Effects of Glutathione on Red Blood Cell Intracellular Magnesium Relation to Glucose Metabolism

Mario Barbagallo, Ligia J. Dominguez, Maria Rosaria Tagliamonte, Lawrence M. Resnick, Giuseppe Paolisso

Abstract—Recent evidence suggests that the endogenous antioxidant glutathione may play a protective role in cardiovascular disease. To directly investigate the role of glutathione in the regulation of glucose metabolism in hypertension, we studied the acute effects of in vivo infusions of this antioxidant (alone or in combination with insulin) on whole body glucose disposal (WBGD) using euglycemic glucose clamp and the effects on total red blood cell intracellular magnesium (RBC-Mg) in hypertensive (n = 20) and normotensive (n = 30) subjects. The relationships among WBGD, circulating reduced/oxidized glutathione (GSH/GSSG) levels, and RBC-Mg in both groups were evaluated. The in vitro effects of glutathione (100 μmol/L) on RBC free cytosolic magnesium (Mg_i) were also studied. In vivo infusions of glutathione (15 mg/min × 120 minutes) increased RBC-Mg in both normotensives and hypertensives (1.99±0.02 to 2.13±0.03 mmol/L, P<0.01, and 1.69±0.03 to 1.81±0.03 mmol/L, P<0.01, respectively). In vitro GSH but not GSSG increased Mg_i (179±3 to 214±5 μmol/L, P<0.01). In basal conditions, RBC-Mg values were related to GSH/GSSG ratios (r = 0.84, P<0.0001), and WBGD was directly, significantly, and independently related to both GSH/GSSG ratios (r = 0.79, P<0.0001) and RBC-Mg (r = 0.89, P<0.0001). This was also true when hypertensive and control groups were analyzed separately. On multivariate analysis, basal RBC-Mg (t = 6.81, P<0.001), GSH/GSSG (t = 3.67, P<0.02), and blood pressure (t = 2.89, P<0.05) were each independent determinants of WBGD, with RBC-Mg explaining 31% of the variability of WBGD. These data demonstrate a direct action of glutathione both in vivo and in vitro to enhance intracellular magnesium and a clinical linkage between cellular magnesium, GSH/GSSG ratios, and tissue glucose metabolism. (Hypertension. 1999;34:76-82.)

Key Words: glutathione • magnesium • hypertension, essential • glucose • insulin resistance • antioxidants

Glutathione in the reduced state (GSH) is present in human plasma and intracellularly, has antioxidant properties to inhibit free radical formation, and functions more generally as a redox buffer.1–3 Recent evidence suggests that GSH may also be important in blood pressure and glucose homeostasis, consistent with the involvement of free radicals in both essential hypertension and diabetes mellitus.3–6 Thus, glutathione infusions both lower blood pressure6 and directly potentiate insulin secretion in subjects with insulin resistance and impaired glucose tolerance.3 However, the mechanism(s) underlying the contribution of GSH to vascular tone and carbohydrate metabolism remains undefined.

We have recently investigated ionic aspects of insulin resistance in diabetes and hypertension and have suggested that the depletion of intracellular free magnesium common to both conditions may help to explain their frequent clinical association,7–12 especially because all kinases and other ATP-related enzymes and channels regulating insulin action11,12 and vascular tone13,14 are magnesium dependent. Because magnesium deficiency is also associated with increased free radical–dependent oxidative tissue damage15–17 and because magnesium supplementation may lower blood pressure18–20 and improve circulating glucose levels and tissue glucose oxidation in subjects with non–insulin-dependent diabetes mellitus (NIDDM),21 we wondered whether changes in intracellular magnesium might mediate the relation of GSH to glucose metabolism.

Therefore, in normal and hypertensive subjects, we studied the direct effects of glutathione on total intracellular magnesium content in red blood cells (RBC-Mg) after in vivo infusions of glutathione alone or in combination with insulin, as well as the relation of RBC-Mg to circulating levels of GSH and oxidized glutathione (GSSG) and to glucose clamp–derived indices of peripheral insulin action. The effects of glutathione on cytosolic free magnesium (Mg_i) in vitro in red blood cells (RBCs) were as well evaluated to determine the...

