An Insulin Sensitizer Improves the Free Radical Defense System Potential and Insulin Sensitivity in High Fructose–Fed Rats

Patrice Faure, Eliane Rossini, Nicolas Wiernsperger, Marie Jeanne Richard, Alain Favier, and Serge Halimi

Recently there has been growing interest in the effects of antioxidants on insulin activity. In the present study, we investigated the effect of metformin on free radical activity and insulin sensitivity in high fructose–fed rats, a diet that leads to insulin resistance. The animals were divided into four groups (n = 16 per group; experiment duration = 6 weeks): the control (C) group received a standard diet; the control metformin (CM) group was fed a control diet and received metformin (200 mg · kg^{-1} · day^{-1} in water); the fructose control (FT) group was fed a diet in which fructose composed 56.8% of the total carbohydrates; and the fructose metformin (FM) group received high-fructose diet and metformin (200 mg · kg^{-1} · day^{-1} in water). The glucose clamp technique was used to determine insulin sensitivity in eight animals per group. Metabolic and oxidative stress parameters were measured in the remaining rats. In the FT rats, insulin resistance, lower red cell CuZn superoxide dismutase activity and lower blood reduced glutathione were observed. Metformin treatment improved both the insulin activity and the antioxidant defense system. In the CM group, metformin had no effect on metabolic parameters, but improved red cell antioxidant enzyme activities and the blood GSH level, which suggests that it has an antioxidant activity independent of its effect on insulin activity. *Diabetes* 48:353–357, 1999

We recently showed that rats fed with high dosages of fructose, which induce insulin resistance, develop oxidative stress and that supplementing them with vitamin E improves their insulin sensitivity (1). An increasing number of studies have focused on the role of antioxidants in insulin sensitivity (2). Metformin is an effective antidiabetic agent (3) that is approved for the management of NIDDM. Because metformin is selective in glycemic regulation, its effect may depend strongly on decreased insulin sensitivity in the liver and probably of the skeletal muscle (1,4). The mechanisms of action of metformin are not yet fully understood. The improvement in glucose uptake by the peripheral tissues could result from increased insulin binding to its membrane receptors (5), from the activation of postreceptor metabolic pathways (6) as well as from a beneficial effect on lipid metabolism.

These effects improve the plasma membrane fluidity (7) and increase the intracellular reduced glutathione, whose importance in the maintenance of the cellular redox state is well known (8–10). In a model of diabetes induced by alloxane, it was shown that metformin decreases lipid peroxidation (11). Although the origin of the antioxidant activity of metformin is not clearly understood, it might result from indirect effects (12). This raises the question of whether metformin has antioxidant activity when given in a model of insulin resistance without fasting hyperglycemia, as we observed in rats fed with high dosages of fructose (1). In that study, rats fed the high-fructose diet developed insulin resistance, hypertriglyceridemia, and hypertension (13), but not fasting hyperglycemia. This diet caused metabolic effects similar to those observed in syndrome X, in which insulin resistance, hypertension, and dyslipidemia are observed among patients with glucose intolerance (14). In light of our previous work showing the beneficial effects of a specific antioxidant molecule on insulin sensitivity, the goal of the present work was to evaluate the antioxidant capability of metformin during a state of insulin resistance in rats. Unlike vitamin E, metformin is not a directly antioxidant molecule—hence the original contribution of this work, which provides a complementary approach to studying the impact that oxidative stress may have on insulin activity.

RESEARCH DESIGN AND METHODS

The animal care complied with the Guide for the Care and Use of Laboratory Rats (15). Male Wistar rats were provided by Iffa Credo (Les Arbresles, France). At the beginning of the experiment, the rats were age 2 weeks. Food intake was recorded daily, and their weight was monitored weekly. The rats were maintained at a constant temperature (23°C), with a fixed 12-h artificial light period. They were divided into four experimental groups (n = 16 per group): the control (C) group received a standard diet; the control metformin (CM) group received the control diet and metformin in water (200 mg · kg^{-1} · day^{-1} in water); the high fructose–fed (FT) group received a diet in which fructose composed 56.8% of the total carbohydrates; and the fructose plus metformin (FM)-treated group received the FT diet and metformin (200 mg · kg^{-1} · day^{-1} in water). The diets are described in Table 1. The rats received the diet and the metformin for 6 weeks. The euglycemic-hyperinsulinemic glucose clamp technique was used to study insulin sensitivity in eight rats per group. In the remaining rats (n = 8), the metabolic and some of the free radical system components were measured. At the end of the experiment, the rats were killed with a lethal dose of pentobarbital (Sanofi Santé Animale, Paris, France).

