Effect of *N*-acetyl-L-cysteine on insulin resistance caused by prolonged free fatty acid elevation

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Abstract

Circulating free fatty acids (FFAs) are elevated in obesity and cause insulin resistance. The objective of the current study was to determine whether the antioxidant *N*-acetyl-L-cysteine (NAC) prevented hepatic and peripheral insulin resistance caused by prolonged elevation of plasma FFAs. Chronically cannulated Wistar rats received saline (SAL), Intralipid plus heparin (IH), IH plus NAC, or NAC i.v. infusion for 48 h. Insulin sensitivity was determined using the hyperinsulinemic–euglycemic clamp with tritiated glucose tracer. IH induced hepatic and peripheral insulin resistance ($P < 0.05$). NAC co-infusion did not prevent insulin resistance in the liver, although it was able to prevent peripheral insulin resistance. Prolonged IH infusion did not appear to induce oxidative stress in the liver because hepatic content of protein carbonyl, malondialdehyde, and reduced to oxidized glutathione ratio did not differ across treatment groups. In alignment with our insulin sensitivity results, IH augmented skeletal muscle protein carbonyl content and this was prevented by NAC co-infusion. Taken together, our results indicate that oxidative stress mediates peripheral, but not hepatic, insulin resistance resulting from prolonged plasma FFA elevation. Thus, in states of chronic plasma FFA elevation, such as obesity, antioxidants may protect against peripheral but not hepatic insulin resistance.

Key Words

- ▶ free fatty acids
- ▶ insulin resistance
- ▶ *N*-acetyl-L-cysteine
- ▶ liver

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Introduction

Obesity is associated with elevated circulating free fatty acids (FFAs) and FFAs cause insulin resistance (Lewis *et al.* 2002, Xiao *et al.* 2008). FFAs induce oxidative stress (Nakamura *et al.* 2009, Yuzefovych *et al.* 2010, Gurzov *et al.* 2014), which occurs when the rate of reactive oxygen species (ROS) production is greater than their removal (Evans *et al.* 2002), and ROS cause insulin resistance (Hansen *et al.* 1999).

High-fat diet feeding in rodents is often characterized by oxidative stress at the tissue level. In the liver, it has been reported that the levels of malondialdehyde (MDA), a marker of lipid peroxidation, and protein carbonyl content are elevated (Carmiel-Haggai *et al.* 2005, Satapati *et al.* 2012). Furthermore, the levels of the antioxidant glutathione (GSH) are reduced and antioxidant enzyme

activity are diminished, while the activity of some enzymes that produce ROS, such as NADPH oxidase, is augmented (Carmiel-Haggai *et al.* 2005). In skeletal muscle, markers of oxidative stress have also been reported to be increased by high-fat diet (Anderson *et al.* 2009, Yokota *et al.* 2009).

In high-fat diet, however, the relative contribution of FFAs vs other factors, such as cytokines, to oxidative stress cannot be distinguished. A reductionist approach to the study of the effects of FFAs on insulin sensitivity is to intravenously infuse lipid emulsions, such as Intralipid, with heparin (IH) to stimulate lipoprotein lipase so that plasma FFAs are elevated. Wang *et al.* (2009) have first shown that the antioxidant *N*-acetyl-L-cysteine (NAC) improves the whole-body insulin resistance and the reduction of circulating GSH caused by short-term (4 h) lipid infusion. We have recently shown that NAC prevents whole-body, hepatic and peripheral insulin resistance caused by short-term (7 h) IH infusion (Pereira *et al.* 2014). The normalization of hepatic insulin sensitivity by NAC co-infusion was accompanied by prevention of IH-induced increase in hepatic protein carbonyl content. Interestingly, we have found that the mechanisms of FFA-induced hepatic insulin resistance differ depending on the duration of plasma FFA elevation. Salicylate, an inhibitor of I κ B α kinase β (IKK β), prevents hepatic insulin resistance following short-term, but not prolonged (48 h), IH infusion (Park *et al.* 2007, Pereira *et al.* 2013). The prolonged lipid infusion model better represents the sustained increased levels of circulating FFAs associated with obesity than does short-term lipid infusion. In the current study, our objective was to determine whether the insulin resistance caused by prolonged plasma FFA elevation was accompanied by oxidative stress in the liver as well as skeletal muscle and whether NAC was effective at preventing the FFA-induced insulin resistance.