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From the Institute of Internal Medicine and Geriatrics, University of Palermo (Italy) (M.B., L.J.D.); Division of Endocrinology, Metabolism and Hypertension, Wayne State University, Detroit, Mich (L.J.D., L.M.R.); and Department of Geriatric Medicine and Metabolic Diseases, II University of Naples (Italy) (M.R.T., G.P.).

Correspondence to Mario Barbagallo, MD, PhD, viale F. Scaduto 6/c, 90144 Palermo, Italy. E-mail mabar@unipa.it
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direct effects of glutathione on Mg, independent of insulin or other circulating hormones and/or metabolic conditions.

Methods

In Vivo Experimental Protocol 1

After an overnight fast, 10 controls and 8 hypertensive subjects (a subset of individuals from protocol 2 below) underwent the following on different days: (1) saline infusion (120 minutes); (2) glutathione infusion (Boehringer) (15 mg/min×120 minutes); (3) insulin infusion with glucose clamp (Humulin R, Ely Lilly) (0.5 mU/kg per minute from 0 to 120 minutes and 1 mU/kg per minute from 121 to 240 minutes); and (4) infusion of glutathione and insulin (same doses as above). The tests were performed in a random order with at least 24-hour intervals. Blood samples for RBC-Mg and plasma glutathione concentration measurements were taken at baseline and at 120 and 240 minutes (end of each test).

In Vivo Experimental Protocol 2

After an overnight fast, a total of 50 subjects (20 hypertensives and 30 normotensives) underwent the following on different days: (1) saline infusion (120 minutes); (2) glutathione infusion (Boehringer) (15 mg/min×120 minutes); (3) insulin infusion with glucose clamp (Humulin R, Ely Lilly) (0.5 mU/kg per minute from 0 to 120 minutes and 1 mU/kg per minute from 121 to 240 minutes); and (4) infusion of glutathione and insulin (same doses as above). The tests were performed in a random order with at least 24-hour intervals. Blood samples for RBC-Mg and plasma glutathione concentration measurements were taken at baseline and during the last 60 minutes of the glucose clamp to estimate the net rate of glucose and lipid oxidation from gas exchange data are those described in Reference 23. A computerized open-circuit system was used to measure gas exchange through a 25-L polycyanochloride plastic canopy (Deltatrac; Datex). Nonoxidative glucose metabolism was calculated as WBGD minus oxidative glucose metabolism calculated by indirect calorimetry.

Euglycemic Glucose Clamp and OGTT

Euglycemic glucose clamp was performed according to De Fronzo et al.22 In brief, with a fixed insulin infusion rate (1 mU/kg per minute), the pump delivered a variable amount of glucose (as 30%) solution supplemented with 0.26 mmol/L KCl to maintain euglycemia and basal plasma potassium levels throughout the experiment, and blood samples were drawn for measurements of glucose and insulin at −20, −5, and 0 minutes and then every 20 minutes until the end of the test. Whole body glucose disposal (WBGD) was calculated during the final 60 minutes of the clamp as glucose infusion rate plus pool correction, in which the pool correction takes into account the change in the whole body glucose pool, as estimated from the change in plasma glucose concentration.22 For OGTT, blood was obtained before and 60, 90, 120, and 180 minutes after oral glucose (75 g) administration. Plasma glucose was determined by the glucose-oxidase method (Beckman Auto-Analyzer), and serum insulin was measured with the use of standard radioimmunoassay techniques.

Indirect Calorimetry

Indirect calorimetry was used at baseline and during the last 60 minutes of the glucose clamp to estimate the net rate of glucose and lipid oxidation and to calculate basal fat-free mass. The constants to calculate glucose and lipid oxidation from gas exchange data are those described in Reference 23. A computerized open-circuit system was used to measure gas exchange through a 25-L polycyanochloride plastic canopy (Deltatrac; Datex). Nonoxidative glucose metabolism was calculated as WBGD minus oxidative glucose metabolism calculated by indirect calorimetry.