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Received for publication 14 July 1997 and accepted in revised form 28 September 1998.

C, control; CM, control metformin; FM, fructose plus metformin; FT, fructose control; GIR, glucose infusion rate; GSH, reduced glutathione; GSSG, oxidized glutathione; R_d, rate of glucose disappearance; Se-GSH-Px, Se-glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance.

The rats were prepared for the euglycemic clamp procedure as previously described (1). The jugular vein for infusing and the carotid artery for sampling were catheterized and ligated securely; the catheters were tunneled subcutaneously and emerged on the dorsal side of the neck. All skin incisions were closed with a 3-0 thread. The catheters were filled with a viscous solution of polyvinylpirrolidone and sealed. The catheters required no more care before study.

The euglycemic clamps were performed 24 h after surgery (10:00 A.M.) on fasting conscious rats. Insulin (Actrapid; Novo Nordisk, Paris, France) and glucose (1 min later) were then infused; the rate of glucose infusion was corrected manually every 5 min to maintain the desired level of glycemia. Altogether, 1,200 µl of blood were collected during the experiment to measure the glucose levels. The analyses were carried out on a glucose analyzer (Yellow Spring Instruments, Columbus, OH) using the glucose oxidase method.

During insulin administration, the increase in glucose uptake by insulin-sensitive tissues was measured as the increase in the rate of glucose disappearance (R_d). Indeed, at a high-insulin infusion rate (13,600 pmol/min), R_d can be determined by the glucose infusion rate (GIR), since hepatic glucose production is completely inhibited at this insulin level (as demonstrated in our previous study) (1). At a constant plasma glucose level, the rate of glucose appearance is equal to R_d and is given by the GIR. Under these conditions, the level of the GIR reflects the insulin sensitivity of the peripheral tissues. Because the hepatic glucose production was completely inhibited at this insulin infusion rate, we did not use labeled glucose in this study (16).

Sample collections for biochemical measurements were performed at the end of the sixth week of diet therapy, after an overnight fast. Laboratory tests were carried out on 10 ml of blood collected in heparinized polypropylene tubes that were free of trace elements (Bioblock Scientific, Illkirch, France).

Plasma triglycerides were measured by an autoanalyzer (BM Hitachi 717) using a Sigma kit (ref. 339-10; Sigma-Aldrich, L' Isle d'Abeau, France). Glucose levels were measured using an autoanalyzer (BM Hitachi 717, Melan, France) fitted with a Boehringer Mannheim kit (ref. 166.391; Meylan, France) by the glucose oxidase method. Fructosamine levels were determined on an autoanalyzer (BM Hitachi 717) using a Boehringer Mannheim kit (ref. 1.101.668; Meylan, France). Plasma insulin levels were measured by radioimmunoassay (Insulin CT; ORIS, Gif sur Yvette, France).

Total glutathione (reduced glutathione [GSH] + oxidized glutathione [GSSG]) levels were determined enzymatically (17) in the acidic protein-free supernatant. The assay of GSSG was performed after having masked GSH by adding 2-vinylpyridine to the deproteinized extract. Thereafter GSSG was also determined enzymatically.

TABLE 1
Diet compositions of group C and group FT

	C diet	FT diet
Glucose	38	15.96
Fructose	—	33.64
Wheat starch	20	8.40
Casein	23	23
Cellulose	6	6
Lard	3	3
Corn oil	1	1
Rape oil	1	1
Salt mixture	7	7
Vitamins	1	1

Data are given in grams per 100 g of dry weight. The salt mixture is expressed in grams per kilogram: $CaHPO_4$, 30 g; KCl, 100 g; NaCl, 100 g; MgO, 10.5 g; $MgSO_4$, 50 g; $Fe2O_3$, 3 g; and $FeSO_47H_2O$, 5 g. Trace elements found at 10 g/kg included the following: Mn, 0.8 g; Cu 0.125 g, 125 g; Co, 0.0009 g; Zn, 0.450 g; and I, 0.0049 g. Vitamins are expressed per kilogram of the vitamin mixture: retinol, 539 mg; cholecalciferol, 6.250 mg; thiamine, 2,000 mg; riboflavin, 1,500 mg; niacin, 7,000 mg; pyridoxine, 1,000 mg; cyanocobalamin, 5 mg; ascorbic acid, 80,000 mg; *dl*-α-tocopherol acetate, 17,000 mg; menadione, 1,000 mg/kg; nicotinic acid, 10,000 mg; *o*-choline, 136,000 mg; folic acid, 500 mg; *p*-aminobenzoic acid, 5,000 mg; and biotin, 30 mg/kg.

