS is critical for insulin action in peripheral tissues and in the liver, it is not directly involved in hepatic insulin clearance, whereas tyrosine phosphorylation of insulin receptor is the first committed step in both hepatic insulin action and clearance. Insulin receptor phosphorylation mediates internalization of the insulin-insulin receptor complex. It also causes the phosphorylation of CEACAM1, which contributes to insulin-insulin receptor endocytosis to increase the cellular transport and clearance of insulin (11, 45). Since in the NAC study IH decreased insulin clearance in accord with our previous studies in rats (32), dogs (54), and humans (9), we measured tyrosine phosphorylation of insulin receptor and CEACAM1 at the end of the clamp in the SAL, IH (full-insulin dose), and IH + NAC liver samples. As Fig. 8 illustrates, IH infusion decreased tyrosine phosphorylation of both insulin receptor and CEACAM1. This effect was partially prevented by NAC coinfusion. IH also decreased CEACAM1 levels, and this effect was completely prevented by NAC.

**DISCUSSION**

We herein demonstrate that NAC and the NADPH oxidase inhibitor prevent FFA-induced hepatic and peripheral insulin resistance and that antisense oligonucleotide against PKCδ prevents FFA-induced hepatic insulin resistance. From liver tissue analysis, a pathway that emerged sequentially involves FFA, PKCδ, NADPH oxidase and oxidative stress, and IKKβ/JNK, leading to impaired hepatic insulin signaling.

**Oxidative Stress Is a Causal Factor in FFA-Induced Insulin Resistance**

During the basal state, IH infusion increased EGP despite increased plasma insulin and glucose. Studies in humans (48) and in 5-h-fasted rats (31) show that IH infusion increases gluconeogenesis but does not affect EGP under basal conditions due to an autoregulatory decrease in glycogenolysis. However, hepatic autoregulation was likely abolished during IH infusion due to the marked extent of plasma FFA elevation. Plasma FFA elevation was less with oleate than with IH infusion, and with oleate peripheral and hepatic insulin resis-
Fig. 6. Effects of IH and NAC on IκBα content, JNK phosphorylation, and serine phosphorylation of IRS in liver tissue (n = 7–10/group). A: IH infusion decreased hepatic IκBα levels, which were restored to control levels by NAC coinfusion. B: IH infusion increased Thr183/Tyr185 phosphorylation of JNK, which was prevented by NAC coinfusion. C: IH infusion increased Ser307-phosphorylated IRS-1, which was prevented by NAC coinfusion. Total IRS-1 was not different among treatments. D: IH infusion increased Ser233-phosphorylated IRS-2, which was prevented by NAC coinfusion. Total IRS-2 was not different among treatments. Liver samples assayed were collected at the end of the basal infusions. Data are means ± SE. *P < 0.05 vs. other treatment groups; #P < 0.01 vs. other treatment groups.
Insulin (2 U/kg in all groups) under anesthesia after the infusion period. Data are means ± SE. IB, immunoblot; IP, immunoprecipitation; p-Tyr, phosphotyrosine.

Irrespective of the differences in basal EGP and insulin clearance, direct infusion of oleate induced hepatic and peripheral insulin resistance as IH did, and NAC, added to either IH or oleate, prevented hepatic and peripheral insulin resistance. The observed insulin resistance with oleate is presumably attributable to FFA itself rather than to the caloric load, as the caloric load of the oleate infusion is negligible.

In the present study, similarly to NAC, the NADPH oxidase inhibitor APO also prevented IH-induced peripheral and hepatic insulin resistance. Our findings are in accord with data in HepG2 cells, which showed that NADPH oxidase-derived ROS were associated with palmitate-induced insulin resistance (17). However, a role for mitochondrial ROS has also been postulated based on in vitro data (41).

