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Metabolism Clinical and Experimental 55 (2006) 691–695

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Insulin decreases intracellular oxidative stress in patients with type 2 diabetes mellitus

Maria Cristina Bravi, Antonietta Armiento, Oriana Laurenti, Maria Cassone-Faldetta, Orietta De Luca, Arianna Moretti, Giancarlo De Mattia*

Department of Internal Medicine, "La Sapienza" University, 00161 Rome, Italy

Received 13 June 2005; accepted 8 January 2006

Abstract

Patients affected by diabetes mellitus have oxidative stress with an impaired glutathione (GSH) redox state. The objective of this study was to determine the influence of insulin on oxidative stress, defined as a reduced intracellular GSH/GSH disulfide (GSSG) ratio and lipid peroxidation by plasma thiobarbituric acid reactive substances (TBARSs) in patients with type 2 diabetes. Two experimental interventions were used: (1) measurement of GSH/GSSG ratio after insulin incubation in erythrocytes from 10 type 2 diabetic patients, and (2) measurement of intraerythrocytic GSH/GSSG ratio and plasma TBARS in 14 type 2 diabetic patients during an in vivo hyperinsulinemic condition obtained from a euglycemic hyperinsulinemic clamp study. We confirmed that our patients underwent oxidative stress as shown by the significant difference in intracellular GSH/GSSG ratio in diabetic patients as compared to controls (13.56 ± 3.84 vs 27.89 ± 8.37, $P < .0001$). We found a significant elevation in the GSH/GSSG ratio after 2 hours of incubation with insulin in erythrocytes from diabetic patients (11.56 ± 1.98 to 15.61 ± 2.62, $P < .001$). During the clamp studies, GSH/GSSG ratio had already increased after 60 minutes and even more after 120 minutes (baseline, 15.04 ± 4.19; at 60 minutes, 19.74 ± 6.33; at 120 minutes, 25.33 ± 11.15; $P < .0001$). On the contrary, no significant changes were observed in plasma TBARS (3.59 ± 0.77 to 3.56 ± 0.83, NS). We conclude that insulin in patients with type 2 diabetes mellitus can reduce intracellular oxidative stress through increased GSH/GSSG ratio.

1. Introduction

Diabetes mellitus is a metabolic disease characterized by hyperglycemia and oxidative stress. The latter results from reactive oxygen species (ROS) overproduction and/or decreased system efficiency of scavengers such as vitamin C, vitamin E, and glutathione [1-4]. During ROS overproduction, intracellular antioxidant GSH (L-γ-glutamyl-L-cysteinylglycine–GSH) is oxidized to GSH disulfide (GSSG), which is then reconverted to GSH by GSH reductase. The GSH/GSSG ratio defines the so-called GSH redox state which plays an important role in cellular activation, gene expression, mRNA stability, protein folding, metabolic regulation, and cell protection against oxidative damage [5-9]. The relationship between oxidative stress and diabetic complications as well as between oxidative stress and insulin action is a research area that is extensively investigated [10-13]. In particular, we found that GSH infusion in patients with type 2 diabetes mellitus increased intracellular GSH/GSSG ratio and insulin sensitivity [14]. Nevertheless, the role exerted by insulin on intracellular oxidative stress is still unclear. In this context, it has recently been shown that patients with type 2 diabetes mellitus have lower GSH levels and higher levels of thiobarbituric acid reactive species (TBARS) as compared with type 1 diabetic patients and that these differences are partially improved by insulin treatment [15].

Thus, to clarify the influence of insulin on oxidative stress, we decided to evaluate the influence of this hormone on the GSH redox state in cells from patients with type 2 diabetes mellitus. Furthermore, to test the in vivo insulin effects on oxidative stress, we also evaluated the GSH/GSSG ratio during euglycemic hyperinsulinemic clamp studies. Because ROS overproduction may result in increased oxidative damage to lipids [16], we measured the levels of TBARS during clamp studies to clarify the relationship between insulin and extracellular ROS overproduction.

