0021-972X/96/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 81, No. 12
Printed in U.S.A.

Does Free Fatty Acid Infusion Impair Insulin Action Also through an Increase in Oxidative Stress?

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ABSTRACT

In vitro studies have demonstrated that free fatty acids (FFA) may enhance oxidative stress. In contrast, no *in vivo* studies have addressed such a relationship. This four-part study aims at investigating the association between FFA and oxidative stress in healthy volunteers. The following experimental procedures were carried out: 1) determination and simple correlations among fasting plasma FFA, glucose, insulin, plasma thiobarbituric acid-reactive substance (TBARS), the ratio of reduced glutathione (GSH) to oxidized GSH, and lipid hydroperoxide (n = 30); 2) time-dependent effect of FFA on plasma TBARS concentrations and GSH/oxidized GSH ratio (n = 10); 3) dose-dependent effect of FFA on plasma TBARS concentrations (n = 9); and 4) relationship among plasma FFA concentrations, plasma TBARS concentrations, and insulin action (n = 11). The results demonstrate that fasting plasma FFA concentrations correlated with fasting plasma TBARS concentrations ($r = 0.65$; $P < 0.001$) and lipid hydroperoxide ($r = 0.79$; $P < 0.001$). The correlation between plasma FFA and TBARS remained significant even after adjustment for age, sex, body mass index, and fasting and 2-h plasma glucose concentrations ($r = 0.43$; $P < 0.01$). In the time-dependent study, plasma TBARS concentrations increased with the rise in plasma FFA concentrations. In the dose-response study, a progressive increase in fasting plasma FFA concentrations was achieved by varying the Intralipid infusion rate, which also caused plasma TBARS concentrations to increase progressively until they reached a plateau between the last two infusion rates (0.3 and 0.4 mL/min). A euglycemic hyperinsulinemic glucose clamp (insulin infusion rate, 10.2 pmol/kg·min for 360 min) was also performed. Simultaneous 10% Intralipid (0.4 mL/min) infusion significantly enhanced plasma TBARS concentrations and inhibited insulin-stimulated whole body glucose disposal (WBGD). GSH infusion (15 mg/min for 360 min) had opposite effects on plasma TBARS concentrations and WBGD. A combined infusion of 10% Intralipid and GSH was associated with a stimulation of WBGD with a magnitude midway between that of 10% Intralipid and GSH infused separately. In conclusion, fasting plasma FFA seems to enhances oxidative stress, which might contribute to the disruptive effects of plasma FFA on insulin-mediated glucose uptake. (*J Clin Endocrinol Metab* **81:** 4244–4248, 1996)

AN ELEVATED plasma free fatty acid (FFA) concentration is known to impair insulin action (1, 2) and to be a risk factor for the development of noninsulin-dependent diabetes mellitus (NIDDM) (3). It has also been suggested that an elevated plasma FFA concentration may inhibit glucose-induced insulin secretion (4, 5), but such data require additional confirmation (6).

A recent study provided evidence that FFA enhance oxidative stress in cultured endothelial cells (7). Because oxidative stress may impair insulin-mediated glucose uptake in healthy subjects and those with NIDDM (8–10), it is possible that FFA may negatively interfere with glucose metabolism at the skeletal muscle level through an increase in plasma free radical concentrations. This hypothesis is particularly intriguing when applied to the pathophysiology of NIDDM. In patients with NIDDM, elevated fasting plasma FFA concentrations (11) and exaggerated oxidative stress (10, 12) coexist and contribute, with different pathophysiological mechanisms, to worsen insulin-mediated glucose uptake (1, 2, 8–11).

In previous studies (8, 9), oxidative stress was evaluated by determining plasma free radical concentrations. Nevertheless, evidence has been accumulated that plasma thiobarbituric acid-reactive substance (TBARS) is a better and more specific index of oxidative stress (13). Thus, in our study, plasma TBARS was used as the main index of oxidative stress.