Glutathione Measurements

Samples for plasma glutathione determinations were collected according to the techniques described by Beutler and Gelbart.2 Plasma total glutathione, GSH, and GSSG levels were determined with the use of an enzymatic assay24 that allows a recovery of GSH >90% and that has no appreciable interference with other thiols present in the plasma or in the reactive mixture.
TABLE 2. In Vivo Effects of Glutathione and Insulin Infusions on Total RBC Intracellular Magnesium

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Saline</th>
<th>Glutathione</th>
<th>Insulin A</th>
<th>Insulin A + Glutathione</th>
<th>Insulin B</th>
<th>Insulin B + Glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensives</td>
<td>1.92±0.03</td>
<td>1.93±0.02</td>
<td>2.13±0.03†</td>
<td>2.26±0.05†</td>
<td>2.37±0.04†</td>
<td>2.48±0.04†</td>
<td>2.55±0.05†</td>
</tr>
<tr>
<td>Hypertensives</td>
<td>1.64±0.01</td>
<td>1.66±0.02</td>
<td>1.81±0.03†</td>
<td>1.87±0.04*</td>
<td>1.83±0.03†</td>
<td>1.92±0.05†</td>
<td>2.01±0.04†</td>
</tr>
</tbody>
</table>

P<0.05 vs baseline. P value for trend was 0.0001 in controls and 0.007 in hypertensives.

Values are mean±SEM, expressed in millimoles per liter. Insulin A: insulin infusion rate=0.5 mU/kg · min×120 min. Insulin B: insulin infusion rate=1 mU/kg · min×120 min. Glutathione infusion rate=15 mg · min×120 min.

*Normotensives vs hypertensives.

Total RBC-Mg Measurements

We used a method previously described in detail elsewhere. Briefly, blood samples were collected into tubes containing heparin; erythrocytes were isolated by centrifugation and washed 3 times with saline solution and subsequently incubated for 90 minutes in a Krebs-Ringer buffer (NaCl 2.5 mmol/L, MgCl2 1.2 mmol/L, and NaHCO3 20 mmol/L), continuously gassed with 95% O2 and 5% CO2 (pH of 7.4 and 37°C). Cells were counted to normalize samples, lyzed by the addition of deionized water, and centrifuged, and the supernatant was kept at −20°C until magnesium determinations in duplicates were made by atomic absorption spectrophotometry (Perkin-Elmer Co).

In Vitro Experimental Protocol

Ten milliliters of heparinized blood was drawn from fasting normotensive, nondiabetic volunteers (n=10) (clinical data are shown in Table 1) at the Division of Endocrinology, Wayne State University Medical Center (Detroit, Mich) between 9AM and noon. Samples were processed with the use of 31P nuclear magnetic resonance (NMR) techniques for analysis of Mg, levels before (basal, time=0 minutes) and 30, 60, and 120 minutes after the in vitro addition (directly into the tube) of 100 µmol/L GSH or GSSG (Sigma). The method for 31P NMR analysis of Mg, has been described in detail elsewhere. In brief, 10 mL of heparinized blood was spun at 2000 rpm for 10 minutes, the plasma was discarded, and the remaining packed cell fraction was decanted into a 12-mm NMR tube. 31P NMR spectra were recorded at 81 MHz and at 37°C for 30 minutes on an XL200 spectrometer (Varian Associates Inc) in the Fourier transform mode and with wide-band proton noise decoupling. Mg, was determined according to the following equation:

\[ M_{\text{g}} = K_{d}(MgATP) \times (\phi)^{-1} - 1 \]

where \( K_{d}(MgATP) \) is the apparent dissociation constant for the reaction MgATP=Mg2+ + ATP=40 µmol/L under physiological conditions at 37°C and at pH 7.2, and \( \phi = (\text{ATP})_{\text{free}}/(\text{ATP})_{\text{total}} \) as determined from the chemical shift difference of the α- and β-phosphoryl group resonances of ATP in the 31P NMR spectrum.

Statistical Analysis

Data are expressed as mean±SEM. Differences between hypertensive patients and controls were assessed by unpaired t tests. One-way ANOVA for repeated measurements was used to compare time-dependent changes in Mg, values before and after the in vitro addition of GSH. Pearson’s correlation coefficients were used to analyze the linear correlations between variables. Stepwise multivariate analysis was used to study the different contribution of basal plasma GSH/GSSG and RBC-Mg to WBGD. Differences were considered to be statistically significant for \( P<0.05 \).