* Corresponding author. Tel. +39 6 4940678; fax: +39 6 4940678.
E-mail address: giancarlo.dematti a@uniroma1.it (G. De Mattia).

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doi:10.1016/j.metabol.2006.01.003

2. Subjects and methods

2.1. Patients and controls

The study population consisted of 24 (15 males, 9 females; mean age, 52 ± 7 years) nonobese, nonhypertensive, type 2 diabetes mellitus outpatients and 24 (15 males, 9 females) healthy, drug-free subjects who were matched for age and body mass index (BMI). All patients and controls had a stable body weight for at least 3 months before the study and good metabolic control (HbA_{1c} <7%). All consumed a standard weight-maintenance diet of 50% to 55% carbohydrates, 15% to 20% proteins, and 30% lipids. Other inclusion criteria were BMI of less than 27 kg/m^2, systolic blood pressure of less than 140 mm Hg, diastolic blood pressure of less than 90 mm Hg, and serum creatinine of less than 100 μmol/L. All patients and controls were white, nonsmokers, and consumed less than 10 g of alcohol per day. None of the subjects were taking vitamin supplements or GSH. The study was approved by our ethical committee, and all participants gave written informed consent.

2.2. Experimental design

In both controls and diabetic patients, GSH redox state was evaluated at baseline. To study insulin effect on intracellular oxidative stress, we evaluated the GSH redox state ex vivo after 2 hours of insulin stimulation in erythrocytes from 10 diabetic patients. Euglycemic hyperinsulinemic clamp studies were performed in 14 other diabetic patients. During the clamp studies, intraerythrocytic GSH redox state and plasma TBARS were measured.

2.3. Clamp studies

Euglycemic hyperinsulinemic clamp was performed according to De Fronzo et al [17]. The study began at 8 AM after an overnight fast of each patient, without the morning dose of oral hypoglycemic agent. Briefly, at 8 AM, an intravenous heparin-lock catheter system was placed in a retrograde fashion in a distal hand vein for blood sampling, while the patients were recumbent in a comfortable air-conditioned room (22°C-24°C). An antecubital vein of the contralateral forearm was intubated for infusions. The hand bearing the blood sampling system was kept in a heating device to allow measurement of arterialized venous blood glucose levels. At time 0, blood samples were taken, then at 10 minutes, a priming of recombinant insulin infusion (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark) was started and was followed by continuous insulin infusion (40 mU/m^2 per minute) over a period of 110 minutes. Plasma glucose concentrations were measured at 5-minute intervals and maintained at euglycemic levels by a variable infusion of 20% D-glucose. Blood samples were taken at 60 and 120 minutes after insulin infusion. Blood pressure and heart rate were monitored every 10 minutes throughout the test by Spacelab Omega 1400 (Spacelab, Redmond, WA).

2.4. Materials

Glutathione reductase, nicotinamide adenine dinucleotide phosphate, and test combination for hemoglobin (Hb) were purchased from Roche (Mannheim, Germany). The GSH, GSSG, 5-sulfosalicylic acid, 5,5′-dithiobis-2-nitrobenzoic acid, triethanolamine, hystopaque-1077, Hanks' balanced salt solution, and insulin were purchased from Sigma Chemical (St Louis, Mo), and 2-vinylpyridine and 1,1,3,3-tetraethoxypropane were purchased from Aldrich (Steinheim, Germany). Radioimmunoassay kits for insulin and C-peptide were obtained from BioChem Immuno Systems (Bologna, Italy). The Beckman glucose analyzer II was obtained from Beckman (Fullerton, Calif). All other reagents were of analytic grade and were obtained from commercial sources.

2.5. Analytical methods

Plasma glucose concentration was determined by glucose oxidase method. Insulin and C-peptide were measured by radioimmunoassay methods.