To the best of our knowledge, no previous study has addressed the potential relationship between FFA and oxidative stress *in vivo*. We, therefore, investigated *in vivo* the relationship between plasma FFA concentrations and oxidative stress in healthy subjects. Further, we examined whether changes in FFA-induced plasma TBARS and insulin-mediated glucose uptake are correlated.

Subjects and Methods

Subjects

A total of 30 healthy volunteers were enrolled in the study. Subjects were not obese and had normal glucose tolerance. Glucose tolerance was determined through a 75-g oral glucose tolerance test and classified as normal using WHO criteria (14). None of the subjects had a personal or family history of diabetes or obesity. All participants consumed a weight-maintaining diet, which included more than 250 g carbohy-

Received March 18, 1996. Revision received June 10, 1996. Rerevision received August 5, 1996. Accepted August 6, 1996.

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drates/day, for at least 3 days before undergoing the metabolic investigations. Subjects were required to refrain from taking any drugs in the 4 weeks preceding the tests. None of the participants was a smoker or consumed alcohol. All individuals gave informed consent before participation.

Experimental design

The subjects were divided into three groups (Table 1). The clinical characteristics of each group were superimposable to those of the whole group (n = 30). All studies were made in random order and were initiated at 0800 h after a 12-h overnight fast. FFA infusions were given as Intralipid (10% triglyceride emulsion, Intralipid, Pharmacia, Milan, Italy) with a simultaneous infusion of heparin (a bolus of 200 U followed by 0.2 U/min·kg BW).

Fasting study. In all subjects (n = 30) the relationship among fasting plasma glucose, insulin, FFA, TBARS concentrations, and the ratio of reduced (GSH) to oxidized (GSSG) glutathione and lipid peroxides (LPO) was studied.

Time-dependent changes. Ten subjects were given a continuous 24-h infusion of Intralipid at the rate of 0.4 mL/min with a simultaneous infusion of heparin to raise plasma FFA concentrations 2- to 3-fold. At baseline, at 6 h, and at the end of the 24 h, plasma TBARS concentrations were determined for each subject. After a wash-out period of 24 h from the end of infusion, plasma TBARS concentration was again determined. The experiment was repeated after 48 h, with an equivalent volume of 0.9% NaCl replacing Intralipid and heparin. Along with Intralipid and saline infusion, subjects were fasted until the tests at 6 h, but then were free to eat lunch and dinner. Subjects were fasted overnight before testing.

Dose-response curve. Nine subjects were given a 6-h Intralipid infusion at 0.1, 0.2, 0.3, and 0.4 mL/min. Each rate was infused on different days and in random order. In all experiments, Intralipid was delivered with a simultaneous infusion of heparin. At the beginning and end of each infusion rate, plasma samples were taken to determine changes in plasma insulin, glucose, FFA and, TBARS concentrations. Differences between basal values and those at the end of each test were used to construct a dose-response curve.

Effect on insulin action. In 11 subjects, a euglycemic hyperinsulinemic glucose clamp (insulin infusion rate, 10.2 pmol/kg·min for 360 min) was performed with simultaneous infusion of 1) 0.9% NaCl, 2) 10% Intralipid (0.4 mL/min with heparin), 3) GSH (Tationil, Boehringer Mannheim, Mannheim, Germany; 15 mg/min for 360 min), or 4) 10% Intralipid (0.4 mL/min with heparin) plus GSH (15 mg/min for 360 min). Glucose was infused as a 20% solution to keep plasma glucose within a narrow range and close to the basal concentration. In the control study, an equal volume of 0.9% NaCl was delivered. All clamps were performed in random order by a physician who was blinded to the study design.