Results

In Vivo Effect of Glutathione and Insulin Infusions on Total Glutathione Concentrations and on RBC-Mg

Baseline fasting plasma total glutathione concentrations were similar in normotensive and hypertensive subjects (0.91±0.15 versus 0.87±0.18 µmol/L, \( P=NS \)). At the end of glutathione infusion, plasma levels of total glutathione were also similar between the 2 groups (1.43±0.32 versus 1.49±0.24 µmol/L, \( P=NS \)). Insulin infusion did not affect plasma total glutathione concentrations when infused alone or with glutathione (data not shown). Glutathione infusion alone or with insulin at both concentrations significantly increased RBC-Mg in both groups. Saline infusion did not alter glutathione levels or RBC-Mg concentrations (Table 2). During insulin infusion, steady state plasma glucose (5.1±0.3 versus 4.9±0.4 mmol/L, \( P=NS \)) and insulin concentrations (218±35 versus 231±42 pmol/L, \( P=NS \) at low insulin dose, and 444±62 versus 484±72 pmol/L, \( P=NS \), at high insulin dose, respectively) were not different between controls and hypertensives. Despite similar metabolic conditions, the effects of insulin alone and insulin plus glutathione on RBC-Mg were stronger in controls than in hypertensives. Glutathione and high insulin infusion produced a significantly greater increase in RBC-Mg in controls than in hypertensives (28±3.1% versus 17±2.2%, respectively, \( P<0.03 \)) (Table 2).

In Vivo Relationship Between Glutathione, Magnesium, and Insulin Action

In addition to elevated blood pressure, hypertensive subjects had lower basal GSH/GSSG ratio (\( P<0.005 \)), lower RBC-Mg (\( P<0.01 \)), and higher fasting plasma insulin levels (\( P<0.02 \)) than normotensive controls (Table 1). In all subjects, higher GSH/GSSG was associated with higher RBC-Mg (\( r=0.84, P<0.0001 \)) (Figure 1). This relationship was also significant for each patient subgroup individually (hypertensives: \( r=0.64, P=0.002 \); normotensives: \( r=0.62, P<0.001 \)). WBGD was significantly lower in hypertensives than in normotensive control subjects (\( P<0.02 \), Table 1), despite the

Figure 1. Relation between glutathione and RBC-Mg. This relation was also significant when hypertensive ○ and normotensive ● subgroups were analyzed individually (normotensives: \( r=0.62, P<0.001 \); hypertensives: \( r=0.64, P=0.002 \)).
narrow range of plasma glucose concentrations (coefficient of variation, 3.9±0.4% versus 4.3±0.3%, P=NS) and equivalent steady state plasma insulin concentrations achieved (518±41 versus 561±53 pmol/L, P=NS). WBGD was significantly related to GSH/GSSG ratios (r=0.79, P<0.0001) and to RBC-Mg (r=0.89, P<0.0001) (Figure 2). These relations were also true when hypertensive and normotensive subgroups were analyzed separately (hypertensives: WBGD vs GSG/GSSG: r=0.47, P<0.01; WBGD vs RBC-Mg: r=0.50, P<0.01; hypertensives: WBGD vs GSH/ GSSG: r=0.60, P<0.01; WBGD vs RBC-Mg: r=0.56, P<0.01). FFM indicates fat-free mass.

Mechanistically, when analyzed during the last hour of the clamp procedure, hypertensive subjects exhibited a blunted inhibition of lipid oxidation (1.6±0.4 versus 9.9±6.0 mol/kg per minute, P<0.01 versus basal at all times) (Figure 3). The effect of GSH was specific because addition of antioxidant compounds such as glutathione in cardiovascular diseases led our group in the past to examine the effects of glutathione on glucose and insulin metabolism and on blood pressure in normotensive and hypertensive subjects.3,4,6 Improved insulin secretion, peripheral insulin action, and decreased blood pressure were consistently observed after GSH

**Figure 2.** Relations of WBGD to glutathione and to RBC-Mg. These relations to WBGD were also true when hypertensive ○ and normotensive ● subgroups were analyzed individually (normotensives: WBGD vs GSG/GSSG: r=0.47, P<0.01; WBGD vs RBC-Mg: r=0.50, P<0.01; hypertensives: WBGD vs GSH/ GSSG: r=0.60, P<0.01; WBGD vs RBC-Mg: r=0.56, P<0.01). FFM indicates fat-free mass.