Materials and methods

Experimental design

Female Wistar rats (250–300 g; Charles River, Saint-Constant, QC, Canada) were kept in the Department of Comparative Medicine, University of Toronto (Pereira *et al.* 2013). Standard chow (Teklad Global Diet #2018; Harland Laboratories, Indianapolis, IN, USA) and water were available *ad libitum*. The Animal Care Committee of the University of Toronto approved all experimental procedures. After 1- to 2-week adaptation, rats were anesthetized with isoflurane and implanted with catheters

in the right jugular vein and left carotid artery (Pereira *et al.* 2013). After rats had recovered from the surgery for 3 or more days, they were put in metabolic cages and assigned to a 48-h i.v. treatment group: saline (SAL, 5.5 μ l/min), IH (20% Intralipid plus 20 U/ml heparin; 5.5 μ l/min), IH plus NAC (0.35 mg/kg per min; Sigma–Aldrich) or NAC. I.v. infusion occurred via the jugular vein while blood samples were obtained from the carotid artery. The rats were fasted overnight and at 44 h of treatment infusion, [3-³H] glucose (Perkin–Elmer, Boston, MA, USA) administration was initiated (8 μ Ci bolus and then 0.15 μ Ci/min), as previously described (Pereira *et al.* 2013). At 46-h treatment infusion, a 2-h hyperinsulinemic (5 mU/kg per min; Humulin R, Eli Lilly, Toronto, ON, Canada) euglycemic clamp begun. A detailed description of the clamp protocol that we followed has been published (Pereira *et al.* 2013). The clamp was begun immediately after a 30-min basal period and the last 30 min of the clamp was the clamp period. Blood samples were collected every 10 min during the basal and clamp periods. Following the clamp, rats were anesthetized; liver was freeze-clamped and soleus muscle was snap frozen in liquid nitrogen. The tissues were stored in a –80 °C freezer until the time of analysis.

Plasma assays

Plasma levels of FFAs, insulin and glucose were measured using a colorimetric assay (Wako Pure Chemical Industries Ltd, Osaka, Japan), RIA (Millipore, Billerica, MA, USA), and a glucose analyzer (Analox, GM9D Analyser, Analox Instruments, Hammersmith, UK) respectively (Pereira *et al.* 2013).

Plasma 8-iso-prostaglandin F2 α was measured on the samples obtained at 44-h treatment infusion using an ELISA Kit (OxiSelect 8-iso-prostaglandin F2 α ELISA kit, Cell Biolabs, Inc., San Diego, CA, USA).

Glucose kinetics

We have previously described the protocol for quantification of plasma [3-³H] glucose and determination of glucose kinetics (Pereira *et al.* 2013). Briefly, a modified Steele's equation that factors in [3-³H] glucose in the glucose infusate was used to determine endogenous glucose production (EGP) and glucose utilization (Rd) (Steele *et al.* 1956, Finegood *et al.* 1987).

Markers of oxidative stress

Protein carbonyl content Briefly, 100 mg of liver or skeletal muscle was homogenized with the following

Table 1 Plasma concentration of free fatty acids (FFA), insulin, and glucose during basal and hyperinsulinemic–euglycemic clamp periods