TABLE 1. Clinical characteristics of the subjects (n = 30)

	Time-dependent study (n = 10)	Dose-effect study (n = 9)	Insulin action study (n = 11)
Age (yrs)	31.2 ± 1.8	32.3 ± 1.6	36.1 ± 0.6
Gender (M/F)	5/5	4/5	5/6
BMI (kg/m^2)	25.5 ± 0.3	23.4 ± 0.6	24.8 ± 0.5
FFM (kg)	41.4 ± 0.8	49.5 ± 1.3	45.9 ± 1.7
FPG (mmol/L)	5.0 ± 0.2	4.9 ± 0.1	5.2 ± 0.3
FPI (pmol/L)	63 ± 2.8	70 ± 4.1	68 ± 3.1
2 hr-pG (mmol/L)	6.0 ± 0.4	6.3 ± 0.2	6.1 ± 0.3
FFA (mmol/L)	398 ± 186	510 ± 211	451 ± 179
LPO μmpl/L)	0.27 ± 0.03	0.26 ± 0.04	0.26 ± 0.03
TBARS (nmol MDA/mL plasma)	0.23 ± 0.07	0.24 ± 0.08	0.22 + 0.06

BMI, Body Mass Index; FFM, Fat Free Mass; FPG; Fasting Plasma Glucose; FPI, Fasting Plasma Insulin; FFA, Free Fatty Acid; LPO, Lipo-Hydroperoxidase; TBARS, Thiobarbituric Acid Reactive Substance. No significant differences among the groups were found.

Blood sampling

Blood samples were collected in prechilled tubes containing diethyl-*p*-nitrophenyl-phosphate (Paroxam, Sigma Chemical, MO) to prevent *in vitro* lipolysis, kept in an ice bath until the end of the procedures, and then immediately centrifuged. The resultant plasma was stored in a deep freezer for use in future hormonal assays. Plasma glucose concentrations were measured by a glucose oxidase method adapted for use with a Beckman glucose analyzer (Fullerton, CA). Plasma insulin concentrations were measured by RIA, as described previously (15). Fasting plasma FFA values were derived using the mean of four different samples drawn at 10-min intervals. FFA concentrations were measured in triplicate on each sample, according to the method of Dole and Meinertz (16). Plasma GSH and GSSG concentrations were determined in the fasting state and at the end of each test, as reported previously (15). Serum oxidative stress was measured as the reaction products of malondialdehyde (TBARS) with thiobarbituric acid (13). In this reaction, to prevent an artificial autooxidation, *t*-butyl-4-hydroxyanisole (Sigma Chemical Co., St. Louis, MO) in a final concentration of 10 μmol/L was added to the specimens. The storage period of the specimens was no longer than 3 weeks before performing the assays. Each value was the result of the mean of three samples, each assayed in triplicate. LPO were measured according to the method of Yagi (17) and adjusted for plasma total cholesterol and triglycerides.

Calculations and statistical analyses

Whole body glucose disposal (WBGD) was calculated as reported previously (18). Preliminary experiments (n = 4) with [3-^{3}H]glucose infusion provided evidence that an insulin infusion rate of 10.2 pmol/kg·min fully inhibited (>94%) hepatic glucose output (HGO) along with 10% Intralipid infusion. In the same tests no negative values of HGO were found. Such data are in agreement with a previous study (19), which showed that HGO was fully inhibited in NIDDM patients when stronger Intralipid (3 mL/min) and lower insulin (7.1 pmol/kg·min) infusion rates were used. Fat-free mass was measured by bioimpedance analysis (20).

Statistical analyses were performed using the SOLO (BMDP, Cork, Ireland) software package. All values are presented as the mean ± SD. Plasma insulin concentrations were log transformed to approximate a normal distribution. Pearson product-moment correlations were used. ANOVA with repeated measures was used to evaluate the effect of time on TBARS concentration during Intralipid infusion. ANOVA with Scheffe's test was used to evaluate the differences among all experimental conditions. Partial correlations allowed evaluation of the relationship between insulin action and oxidative stress (as the TBARS concentration) independently of the main confounding covariates. $P < 0.05$ was chosen as the level of significance.