2.6. Preparation of samples

Heparinized venous blood samples from each patient and controls were immediately chilled at 4°C; blood was diluted with NaCl 154 mmol/L and layered onto Hystopaque-1077. After 30 minutes of centrifugation at 20°C, plasma and buffy coat were carefully removed. The erythrocytes were washed 3 times with phosphate buffered saline (pH 7.4). To remove the platelets, the samples underwent an additional 15 minutes of centrifugation at 800 rpm. Cells obtained from 14 patients during clamp studies were immediately treated for GSH and GSSG measurements, whereas those obtained from 10 other patients were used for cell cultures.

2.7. Cell cultures

Erythrocytes were cultured at 37°C in 5% CO_2 in Hanks' balanced salt solution, which had been previously adjusted to pH 7.4. Cells were treated with insulin (100 nmol/L) for 2 hours. Then, the cells were washed 3 times with ice-cold phosphate buffered saline and treated for GSH measurement.

2.8. Measurement of GSH

For determination of GSH levels, 0.1 mL of packed erythrocytes was diluted for 0.1 mL NaCl 154 mmol/L and 0.5 mL HCl 10 mmol/L. Then, erythrocytes were lysed in dry-ice acetone, thawed 3 times, and centrifuged for 10 minutes at 4°C. Supernatant was deproteinized with 5-sulfosalicylic acid (10%). The mixture was centrifuged at 13 000 × g in a microfuge (Beckman Instruments, Palo Alto, Calif) and used to total GSH determination (GSH + GSSG) by the enzymatic method described by Anderson [18]. For GSSG determination [19], 0.1 mL of deproteinized supernatants was treated with 2 μL of 2-vinylpyridine and neutralized with triethanolamine at a final pH of 6.5. After 60 minutes of incubation at room temperature, the supernatant was used for GSSG

Table 1
Clinical and laboratory features of patients with type 2 diabetes mellitus and a control group matched by age, sex, and BMI

Features	Controls (n = 24)	Type 2 diabetic patients (n = 24)
Age (y)	48 ± 10	52 ± 7
BMI (kg/m^2)	24.8 ± 1.6	25.3 ± 0.8
Sex (male/female)	15/9	15/9
Fasting blood glucose (mmol/L)	4.9 ± 0.3	6.7 ± 0.3*
HbA_{1c} (%)	4.1 ± 0.7	6.1 ± 0.7*
Systolic blood pressure (mm Hg)	130 ± 10	122 ± 18
Diastolic blood pressure (mm Hg)	82 ± 7	87 ± 3
GSH (μmol/Hb)	7.87 ± 1.26	5.57 ± 1.02*
GSSG (μmol/Hb)	0.300 ± 0.09	0.440 ± 0.14*
GSH/GSSG	27.89 ± 8.37	13.56 ± 3.84*

* $P < .0001$ vs controls.

measurement. Recovery for both procedures ranged from 96% to 102% (mean, 98%). Interassay and intra-assay variabilities were less than 10%. The results were expressed as micromoles per gram of hemoglobin.

2.9. Measurement of GSH reductase activity on cultured cells

Glutathione reductase activity was measured in the cell extract from erythrocytes previously incubated with insulin (100 nmol/L) for 2 hours. Glutathione reductase activity was followed by a decrease in absorbance at 340 nm. The assay was performed according to Beutler [20]. One enzyme activity unit is defined as the amount of enzyme which reduces 1 μmol of GSSG per minute. Specific activity is expressed as units of enzyme per gram of hemoglobin.

2.10. Assay of TBARS

Thiobarbituric acid reactive substances were measured according to a modified version of Yagi's method [21] by Richard et al. [22] in 14 type 2 diabetic patients who underwent the euglycemic hyperinsulinemic clamp study. Results are expressed as micromoles per liter of TBARS.

2.11. Statistical analysis

Data are expressed as mean ± SD. For cultured cells, each cell preparation was derived from 1 subject and triplicate plates were used for each condition. The mean of each triplicate from 1 experiment was considered (n = 1). Statistical tests used were Student *t* test, variance analysis, and Pearson's coefficient. Regression and correlation techniques were used to assess the linear relationships between variables. Repeated measures were tested by analysis of variance followed by post hoc analysis (Bonferroni). Statistical significance was considered as $P < .05$. All calculations were made using the computer program StatView II (Abacus Concepts, Berkeley, Calif).