Plasma tocopherol was measured by high-performance liquid chromatography (Kontron Instruments, Rotkreuz, Switzerland) using α-tocopherol acetate as the internal standard (18).

Trace elements were analyzed with atomic absorption spectrophotometry. Zinc and copper concentrations were measured using flame atomic absorption spectrophotometry (Model 460; Perkin-Elmer/Cetus, Norwalk, CT), as described previously (19, 20), and selenium was measured by flameless atomic absorption (21).

Se-glutathione peroxidase activity (Se-GSH-Px; EC 1.11.1.19) was determined by the modified method of Gunzler et al. (22), using *tert*-butyl hydroperoxide as substrate. CuZn superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined by monitoring the auto-oxidation of pyrogallol using the Marklund method (23).

Lipid peroxidation intermediates were measured by the plasma thiobarbituric acid reactive substances (TBARS). TBARS are products of the oxidative degradation of polyunsaturated fatty acids, in particular malondialdehyde. We used the modified method of Ohkawa et al. (24), as described previously (25).

Statistical analysis. Analysis of variance was used to compare multiple group means, followed by the Newman-Keuls test (26) to determine statistical significance between two groups. When the data were not normally distributed, the Kruskal-Wallis test was performed to compare multiple group means, followed by the Mann-Whitney test. Differences were considered significant at $P < 0.05$. All statistical analyses were performed on an IBM computer set up with PCSM software package (Personal Computer Software, Meylan, France). All data are presented as means ± SD.

RESULTS

Food consumption and weight of the rats. Throughout the experiment, all groups had similar food intake. After 6 weeks, the body weight of the rats was similar in all groups (group C, 242 ± 11 g; group CM, 251 ± 13 g; group FT, 249 ± 14; group FM, 251 ± 12). The hematocrit levels were similar in all groups (data not shown).

Insulin sensitivity and metabolic studies. Group FT developed significant insulin resistance, as shown by its lower GIR ($P < 0.001$) (Table 2). Group FM had a dramatically higher GIR than the FT group, though still lower than that of groups C and FM. Metformin did not increase the GIR of group CM in comparison with group C. Group FT had a much higher triglyceride level than the control groups. This parameter was significantly lower in group FM. Glycemia was the same in all groups. Fructosamines were significantly higher in group FT than in all other groups. Plasma insulin did not differ among groups.

Lipid peroxidation and antioxidant system components. Group FT showed a significant increase in TBARS compared with the other three groups, which all had equivalent values (Table 3). Red cell CuZn SOD activity was dramatically lower in group FT than in the other groups ($P < 0.001$). A significant increase in the activity of this enzyme was observed in the groups given metformin. The activity of Se-GSH-Px also improved in the groups given metformin ($P < 0.01$), and was thereby significantly higher in group CM than in group FM. All the high fructose–fed rats had a higher blood GSSG. A significantly higher blood GSH was noticed in the groups given metformin.

DISCUSSION

The results of this study showed that metformin therapy increases insulin sensitivity in high fructose–fed rats and lowers plasma triglyceride levels. Simultaneously, metformin enhances antioxidant defense. This improvement is independent of metformin's effect on insulin sensitivity, since its antioxidant activity was also observed in group CM.

Several explanations for the insulin resistance in high fructose–fed rats have been put forward (27), such as an

TABLE 2
Insulin sensitivity and plasma metabolic status of the control, fructose, and fructose + metformin-treated rats

	Control	Control + metformin	Fructose	Fructose + metformin
GIR (μmol · kg^{-1} · min^{-1})	170.21 ± 15.01^{c}	161 ± 12.3^{c}	83.10 ± 8.34^{a}	129.11 ± 10.60^{b}
Glucose (mmol/l)	4.5 ± 0.3	4.8 ± 0.5	4.9 ± 0.2	5.3 ± 0.4
Fructosamines (μmol/l)	122.1 ± 11.3^{a}	125 ± 12.3^{a}	134.7 ± 12.5^{b}	122.3 ± 16^{a}
Triglycerides (mmol/l)	0.45 ± 0.022^{a}	0.51 ± 0.019	2.30 ± 0.34^{c}	0.68 ± 0.080^{b}
Insulinemia (pmol/l)	340 ± 100	410 ± 100	420 ± 110	410 ± 181