Results

Fasting study

Fasting and 2-h plasma glucose, insulin, FFA, GSH/GSSG ratio, and TBARS concentrations were all correlated. In particular, we found a strong correlation between fasting plasma FFA and concentrations of TBARS and LPO (Table 2). The plasma TBARS concentration was also significantly correlated with LPO. After adjustment for age, sex, body mass index, and fasting and 2 h plasma glucose concentrations, the relationship between FFA and TBARS (r = 0.43; $P < 0.01$) was still significant.

Time-dependent changes

Compared to baseline concentrations, the Intralipid infusion caused a 3-fold raise in plasma FFA concentrations. In particular, basal fasting plasma FFA (412 ± 98 μmol/L) rose to 1247 ± 215 μmol/L ($P < 0.001$ *vs.* basal) and 1389 ± 313 μmol/L ($P < 0.001$ *vs.* basal) after 6 and 24 h, respectively. No difference between FFA values at 6 and 24 h was found.

TABLE 2. Correlation matrix between fasting plasma metabolites and oxidative stress indices (n = 30)

	FFA	Glucose	Insulin	TBARS	GSH/GSSG	LPO
FFA		0.43 ($P < 0.03$)	0.60 ($P < 0.001$)	0.65 ($P < 0.001$)	−0.65 ($P < 0.001$)	0.60 ($P < 0.005$)
Glucose	0.43 ($P < 0.02$)		0.47 ($P < 0.008$)	0.54 ($P < 0.002$)	−0.49 ($P < 0.007$)	0.40 ($P < 0.05$)
Insulin	0.60 ($P < 0.001$)	0.47 ($P < 0.008$)		0.50 ($P < 0.005$)	0.12 ($P <$ NS)	0.51 ($P < 0.005$)
TBARS	0.65 ($P < 0.001$)	0.54 ($P < 0.002$)	0.50 ($P < 0.005$)		−0.75 ($P < 0.001$)	0.79 ($P < 0.001$)
GSH/GSSG	−0.65 ($P < 0.001$)	−0.49 ($P < 0.007$)	0.12 (P = NS)	−0.75 ($P < 0.001$)		−0.70 ($P < 0.001$)
LPO	0.60 ($P < 0.005$)	0.40 ($P < 0.05$)	0.51 ($P < 0.005$)	0.79 ($P < 0.001$)	−0.70 ($P < 0.001$)	

FFA, Free Fatty Acids; TBARS, Thiobarbituric Acid Reactive Substance; GSH/GSSG, reduced/oxidized glutathione; LPO, Lipo-Hydroperoxidase; NS, Not significant.

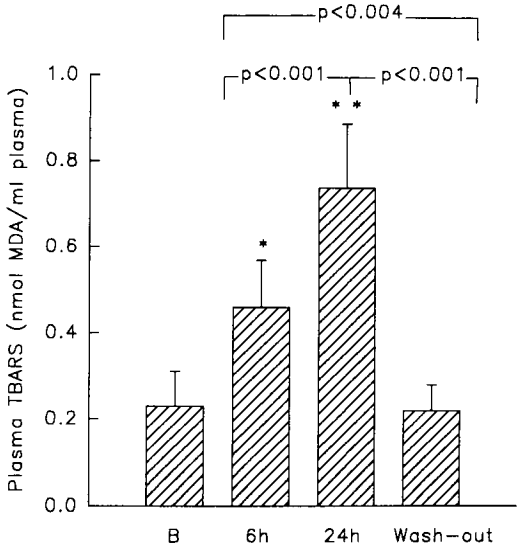

FIG. 1. Time-dependent change in plasma TBARS concentrations along with Intralipid infusion. Wash-out determination was made after 24 h of 0.9% NaCl infusion. Statistically significant differences *vs.* baseline: *, $P < 0.01$; **, $P < 0.001$.