The relationship between oxidative stress and insulin sensitivity is complex. First, solid in vitro and animal studies have linked oxidative stress with insulin resistance of obesity and diabetes (2, 26), and a few studies have demonstrated that antioxidants improve insulin action in humans (8, 47), but human studies are characterized by heterogeneous populations (16) and different routes of antioxidant administration. Intravenous glutathione partially prevented IH-induced insulin resistance in humans, although tracer studies were not performed to separately assess hepatic and peripheral insulin sensitivity (43). We have also shown that oral taurine, but not NAC, which has extensive first-pass hepatic metabolism, partially prevents IH-induced whole body insulin resistance in humans (56). However, oral NAC improved insulin clearance, suggesting that it was likely effective in the liver. Although it has been reported that the improvement in whole body insulin sensitivity induced by intravenous reduced glutathione is accompanied by an improvement in circulating markers of oxidative stress in patients with T2DM (13), others found that oral vitamin C does not ameliorate whole body insulin sensitivity in T2DM, possibly because plasma levels of vitamin C were not normalized by the treatment (10). Likewise, oral vitamin E has failed to prevent T2DM (35). Intravenous NAC prevented insulin resistance induced by lipid infusion in rat skeletal muscle (4), but hepatic insulin resistance was not assessed. Second, although Ristow et al. (46) found that in healthy individuals vitamins C plus E block exercise-stimulated elevation in whole body insulin sensitivity, it was subsequently reported that this antioxidant combination does not impair the ability of exercise to augment insulin sensitivity (57). Third, ROS have been found to promote insulin action, but this effect is localized to the periphery and not the liver (36). It has been suggested that the
nature of the relationship between oxidative stress and insulin sensitivity rests on the duration as well as the magnitude of oxidative stress (36). Herein, we show that in the liver elevations in markers of oxidative stress are accompanied by impaired insulin signaling and not improved insulin action. Therefore, we propose that this magnitude and duration of FFA-induced oxidative stress causes hepatic insulin resistance.

PKCδ Is a Link Between FFA and Oxidative Stress in Hepatic Insulin Resistance via NADPH Oxidase

PKCδ activation can induce hepatic insulin resistance (6); however, whether PKCδ mediates hepatic insulin resistance induced selectively by fat has not been explored. PKCδ may directly phosphorylate IRS-1 on serine/threonine residues, thereby diminishing its tyrosine phosphorylation (23, 28), although it has not been reported to directly phosphorylate IRS-1 on Ser307 (equivalent to Ser312 in humans). Alternatively, PKCδ may activate other serine/threonine kinases that phosphorylate Ser307, such as IKKβ and JNK (20), either directly (21, 37) or indirectly via oxidative stress (18, 27). In the present study, NAC, which prevented IH-induced increase in protein carbonyl content, a marker of oxidative stress, completely abolished IH-induced hepatic insulin resistance without affecting PKCδ membrane translocation. This suggests that PKCδ translocation occurs upstream of or is unrelated to oxidative stress. Our findings that IH did not increase Tyr311-phosphorylated PKCδ, which may be increased by ROS (30), suggest that PKCδ activation does not occur downstream of oxidative stress in our model. Irrespective of how activated, PKCδ is causal in IH-induced hepatic insulin resistance, as indicated by our finding that this was abolished by PKCδ ASO. In contrast, PKCε, which mediated hepatic insulin resistance caused by

Fig. 8. Effect of IH and NAC on signaling molecules involved in hepatic insulin clearance (n = 4–5/group).
A: IH infusion decreased tyrosine phosphorylation of insulin receptor (IR), which was partially prevented by NAC coinfusion. B: IH infusion markedly decreased phosphorylated carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1, which was partially prevented by NAC coinfusion. IH infusion also decreased hepatic CEACAM1 content, which was totally prevented by NAC coinfusion. Samples were collected at the end of the hyperinsulinemic clamp (insulin infusion rate 5 mU·kg⁻¹·min⁻¹ in all groups). Data are means ± SE. ReIB, reprobed immunoblot; α, anti. *P < 0.05 vs. SAL, **P < 0.01 vs. SAL, ***P < 0.001 vs. SAL; #P < 0.05 vs. IH; &P < 0.01 vs. IH.
3 days of high-fat diet (49), was not translocated by 7-h lipid infusion.