**Figure 3.** In vitro, direct effects of GSH and GSSG on Mg.

**In Vitro Effects of Glutathione on Mg**

In parallel with the in vivo results above, GSH significantly increased Mg levels in vitro. Basal Mg levels were 179.0±2.8 μmol/L. Addition of GSH significantly increased Mg to 192.0±2.2, 221.8±10.6, and 213.9±5.4 μmol/L at 30, 60, and 120 minutes (P<0.01 versus basal at all times) (Figure 3). The effect of GSH was specific because addition of an equal amount of oxidized glutathione, GSSG, did not significantly altered Mg levels (178.0±11.6, 180.0±10.6, 177.7±12.3, and 171.1±5.4 μmol/L at 0, 30, 60, and 120 minutes, P=NS versus basal at all times) (Figure 3).

**Discussion**

Current interest in the protective role of endogenous antioxidant compounds such as glutathione in cardiovascular diseases led our group in the past to examine the effects of glutathione on glucose and insulin metabolism and on blood pressure in normotensive and hypertensive subjects.3,4,6 Improved insulin secretion, peripheral insulin action, and decreased blood pressure were consistently observed after GSH
intracellular magnesium concentrations, 16 and antioxidants to regulate cellular metabolism in general and, in particular, actions of magnesium, which we observed to directly follow insulin action in NIDDM patients. 4 Conversely, membrane-penetrating thiol oxidants impair insulin secretion. 37 Mechanistically, that magnesium exerts similar protective effects, that glutathione directly stimulates intracellular magnesium both in vivo and in vitro, and that there was such a close correspondence in vivo between GSH/GSSG ratios and RBC-Mg all suggest magnesium as one potential factor mediating the insulin-sensitizing effects of GSH reported. Accordingly, previous studies have indicated a role for magnesium in insulin action 7–12,21,25–27,38 The ability of insulin to elevate cellular magnesium levels 25,27,39 and decreased magnesium responsiveness to insulin in cells from subjects with hypertension 25,27,39 has been demonstrated with the use of different techniques and cellular models. Furthermore, altered ionic actions of insulin in hypertension were linked with parallel alterations of insulin-mediated glucose uptake. 25 The insulin-induced changes in magnesium are directly proportional to the initial intracellular magnesium level, depleting normal cells of magnesium renders them “insulin resistant,” 27 and dietary-induced magnesium deficiency is as well associated with a decrease in insulin action. 38 These observations emphasize the potential contribution of altered cellular magnesium as an independent determinant of insulin action. It is noteworthy that in the present study glutathione was as potent as insulin in elevating intracellular magnesium.

Our results are also consistent with previous observations in the literature. Increased oxygen free radical production, which may contribute to several human diseases, 35 is associated with both low plasma GSH/GSSG ratios* and with low intracellular magnesium concentrations, 16 and antioxidants drugs or nutrients such as α-tocopherol, 15,32,33 probucol, and captopril 13 have been demonstrated to protect against magnesium deficiency—associated cerebral vascular damage 32 and magnesium deficiency—induced myocardial injury. 15,17,33 Conversely, prior magnesium depletion renders cells more sensitive to oxidative damage. 15,17 Furthermore, magnesium may itself possess antioxidant properties, scavenging oxygen radicals, possibly by affecting the rate of spontaneous dismutation of the superoxide ion. 34 Chronic hypomagnesemia results in excessive production of oxygen-derived free radicals, 33 supporting a role for magnesium in altering the threshold antioxidant capacity.