A 24-h wash-out period was associated with a decline in the fasting plasma FFA concentration to 397 ± 77 μmol/L (P = NS), which was not different from the basal value. The plasma TBARS concentration increased at 6 h, peaked at 24 h, and then returned to basal values (Fig. 1). However, 24 h after the end of Intralipid infusion, the plasma TBARS concentration returned to preinfusion levels. Along with Intralipid infusion, the basal GSH/GSSG ratio (1.2 ± 0.4) was also significantly reduced after 6 h (0.5 ± 0.2; $P < 0.01$) and 24 h (0.9 ± 0.3; $P < 0.01$); the decline was particularly strong at 6 h ($P < 0.01$). After a 24-h wash-out period, the fasting plasma GSH/GSSG ratio returned to basal values (1.1 ± 0.3; P = NS *vs.* basal). In the control study, an equivalent volume of 0.9% NaCl in place of Intralipid was infused. In this latter test, fasting plasma FFA and TBARS concentrations and the GSH/GSSG ratio were unchanged throughout the study (data not shown).

Dose-response curve

Intralipid infusion at the different rates produced a significant increase *vs.* baseline values in plasma FFA, insulin, glucose, and TBARS levels (Fig. 2). Interestingly, despite a significant trend for glucose, insulin, and lipid concentrations to increase at any Intralipid infusion rate, plasma TBARS concentrations achieved similar values at 0.3 and 0.4 mL/min infusion rates ($P < 0.50$). The plasma GSH/GSSG ratio declined with the increase in plasma lipids (data not shown). Again, no difference in the plasma GSH/GSSG ratio between 0.3 and 0.4 mL/min infusion rates was found.

Effect on insulin action

Fasting plasma glucose and insulin concentrations were similar in all four experimental conditions. Along with the insulin infusion, fasting plasma glucose concentrations were kept within a narrow range (coefficient of variation was >5% in all experimental conditions) and close to basal values. At the steady state, plasma insulin levels achieved similar values under the different experimental conditions. Compared to 0.9% NaCl, FFA infusion was associated with an increase in the plasma TBARS concentration and a decline in WBGD (Fig. 3). In contrast, GSH had opposite effects. Interestingly, simultaneous infusion of FFA and GSH was associated with changes in the plasma TBARS concentration and WBGD that fell midway between the changes associated with FFA and GSH infused separately (Fig. 3).

Correlations

Differences in plasma FFA concentrations between control tests and those in which Intralipid was delivered correlated with changes in WBGD ($r = -0.77$; $P < 0.003$), plasma TBARS concentrations ($r = 0.64$; $P < 0.03$), and the GSH/GSSG ratio ($r = -0.68$; $P < 0.01$). The changes in WBGD correlated with the changes in plasma TBARS concentrations ($r = 0.60$; $P < 0.04$).

Discussion

Our study demonstrates that high plasma FFA concentrations are associated with increases in plasma TBARS and LPO concentrations and a decrease in the plasma GSH/GSSG ratio. Such findings are compatible with, but do not prove, a cause and effect relationship between plasma FFA and oxidative stress. Nevertheless, the results showing that the effects of FFA were time and dose dependent as well as the fact that the inhibitory effect of FFA on insulin action