	Basal period				Clamp period			
	SAL	IH	IH+NAC	NAC	SAL	IH	IH+NAC	NAC
FFA ($\mu\text{Eq/l}$)	497 $\pm$ 32	862 $\pm$ 55 ^{*,†}	740 $\pm$ 60 ^{*,†}	530 $\pm$ 51	129 $\pm$ 22	556 $\pm$ 55 ^{*,†}	453 $\pm$ 50 ^{*,†}	132 $\pm$ 34
Insulin (pM)	83 $\pm$ 10	99 $\pm$ 5	82 $\pm$ 15	61 $\pm$ 12	551 $\pm$ 25	521 $\pm$ 17	585 $\pm$ 41	569 $\pm$ 40
Glucose (mM)	6.55 $\pm$ 0.18	6.18 $\pm$ 0.31	6.26 $\pm$ 0.28	6.57 $\pm$ 0.12	6.54 $\pm$ 0.14	6.04 $\pm$ 0.31	6.12 $\pm$ 0.22	6.38 $\pm$ 0.12

Treatments: SAL, saline; IH, Intralipid plus heparin; IH+NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; $n=6-10/\text{group}$. * $P<0.05$ vs SAL and $^{\dagger}P<0.05$ vs NAC.

buffer: 10 mM HEPES (pH 7.4), 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 , 0.6 mM MgSO_4 , 1.1 mM EDTA, 0.5 $\mu\text{g/ml}$ leupeptin, 0.7 $\mu\text{g/ml}$ pepstatin, 40 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride, and 0.5 $\mu\text{g/ml}$ aprotinin. Homogenates were centrifuged. The supernatant was treated with streptomycin sulfate at a final concentration of 1% in the sample to remove nucleic acids. Protein carbonyl amount per milligram protein was calculated based on protein concentration and absorbance at 365 nm (protein-hydrazone) (protein carbonyl colorimetric assay kit, Cayman Chemical Company, Ann Arbor, MI, USA).

Malondialdehyde A TBARS assay kit from Cayman Chemical Company was utilized to measure MDA in the liver.

GSH and oxidized glutathione Measurement of reduced GSH and oxidized glutathione (GSSG) in liver tissue was carried out using a microplate assay (Oxford Biomedical Research, Oxford, MI, USA) according to the specified instructions.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. and were analyzed using one-way ANOVA with *post hoc* analysis consisting of Tukey's test (IBM SPSS Statistics, Version 22, IBM Corporation, Armonk, NY, USA) $\alpha=0.05$.

Results

IH and IH+NAC treatment groups showed increased plasma FFAs compared with SAL and NAC treatment groups, at baseline and during the hyperinsulinemic–euglycemic clamp, as expected (Table 1, $P<0.05$). Plasma insulin concentrations were similar between groups during the basal period, increased as a result of insulin infusion, and remained similar between groups during the clamp (Table 1). The concentration of glucose in plasma

did not differ between groups during basal or clamp periods (Table 1).

At baseline, EGP was increased in the IH plus NAC group vs SAL and NAC groups (Fig. 1A, $P<0.05$). Insulin-stimulated suppression of EGP was impaired in the IH group, indicating that IH induced hepatic insulin resistance and NAC co-infusion did not prevent this effect (Fig. 1A and B, $P<0.05$ vs SAL and NAC). In contrast, NAC co-infusion prevented the IH-induced peripheral insulin

Figure 1

(A) Endogenous glucose production during basal and clamp periods. (B) Percent suppression of endogenous glucose production in the clamp period compared with the basal period. Basal period refers to the last 30 min before the start of the hyperinsulinemic–euglycemic clamp, while the clamp period is the last 30 min of the hyperinsulinemic–euglycemic clamp. Treatments: SAL, saline; IH, Intralipid plus heparin; IH+NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; $n=6-10/\text{group}$. One-way ANOVA with Tukey's test for *post hoc* analysis. * $P<0.05$ vs SAL; $^{\dagger}P<0.05$ vs NAC.