3. Results

3.1. Physiological parameters of subjects and patients

Fasting plasma glucose and HbA_{1c} were different between controls and diabetic patients ($P < .0001$) (Table 1). In diabetic patients, GSH and GSH/GSSG ratios were decreased by 25.22% ($P < .0001$) and 51.10% ($P < .0001$), respectively, whereas GSSG was increased by 45.79% ($P < .0001$). The other parameters showed no between-group differences, and there was no significant change in these measures during the study.

3.2. Insulin effect on intracellular oxidative stress in cultured erythrocytes from diabetic patients

Insulin increased the GSH/GSSG ratio (baseline, 11.56 ± 1.98; insulin, 15.61 ± 2.62; $P < .001$) and GSH (baseline, 5.43 ± 0.73; insulin, 5.76 ± 0.87 μmol/g Hb; $P < .05$). On the contrary, the hormone reduced GSSG levels (baseline, 0.480 ± 0.09; insulin, 0.370 ± 0.06 μmol/g Hb; $P < .001$). Glutathione reductase activity was measured after stimulation with insulin (100 nmol/L). The mean activity was unchanged (5.56 ± 0.49 to 5.61 ± 0.37 IU/g Hb, NS) during insulin incubation. Similar increases in the GSH/GSSG ratio were also observed with red cells from non-diabetic donors (baseline, 20.19 ± 2.48; insulin, 27.38 ± 2.29; n = 3, $P < .05$).

3.3. Clamp studies

3.3.1. Glutathione redox state

During the clamp studies, euglycemic steady state (5.02 ± 0.10 mmol/L) and hyperinsulinemia (617.21 ± 130.92 pmol/nL) were achieved (Table 2). Intracellular GSH/GSSG ratio increased by 30.51% after 60 minutes and 60.20% after 120 minutes ($P < .0001$). Glutathione levels increased by 8.78% after 60 minutes and 11.61% after 120 minutes ($P < .05$). On the contrary, GSSG levels decreased by 13.80% after 60 minutes and 28.67% after 120 minutes ($P < .005$). Furthermore, a correlation between basal insulin levels and GSH/GSSG ratio was found ($r = 0.704$, $P < .05$) as well as between plasma insulin and GSH/GSSG ratio at 120 minutes ($r = 0.725$, $P < .005$). Increased in the GSH/GSSG ratio was also observed in clamp studies with control subjects (baseline, 27.32 ± 6.80; after 120 minutes, 32.17 ± 5.71; n = 5, $P = .0014$).

Table 2
Glutathione redox state and plasma insulin levels during the euglycemic hyperinsulinemic clamp in 14 type 2 diabetic subjects

	Baseline	60 min	120 min	*P*
GSH (μmol/Hb)	5.67 ± 1.21	6.36 ± 1.09	6.36 ± 1.45	<.05
GSSG (μmol/Hb)	0.407 ± 0.161	0.347 ± 0.133	0.300 ± 0.125	<.005
GSH/GSSG	15.04 ± 4.19	19.74 ± 6.33	25.33 ± 11.15	<.0001
Insulin (pmol/L)	107.42 ± 55.57	623.30 ± 175.05	617.21 ± 130.92	<.001

Data are expressed as mean ± SD.

3.3.2. Plasma TBARS

At baseline, the mean plasma TBARS concentration was 3.59 ± 0.77 μmol/L. During euglycemic hyperinsulinemic clamp, the mean plasma TBARS was unchanged (3.36 ± 0.78 μmol/L at 60 minutes, NS; 3.56 ± 0.83 μmol/L at 120 minutes, NS) as well as at 60 minutes until the end of the clamp study (3.42 ± 0.72 μmol/L, NS).