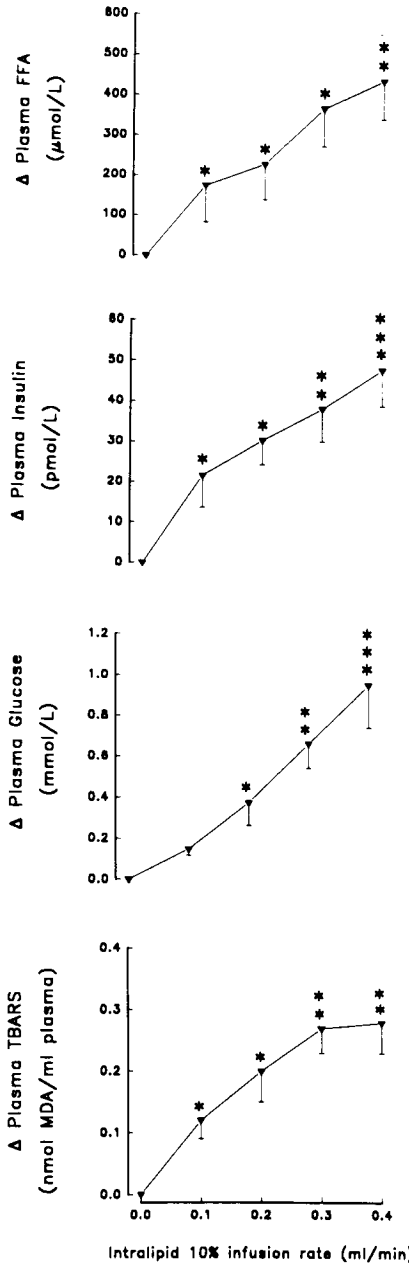

FIG. 2. Dose-dependent effect of different Intralipid infusion rates and changes in plasma glucose, insulin, FFA, and TBARS concentrations. Each experiment lasted 6 h. Statistically significant differences *vs.* baseline: *, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.001$.

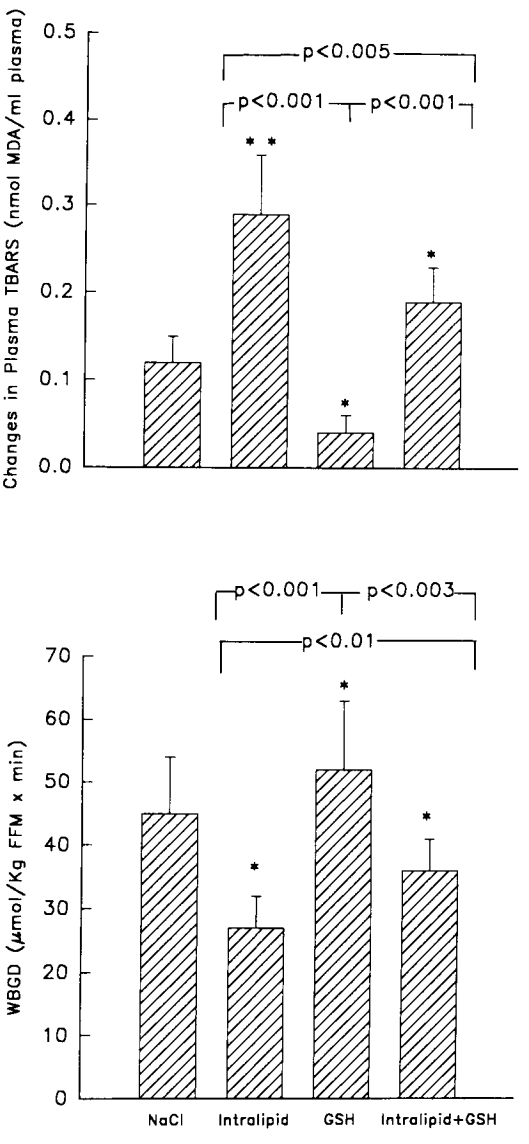

FIG. 3. Changes in plasma TBARS concentrations (*top*) and WBGD (*bottom*) after insulin infusion with simultaneous delivery of 0.9% NaCl, Intralipid (FFA) (0.4 mL/min), GSH (15 mg/min), and Intralipid plus GSH. Each experiment lasted 6 h. Statistically significant differences *vs.* 0.9% NaCl: *, $P < 0.01$; **, $P < 0.001$.

seems to be partially mediated by the rise in the plasma free radical concentration support the hypothesis that plasma FFA may affect oxidative stress.

FFA are known to inhibit oxidative and nonoxidative glucose metabolism through the glucose-fatty acid cycle (1, 2). Nevertheless, several studies have demonstrated that FFA derived from triglyceride-rich lipoproteins may cause cell damage, probably through a rise in the plasma free radical concentration (21). Because it has been shown that oxidative stress correlates with impaired insulin action (8–11), it is possible that FFA may disrupt insulin-mediated glucose uptake by an increasing oxidative stress.