With regard to glucose metabolism, the mechanisms underlying the relationship between elevated plasma free radical concentration and poor insulin-mediated glucose uptake are still unclear. 1–4,35 but glutathione may improve glucose metabolism, 34,36 enhancing glucose-induced insulin secretion in aged patients with impaired glucose tolerance 3 and increasing insulin action in NIDDM patients. 4 Conversely, membrane-penetrating thiol oxidants impair insulin secretion. 37 Mechanistically, that magnesium exerts similar protective effects, that glutathione directly stimulates intracellular magnesium both in vivo and in vitro, and that there was such a close correspondence in vivo between GSH/GSSG ratios and RBC-Mg all suggest magnesium as one potential factor mediating the insulin-sensitizing effects of GSH reported. Accordingly, previous studies have indicated a role for magnesium in insulin action 7–12,21,25–27,38 The ability of insulin to elevate cellular magnesium levels 25,27,39 and decreased magnesium responsiveness to insulin in cells from subjects with hypertension 25,27,39 has been demonstrated with the use of different techniques and cellular models. Furthermore, altered ionic actions of insulin in hypertension were linked with parallel alterations of insulin-mediated glucose uptake. 25 The insulin-induced changes in magnesium are directly proportional to the initial intracellular magnesium level, depleting normal cells of magnesium renders them “insulin resistant,” 27 and dietary-induced magnesium deficiency is as well associated with a decrease in insulin action. 38 These observations emphasize the potential contribution of altered cellular magnesium as an independent determinant of insulin action. It is noteworthy that in the present study glutathione was as potent as insulin in elevating intracellular magnesium. However, only a part of the influence of glutathione on WBGD may be mediated by magnesium, because on multivariate analysis a residual contribution of GSH/GSSG ratios to WBGD independent of RBC-Mg was observed. Thus, other mechanisms, such as a beneficial effect of plasma GSH on membrane fluidity, 4 may also be relevant.

Also consistent with the literature, 7,8,12,40 essential hypertensive subjects in the present study displayed a reduced glucose disposal together with a lower GSH/GSSG ratio compared with normotensive controls. An increased free radical activity and alterations of antioxidant status have been previously reported in essential and pediatric hypertension, 3,41 in women with pregnancy-induced hypertension, 42 and in other cardiovascular diseases. 35,43 Conversely, Ceriello et al 6 reported that glutathione infusion lowered blood pressure in hypertensive but not in diabetic hyperglycemic subjects. 34 Although the mechanisms of the vasodilatory action of this antioxidant have not been elucidated, these data are potentially explainable by the direct relation we observed in vivo between cellular magnesium status and circulating GSH/GSSG ratios, and the well-known, direct vasodilating actions of magnesium, which we observed to directly follow provision of glutathione. The effect of hyperglycemia on intracellular magnesium is the opposite of the effect of glutathione, with glucose decreasing intracellular free magnesium and increasing intracellular calcium. 8,10,27 This counteracting ionic action may also contribute to the absence of the hypertensive effect of glutathione observed in diabetic subjects with hyperglycemia. 44 A possible caveat of the present study is that all the cellular data have been obtained in nonnucleated RBCs and may not apply to nucleated cells. However, our group has previously obtained similar ionic effects with insulin and glucose in nucleated and nonnucleated cells. Although RBC is not an
insulin-sensitive tissue, RBCs are insulin and glucose responsive in ionic terms. Thus, insulin has ionic effects, increasing intracellular calcium and/or magnesium in the same fashion in nucleated cells such as adipocytes, vascular smooth muscle cells, and lymphocytes as well as in nonnucleated RBCs. Glucose also stimulates intracellular calcium in both vascular smooth muscle cells and RBCs. In addition, our group has recent experience with 31P NMR spectroscopic measurement of intracellular free magnesium in the brain and in the muscle. Not only were very high correlations found when magnesium was measured in insulin-sensitive (muscle) or in insulin-insensitive (brain or RBC) tissue, but the effect of glucose ingestion in vivo on intracellular magnesium with the use of 31P NMR spectroscopy is similar in any of the tissues where magnesium was measured (skeletal muscle, brain, or RBC).

In conclusion, our data demonstrate for the first time a direct in vivo and in vitro action of glutathione to enhance intracellular magnesium content and show significant and independent positive relationships in vivo between intracellular magnesium content, GSH/GSSG ratios, and insulin-mediated glucose disposal. Thus, our data are consistent with a role of magnesium in mediating the effects of glutathione on peripheral insulin action.

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