Figure 2

(A) Glucose utilization during basal and clamp periods. Basal period refers to the last 30 min before the start of the hyperinsulinemic–euglycemic clamp while the clamp period is the last 30 min of the hyperinsulinemic–euglycemic clamp. (B) Glucose infusion rate during the last 30 min of the hyperinsulinemic–euglycemic clamp. Treatments: SAL, saline; IH, Intralipid plus heparin; IH + NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; $n=6-10$ /group. One-way ANOVA with Tukey's test for *post hoc* analysis. ^{*} $P<0.05$ vs SAL; [†] $P<0.05$ vs NAC; [§] $P<0.05$ vs other treatment groups.

resistance, namely the decrease in insulin-stimulated glucose utilization caused by IH ($P<0.05$ for IH vs other groups, Fig. 2A). Glucose infusion rate during the hyperinsulinemic–euglycemic clamp, which is a measure of whole-body insulin sensitivity, was blunted by IH and partially normalized by NAC co-infusion (Fig. 2B).

We assessed markers of oxidative stress in the liver and skeletal muscle. The latter is an important determinant of peripheral insulin sensitivity. IH infusion did not affect hepatic content of protein carbonyl or of MDA, which is indicative of tissue lipid peroxidation; parameters did not differ across treatment groups (Fig. 3A and B). Moreover, hepatic levels of GSH and GSSG as well as the ratio of GSH to GSSG in the liver were not significantly altered by IH treatment (Fig. 4). In skeletal muscle, IH increased protein carbonyl content and this was prevented by NAC co-infusion ($P<0.05$ for IH vs other groups, Fig. 5A). Lastly, although plasma 8-iso-prostaglandin F_{2α}, a circulating

marker of oxidative stress, was approximately fourfold more elevated in the IH infusion group, the difference did not reach significance ($P=0.219$ vs SAL).

Discussion

We found that prolonged IH infusion augmented markers of oxidative stress in skeletal muscle, but not in the liver. We assessed three different markers of oxidative stress in the liver that were in agreement with each other: protein carbonyl content, MDA content, and GSH as well as GSSG levels. Accordingly, the antioxidant NAC was able to prevent IH-induced peripheral insulin resistance in parallel with IH-induced increased skeletal muscle protein carbonyl content, but not hepatic insulin resistance caused by IH.

Clinically, the ability of antioxidants to improve insulin sensitivity in obesity and type 2 diabetes mellitus is still somewhat controversial (Caballero 1993, De Mattia *et al.* 1998, Chen *et al.* 2006, Rizzo *et al.* 2008, Zhang *et al.* 2011). Infusion of IH lasting 6 and 24 h in humans results

Figure 3

(A) Hepatic protein carbonyl content. $n=4-9$ /group. (B) Hepatic malondialdehyde (MDA) content. $n=4-8$ /group. Treatments: SAL, saline; IH, Intralipid plus heparin; IH + NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; there was no significant difference on one-way ANOVA.

Figure 4

(A) Hepatic content of reduced glutathione (GSH). (B) Hepatic content of oxidized glutathione (GSSG). (C) Hepatic ratio of GSH to GSSG content. Treatments: SAL, saline; IH, Intralipid plus heparin; IH + NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; $n=6-9/\text{group}$; there was no significant difference on one-way ANOVA.

in increased levels of circulating markers of oxidative stress and co-infusion of GSH partially prevents whole-body insulin resistance and thwarts the elevations in oxidative stress markers caused by 6 h of FFA elevation (Paolisso *et al.* 1996). Our collaborative studies in humans (Xiao *et al.* 2008) show that oral taurine prevents whole-body insulin resistance caused by prolonged (48 h) IH infusion and this is accompanied by a reduction in the plasma levels of markers of oxidative stress, namely MDA and 4-hydroxynonenal. In contrast, oral NAC was not effective at improving insulin sensitivity, likely as a result of NAC being metabolized by the liver due to its route

of administration (Xiao *et al.* 2008). These studies, however, did not distinguish the role of oxidative stress in individual tissues in FFA-induced insulin resistance.