The oxidizability of FFA is linearly related to the number of double alylic positions in the molecule (22), even if their biological effects are not reflected fully by the degree of unsaturation. Recently, Toborek and Hennig (7) showed that fatty acids cause an increase in oxidative stress in cultured endothelial cells and an initial decrease in reduced GSH concentrations after 6-h exposure in the incubation medium. Among the 18-carbon fatty acids used, 18:2 (linoleic acid) was the most effective. During peroxidation of 18:2 (linoleic acid), several cytotoxic metabolites were generated, such as linoleic acid hydroperoxides (23) and 4-hydroxy-2-(E)-noneal (24). Linoleic acid hydroperoxide is also known to increase the concentration of diacylglycerol, an activator of protein kinase C (25, 26). Activation of this kinase may disturb plasma membrane barrier function by increasing the phosphorylation of cytoskeletal proteins (26). Linoleic acid has been

shown to induce fatty acid β-peroxidation more significantly than 18:0, 18:1, or 18:3. Furthermore, H_2O_2, one of the final products of this pathway, is known to be involved in lipid peroxidation, and linoleic acid has been reported to affect endothelial membrane-bound enzymes such as Ca^{2+}-adenosine triphosphatase (27). Changes in Ca^{2+}-adenosine triphosphatase could result in disturbances in electrical and ionic gradients across plasma membranes (mainly enhanced Ca^{2+} permeability), which then could induce lipid preoxidative processes (7, 24). Also, linoleic acid has been found to affect cellular energy balance in cultured endothelial cells, and energy depletion is known to be correlated with impaired endothelial function (7). In addition, linoleic acid markedly increases albumin transfer across endothelial cells (28), another phenomenon linked to cell damage due to oxidative stress.

In the present study, plasma FFA concentrations were raised by Intralipid infusion, which contains linoleic acid as its major fatty acid component. Thus, in light of the previous studies, Intralipid seems an appropriate medium to investigate the relationships among FFA, oxidative stress, and insulin-mediated glucose uptake.

The role of oxidative stress on insulin action has been reported previously. Briefly, changes in the plasma membrane chemical-physical state, intracellular calcium content, and nitric oxide concentration should be taken into account (8–11).

With regard to the changes in the chemical and physical states of the plasma membrane, evidence is accumulating that the fatty acid composition of membrane lipids influences the action of insulin in many tissues. The chemical-physical properties of membranes are largely determined by the nature of the fatty acids within the phospholipid bilayer, which, in turn, may influence diverse cellular functions, including insulin-mediated glucose uptake. Interestingly, Borkman *et al.* (29) provided evidence that changes in the fatty acid composition of the skeletal muscle plasma membrane are responsible for concurrent changes in insulin-mediated glucose uptake.

An oxidative stress-mediated increase in intracellular calcium content (10) may also play a role. It has been suggested that an enhanced intracellular calcium content down-regulates insulin-mediated glucose uptake at both receptor and postreceptor steps (30).

A strong relationship between free radicals and nitric oxide has been demonstrated. Abnormalities in nitric oxide/superoxide radicals would tip the balance in favor of vasoconstriction (31), with a secondary decline in insulin action (10, 31).

A recent study demonstrates that an appropriate plasma GSH/GSSG ratio also plays a role in modulating insulin action in healthy subjects and NIDDM patients (15), thus suggesting that a rise in plasma FFA might impair insulin action through a decline in the plasma GSH/GSSG ratio.

In conclusion, an acute rise in the plasma FFA concentration is associated with an increase in plasma free radical concentrations. Nevertheless, whether a cause-effect relationship occurs remains to be determined. Furthermore, studies in NIDDM patients should be made to investigate the possible relationship among high plasma FFA concentrations, oxidative stress, and impaired insulin-mediated glucose uptake.

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