4. Discussion

Oxidative stress is present in pathological conditions such as atherosclerosis and diabetes mellitus [23,24]. Many papers on diabetes have demonstrated that hyperglycemia, through glucose autoxidation and/or nonenzymatic protein glycosylation, generates ROS and oxidative stress [11]. Besides this, other studies have shown decreased levels of antioxidants such as vitamin C and GSH [25,26]. In the present study, we demonstrated that in erythrocytes from type 2 diabetic patients, insulin decreases intracellular oxidative stress by an elevation of GSH/GSSG ratio. Recently, the existence of an active insulin receptor in human erythrocytes has been documented, and in these cells insulin is suggested to regulate glycolysis [27]. Glutathione/GSSG increase was not related to elevated GSH reductase activity because we found that this enzyme activity was unchanged during insulin stimulation. However, because other enzymes such as thioredoxin, protein disulfide isomerase, and thioltransferase may influence intracellular GSH redox state [28-30], we cannot exclude that the increase in GSH/GSSG ratio was due to the activation of other enzymatic mechanisms.

As for the euglycemic hyperinsulinemic clamp study, it is well known that during this procedure, elevated plasma insulin levels are maintained (617.21 ± 130.92 pmol/l) in a euglycemic state and, consequently, without counterregulatory hormone activation. Thus, in this case, a "pure" hyperinsulinemia can be generated. We found that GSH/GSSG ratio had already increased within 60 minutes from the beginning of the clamp, and even more after 120 minutes. At this time, we also found a direct correlation between GSH/GSSG ratio and plasma insulin levels, indicating that the increase in GSH/GSSG ratio was directly due to the hormone effect. Furthermore, it is noteworthy to consider that insulin was able to increase GSH/GSSG ratio to near normal levels. In this context, the hyperinsulinemic state seems to improve the intracellular GSH redox state in subjects with diabetes in which oxidative stress generates GSH impairment.

In this study, we also measured plasmatic TBARS during clamp studies. Thiobarbituric acid reactive substances result from the interaction between ROS and polyunsaturated fatty acids of cellular membranes [2]. As a consequence, measurement of TBARS is therefore an index of free radical activity and oxidative stress. We demonstrated that insulin does not induce lipid peroxidation, as indicated by the unchanged plasmatic TBARS during clamp studies. Thus, we can exclude extracellular insulin-mediated oxidative stress. These data are in contrast with the results previously reported which showed a direct correlation between plasma ROS levels and fasting insulin concentration in patients with type 2 diabetes [31]. The reasons leading to the above discrepancy are unknown, but one can argue that in type 2 diabetic patients, elevated plasma insulin levels reflect an insulin resistance state such that inducing higher plasma glucose levels results in increased ROS production [32]. In this study, we can exclude the glucose effect on ROS production because the clamp study was conducted under euglycemic conditions. Thus, in our case, the lack of correlation between plasma TBARS and insulin levels confirms that elevated plasma glucose levels, rather than insulin levels, are a determinant in ROS overproduction.

The physiological significance of our results is unknown, but it can be supposed that an improvement in the intracellular redox state obtained with insulin stimulation could influence the enzymatic activity involved in glucose metabolism such as hexokinase which reduces glucose to glucose-6-phosphate. In fact, the oxidation of cysteinyl residues leads to inactivation of hexokinase [33]. In this context, an increase in the insulin-induced GSH/GSSG ratio could represent a mechanism by which insulin influences the altered cellular metabolism in patients with oxidative stress. Recently, some studies indicate that insulin stimulation of hepatoma and adipose-like cells causes intracellular production of ROS with activation of phosphatidylinositol 3′-kinase, protein kinase Akt, and glucose transport; these reports provide new intriguing evidence for an oxidant signal in the regulation of insulin signaling [34,35]. Other studies have demonstrated that insulin exerts a beneficial effect in conditions such as apoptosis or inflammation [36,37]. Here, we have shown that insulin decreases intracellular oxidative stress in type 2 diabetic patients, and we believe that this could be considered as a result of the signaling activation of insulin. Further studies are required to define the molecular mechanisms by which insulin influences the intracellular GSH redox state.

Acknowledgment

The present work was supported by a grant from the Andrea Cesalpino Foundation.

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