Skeletal muscle plays an important role in insulin-stimulated glucose disposal. We found that NAC prevented the diminished insulin-stimulated glucose utilization as well as the increased skeletal muscle protein carbonyl content caused by prolonged (48 h) IH infusion. Our results agree with other reports indicating that oxidative stress in skeletal muscle leads to insulin resistance. Short-term (<7 h) lipid infusion in rodents causes an increase in oxidative stress markers and insulin resistance in skeletal muscle that are averted by antioxidants, including NAC (Kim *et al.* 2009, Barazzoni *et al.* 2012). We did not find significant differences in plasma 8-iso-prostaglandin F2 α levels across treatment groups, but the values were approximately fourfold higher in the IH group than the other groups, consistent with a substantial contribution of muscle to circulating levels in conditions of oxidative stress (Awad *et al.* 1994).

Figure 5

(A) Skeletal muscle protein carbonyl content. $n=6-9/\text{group}$. One-way ANOVA with Tukey's test for *post hoc* analysis. [§] $P<0.05$ vs other treatment groups. (B) Concentration of 8-iso-prostaglandin F2 α in plasma (normalized to control). Treatments: SAL, saline; IH, Intralipid plus heparin; IH + NAC, IH plus *N*-acetyl-L-cysteine (0.35 mg/kg per min); NAC, *N*-acetyl-L-cysteine; $n=4-7/\text{group}$; there was no significant difference on one-way ANOVA.

Studies of the effect of antioxidants on FFA-induced insulin resistance have generally not focused on the liver. We have reported that the pathway in the liver through which short-term plasma FFA elevation causes hepatic insulin resistance is as follows: protein kinase C delta (PKC δ) activation $\rightarrow$ NADPH oxidase activation $\rightarrow$ oxidative stress $\rightarrow$ blunted insulin signaling (Pereira *et al.* 2014). Taken together with the results shown herein, we can conclude that as the duration of plasma FFA elevation increases from 7 to 48 h, oxidative stress in the liver is normalized. The underlying mechanism is unclear, but one possibility is that oxidative stress near the beginning of lipid infusion activates the transcription factor NRF2, which increases antioxidant enzyme expression (Cullinan & Diehl 2006), such that the oxidative stress is overcome by 48 h. Another potential explanation has hepatic PKC δ as a key player. Both short-term (Pereira *et al.* 2014) and prolonged (Pereira *et al.* 2013) IH infusions lead to PKC δ activation in the liver. Short-term IH infusion activates ERK1/2 in the liver (data unpublished) and it has been reported that PKC δ activates ERK (Ueda *et al.* 1996). It has also been shown that hepatic ERK2 alleviates oxidative stress, probably as a result of attenuated endoplasmic reticulum (ER) stress (Kujiraoka *et al.* 2013). Short-term lipid infusion also activates p38 MAPK (data unpublished), which has also been reported to attenuate ER stress (Lee *et al.* 2011). It is therefore possible that the persistent activation of hepatic PKC δ caused by lipid infusion leads to ERK/p38 MAPK activation in the liver, which in turn results in counteraction of oxidative stress.

In our previous study using prolonged lipid infusion, we have found that markers of IKK β activation are not increased in the liver, similar to oxidative stress markers in the present study (Pereira *et al.* 2013). Accordingly, the IKK β inhibitor salicylate does not prevent hepatic insulin resistance similar to NAC. However, in the prolonged lipid infusion model, markers of IKK β activation (Pereira *et al.* 2013) as well as markers of oxidative stress are elevated in muscle, and both salicylate and NAC are effective in preventing peripheral insulin resistance. Given that oxidative stress activates IKK β (Kamata *et al.* 2002), oxidative stress in muscle but not in liver may explain i) the elevation of markers of IKK β activation in muscle but not in liver and ii) the efficacy of both NAC and salicylate in preventing insulin resistance induced by prolonged lipid infusion in the periphery but not in liver. Taken together, both studies suggest that in states of chronic plasma FFA elevation, such as obesity, antioxidant and anti-inflammatory therapies may prevent peripheral but not hepatic insulin resistance.

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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