Accumulation of intracellular lipid metabolites, such as DAG and LCFA-CoA, is associated with PKC activation (7, 58). However, hepatic content of LCFA-CoA or ceramide was not significantly increased by IH in our model. In accord with other reports (7), DAG and TG contents were increased by IH, albeit modestly, and, as expected, the increase was not prevented by NAC. It is possible that these metabolites were elevated to a greater extent before the 7-h point, as it has been shown that intracellular fat levels peak earlier, at least in skeletal muscle (58). It is also possible that PKC activation occurs mainly via Toll-like receptors (TLR) and only partly via intracellular lipid metabolites. In this respect, it has been demonstrated that FFA activate IKKβ and cause insulin resistance in vivo via TLR4 (50), which can activate PKCδ (29).

PKC activation can increase ROS via PKC-dependent activation of NADPH oxidase (53). IH increased the membrane content of Ser435-phosphorylated NADPH oxidase subunit p47phox, a marker of NADPH oxidase activation (12), which was abolished by PKCδ ASO. Our finding that apocynin, which inhibits NADPH oxidase, prevents IH-induced hepatic and peripheral insulin resistance suggests a causal role of NADPH oxidase-derived ROS.

Proinflammatory and Stress-Activated Pathways Are Downstream Mediators of FFA-Induced Impairment of Hepatic Insulin Signaling

Potential mediators of FFA-induced hepatic insulin resistance downstream of oxidative stress include IKKβ and JNK. Consistent with previous reports that ROS activates IKKβ (27), NAC abolished the IH-induced decrease in hepatic IκBα content, suggesting that IKKβ is implicated in the pathway mediating FFA-induced hepatic insulin resistance downstream of oxidative stress. Similar to IKKβ, JNK phosphorylates IRS on serine residues (1). In the present study, NAC prevented IH-induced phosphorylation of JNK, suggesting that lipid infusion results in activation of JNK via oxidative stress. NAC also prevented the IH-induced increase in serine phosphorylation of IRS-1 and IRS-2, the decrease in their tyrosine phosphorylation, and the IH-induced decrease in serine phosphorylation of Akt.

In addition to normalizing insulin-induced EGP suppression, NAC normalized basal EGP, which may have resulted from the fact that NAC prevented the IH-induced insulin resistance without affecting the IH-induced increase in insulin levels. The latter finding was expected, since FFA acutely increase insulin secretion independently of oxidative stress. Unexpectedly, however, NAC also appeared to abolish the FFA-induced decrease in insulin clearance. Further studies are required to determine the exact mechanisms for this effect of NAC. Complete prevention of the IH-induced decrease in the hepatic content of CEACAM1, which promotes insulin removal in liver (14), and partial prevention of the IH-induced decrease in tyrosine phosphorylation of both insulin receptor and CEACAM1, could contribute to the effect of NAC. Preservation of CEACAM1 expression level by NAC may be explained by improvement of hepatic insulin action, as insulin is known to increase the promoter activity of the CEACAM1 gene (40). Partial prevention of the decrease in the tyrosine phosphorylation of the insulin receptor and CEACAM1, which is a substrate of the insulin receptor kinase, may imply that NAC improves insulin signaling at sites other than IRS, perhaps by affecting IH-induced serine phosphorylation of the insulin receptor.

In conclusion, we have demonstrated that antioxidant treatment (both NAC and apocynin) prevents FFA-induced hepatic and peripheral insulin resistance. We have also provided evidence that PKCδ knockdown prevents FFA-induced hepatic insulin resistance. Furthermore, we have shown that activation of IKKβ and JNK is downstream whereas PKCδ activation is likely upstream of oxidative stress. Hence, we propose the following sequence of events leading to FFA-induced hepatic insulin resistance: FFA, via activation of PKCδ and NADPH oxidase, induce oxidative stress, which leads to IKKβ- and JNK-mediated serine phosphorylation of IRS-1 and IRS-2, and subsequent impairment of hepatic insulin signaling.

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DISCLOSURES

S. Bhanot owns stocks/holds stock options in ISIS Pharmaceuticals.

AUTHOR CONTRIBUTIONS


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