Data are means ± SD; n = 8. Samples were collected in anesthesized rats by heart puncture after overnight food deprivation. Means in rows followed by different superscript letters are significantly different, $P < 0.05$.

impaired activity of the hepatic enzymes involved in carbohydrate metabolism, as well as lipid metabolism abnormalities (28,29). The role of oxidative stress has also been suggested, since a free radical scavenger like vitamin E improves insulin sensitivity in high fructose–fed rats (1). The relationship between oxidative stress and the complications of diabetes has been extensively studied (30,31). In contrast, although it is sometimes mentioned (32), the relationship between oxidative stress and insulin action has been neglected. In light of our results, we can draw a parallel between the antioxidant activity of metformin and its mechanism of action on insulin activity. In fact, the abnormalities observed in the different antiradical defense mechanisms in high fructose–fed rats and their improvement by metformin may influence insulin activity.

The levels of the lipid peroxidation marker TBARS significantly increased in group FT, but significantly decreased in group FM, indicating a decrease in lipid peroxidation. When considering the TBARS/triglycerides ratio, fructose seems to have a rather antioxidant activity. However, in the diabetic state, plasma triglycerides are not the main source of TBARS and are independent of the total amount of circulating lipids (33). In the cell membranes, enhanced free radical production can move the free radical species to another polyunsaturated lipid free radical and generate hydroperoxides (34). This latter event is an important source of aldehydes, which are able to migrate from membranes to plasma. Under these conditions, red cell membranes are an important source of plasma TBARS, especially if the enzymatic system of antioxidant defenses is impaired. CuZn SOD activity plays a key role in antioxidant defense mechanisms, particularly in hyperglycemic states or states of insulin resistance. In fact, glucose can enolize; therefore, the oxidation of this enolized form of glucose reduces molecular oxygen and yields oxidizing intermediates such as superoxide anions (35). CuZn SOD activity was much weaker in the red cells of FT rats, but normalized in group FM. This enzyme catalyzes the dismutation of the superoxide anion into hydrogen peroxide. A negative correlation between its activity and blood glucose in glyburide-treated rats has been established (36). Indeed, chemical-physical damage in membranes can trigger a decrease in the insulin receptor activity. Hydrogen peroxide resulting from the activity of the CuZn SOD activity can generate hydroxyl radicals during the Fenton reaction, unless it is reduced by Se-GSH-Px. The activity of this enzyme did not decrease in group FT in comparison with group C, but did increase in groups CM and FM. The increase in Se-GSH-Px could result from an increase in hydroxyl radical production and the subsequent induction of this enzyme activity (37). Hydroxyl radicals can damage cell membranes in that the coordinated function of enzymes and receptors they contain are lost. GSH is the co-factor of Se-GSH-Px. It is worth noting that GSH was increased in the blood of groups CM and FM in comparison with the other two groups. In particular, GSH substantially increased in group CM. Although the biological significance of the GSH/GSSG ratio is hotly debated (38), it is interesting to observe that this ratio was higher in group CM than in group C. This confirms that the antioxidant improvement due to metformin was independent of metformin's effect on insulin activity. A parallel can be drawn between the significant rise of this ratio in group FM in comparison with group FT and the increase in Se-GSH-Px activity (Table 3). Oxidation of glu-

TABLE 3
Oxidative defense system components and lipid peroxidation intermediates in control, fructose, and fructose + metformin-treated rats

	Control	Control + metformin	Fructose	Fructose + metformin
TBARS (μmol/l)	2.25 ± 0.28^{b}	2.18 ± 0.21^{b}	2.63 ± 0.39^{a}	2.13 ± 0.13^{b}
Red cell CuZn SOD activity (μg/mg Hb)	1.29 ± 0.17^{b}	1.50 ± 0.1^{a}	0.87 ± 0.20^{c}	1.59 ± 0.30^{a}
Red cell Se GSH-Px (U/g Hb)	156.7 ± 15.5^{c}	320 ± 20.7^{a}	165 ± 24.6^{c}	266 ± 47^{b}
Blood GSSG (μmol/l)	5.25 ± 1.91^{b}	6.2 ± 4.5^{b}	7.87 ± 2.50^{a}	8.5 ± 1.87^{a}
Blood GSH (μmol/l)	791.2 ± 76.1^{c}	1,170 ± 143.9^{a}	780.5 ± 123^{c}	941 ± 97^{b}
GSH/GSSG	151.1 ± 19.1^{c}	188.8 ± 42.2^{d}	96.2 ± 13^{a}	111 ± 17^{b}
Cu (μmol/l)	16.5 ± 0.9	15.6 ± 0.8	16.8 ± 1.7	17.4 ± 1.8
Zn (μmol/l)	16.8 ± 1.7^{b}	16.2 ± 1.9^{b}	15.1 ± 1.5^{a}	15.6 ± 0.9^{a}
Se (μmol/l)	3.90 ± 0.10^{a}	3.11 ± 0.12^{c}	3.33 ± 0.39^{b}	3.08 ± 0.17^{c}

Data are means ± SD; n = 8. Samples were collected in anesthetized rats by heart puncture after overnight food deprivation. Means in rows followed by different superscript letters are significantly different, $P < 0.05$.

tathione occurs during the process of peroxide reduction catalyzed by Se-GSH-Px (38). The fall in glutathione level is often observed in diabetes, and all the more so when there are linked complications (39). The increase in GSH in rats treated with metformin improves the protection of cell membranes against free radical damage (40), which also contributes to increased insulin sensitivity. Reduced glutathione content in erythrocytes from diabetic subjects showed a negative correlation with metabolic control of diabetes as assessed by HbA_{1c} levels (41), suggesting an important role in insulin activity. That is why we put forward the hypothesis that a partial effect of metformin on insulin activity confers better membrane protection, which may be achieved by a higher GSH level. On the other hand, glutathione might favor the transcription of the insulin receptor. Indeed, the GSH level of the cell enhances the activity of some transcription factors, such as Sp1, which is implicated in the insulin receptor transcription (42).

Some trace elements play an important role in the free radical protection. In this study, plasma zinc and selenium concentrations were significantly lower in both fructose-fed groups. Zinc is a biological antioxidant (43) and its depletion can lead to oxidative stress (44) and reduced insulin sensitivity (45). Selenium exerts its antioxidant effects through the co-factor of Se-GSH-Px. However, metformin treatment decreased plasma selenium concentrations. Tissue measurements of trace elements will be required to support hypotheses on the contribution of zinc and selenium to the data provided by this study.

In conclusion, the present results provided additional evidence that feeding rats with high dosages of fructose leads to insulin resistance and oxidative stress. Furthermore, our results provided direct evidence that metformin has a beneficial effect on some components of the antioxidant defense system; it is tempting to link this effect to the potentiation of insulin action by the molecule. Further investigations are necessary to determine the specific role of antidiabetic agents in the oxygen free radical protection. Our findings are of interest in the field of diabetology, since they contribute to the understanding of the mechanisms of action of antidiabetic agents and indirectly to the clarification of the implications of enhanced free radical activity on insulin receptor function.

ACKNOWLEDGMENTS

The authors would like to acknowledge Dr. Denis Barclay for his assistance in the redaction of this manuscript.

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Author Queries (please see Q in margin and underlined text)

Q1: Addition of “a diet that leads” okay?
Q2: In remainder of text, FT was used instead of FC for this group; change was made here to FT also. Change okay? Also, is this a fructose control or fructose treatment group? See second paragraph under Results in which you compare FT group with “the control groups.”
Q3: Revisions to sentence describing diets okay (e.g., change from FT to FT diet)?
Q4: Group FVE was not discussed in text, nor is there any data for this group in the text. Delete reference in title to this group? Also, in table footnote, revisions to statement regarding trace elements okay? In this same statement, correct that amount of CU found is 0.125g (originally 0,125 g)?
Q5: Please give name and location of manufacturer of the autoanalyzer and give location for Sigma.
Q6: In Tables 2 and 3, could all superscripted letters be replaced with an asterisk? If not, please specify the significance of each letter.
Q7: Please spell out PCSM.
Q8: OK to move reference to Table 2 here? If not, please place it in the correct position.
Q9: In sentence beginning “It is worth noting,” correct that “it” referred to GSH?

Table 1: (footnote to table 1) Is “Cu 0” correct?
Ref 17: Correct that this is a reference to a journal, and not to a book, as originally listed?
Ref 21: Does “4” refer to volume 4?
Ref 26: Please confirm name of editor and page range are correct.
Ref 28: Is journal title correct now (was *Proc Biochem Pharmacol*)?
Ref 34: Is this the same journal as *Biochem Biophys Res Commun*?
Ref. 38: Please list publisher name.
Ref 40: Please clarify “F,rstenberger.”
Ref 43: Is this journal *Free Radic Biol Med* or *Free Radic Res* (formerly *Free Radic Res Commun*)?