

EFFECTS ON THE GLUTATHIONE POOL OF THE INSULIN-INDUCED HYPOGLYCAEMIA TEST

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The growth hormone (GH) stimulation test shows that hypoglycaemia can cause the generation of free radicals, or reactive oxygen species (ROS), together with the migration of amino acids, glutathione and various ions to the interior of fat or muscle cells. The aim of the present study is to evaluate the splitting of plasma glutathione into its two fractions, oxidized (GSSG) and reduced (GSH), after the induction of hypoglycaemia with insulin in the course of the GH stimulation test. We studied 41 short children (47% boys and 53% girls) at the Paediatric Department of the San Cecilio Hospital (Granada, Spain) to evaluate their size and growth. A GH stimulation test using insulin-induced hypoglycaemia was carried out, and GSSG and GSH values in plasma were determined. The glutathione level is associated with the level of glucose reached at 30 min after initiating the test. This provoked an initial reduction in the GSH/GSSG ratio, which fell to a minimum at 30 min after starting the test, although the values rose again at 60 min. The results obtained show that the insulin-induced GH stimulation test produces a decrease in plasma levels of the glutathione pool, that persists at least for 2 hours following the beginning of the test.

The insulin-induced hypoglycaemia test stimulates the secretion of growth hormone (GH) in healthy children. This test is habitually used to diagnose GH secretion defects in short children. Significant rises in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities have been reported immediately after episodes of insulin-induced hypoglycaemic coma (1). However, the hypoglycaemia produced by the insulin-induced hypoglycaemia test is not very marked and does not normally originate coma. This hypoglycaemia has been related with a greater production of free radicals and their metabolites (reactive oxygen species - ROS), which are continually produced by

aerobic mechanisms as a consequence of cellular respiration.

Oxidative stress gives rise to the interaction of ROS with the lipids of the cell membranes and the generation of lipoperoxides, among the consequences of which are increased membrane permeability, lysis and cellular apoptosis (2). Under normal conditions, the organism possesses a system of plasma and cellular antioxidants that protect it from oxidative stress. Among the situations that increase the production of ROS are physical exercise, hyperglycaemia and hyperinsulinism (3-4). Both hypoglycaemia and ischaemia may inhibit the transport of electrons, with resulting loss of mitochondrial membrane potential, which leads to

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reduced synthesis of ATP and to a massive entry of Ca^{2+} into the cell (5), enabling the release of neurotransmitters, including glutamate. Moreover, when hypoglycaemia takes place, other hormones with a hyperglucaemic effect are also released, including catecholamines, which participate in the formation of lipid peroxides. The production of hypoglycaemia-induced ROS in the mitochondrion has been observed to be an initial mechanism in the induction of apoptosis (6-7). This process is regulated by various antioxidant systems active in the mitochondrion, including the enzyme manganese superoxide dismutase (Mn-SOD), catalase, glutathione peroxidase and the local concentration of GSH (8).

Oxygen-free radicals, especially the superoxide anion radical (O_2^-), the hydroxyl radical (OH^\cdot) and the alkylperoxyl radical ($^\cdot\text{OOCR}$), are potent initiators of lipid peroxidation. A free radical overload damages many cellular components, such as cellular proteins, DNA and membrane phospholipids. Thus, lipid peroxidation (LPO) is one consequence of oxygen free radicals, the role of which in the pathogenesis of a wide range of diseases is well established. Numerous publications describing the enhancement of LPO in diabetes have addressed the question of hyperglycaemia (9), but very few (and in studies of mice) have considered oxidative stress under conditions of hypoglycaemia (10-11).

The aim of the present study is to evaluate the modifications of the glutathione pool after a hypoglycaemia-induced test with insulin in the course of a GH stimulation test.

MATERIALS AND METHODS

Description of the sample

The study was carried out on a sample of paediatric patients seen over a period of two consecutive years at the San Cecilio University Clinical Hospital (Granada, Spain) in order to evaluate their growth rate and body size. A total of 41 children were examined (47% boys and 53% girls). The mean age of the boys was 10.7 years (95% CI: 9.4 - 11.9 years) and that of the girls was 10.4 years (95% CI: 9.6 - 11.2 years). The following criteria were used in selecting the patients: a) body size less than the 3rd percentile, according to the Tanner and Whitehouse classification tables (12); b) growth rate below the 25th percentile. In such cases, the GH stimulation test was applied. One of the tests routinely used in our hospital

to evaluate the stimulated secretion of GH is the insulin-induced hypoglycaemia test. On finalising the study, the definitive diagnoses were of short family stature in 39% of cases, constitutional delayed growth in 14.6% of cases and GH deficit in 46.3% of cases.

In every case, informed consent was obtained from the parent or guardian, and the study protocol was approved by the Hospital's Ethics Committee.

Insulin-induced hypoglycaemia test

The insulin-induced hypoglycaemia test is used to evaluate the secretion of GH from the hypophysis in short children. The children attended the Infant Endocrinology Department at our hospital at 8 a.m. having taken no food or medication since the night before, and having drunk only water. Until 9 a.m., when the test was applied, and during the test, the patients remained in a prone position in a comfortable atmosphere. The insulin-induced hypoglycaemia test was carried out by the intravenous administration of a standard insulin pellet (Actrapid, Novo-Nordisk ®) at a dose of 0.15 U/Kg. Blood samples were taken for analysis at the following times: basal, 30, 60, 90 and 120 min after the insulin infusion.

Measurement of glutathione level

The blood samples, extracted by antecubital puncture of the arm, were collected in tubes with ethylenediamine tetraacetic acid (EDTA) and centrifuged at 2000 g for 10 min, and the packed blood cells were separated from the plasma. The levels of GSH, GSSG and total glutathione (GSt) in the plasma were measured in accordance with the technique described by Anderson (13). In order to measure the GSH, 25 μl of plasma were added to 700 μl of phosphate buffer, 100 μl of 6 mM DTNB [5,5'-Dithiobis (2-nitrobenzoic acid)] and 175 μl of water. After mixing, the plasma was incubated at 30°C for 15 min. At the start of the assay, 10 μl of reductase glutathione (Sigma, St. Louis, MO, USA) were added and the mixture was shaken. The formation of TNB (5-thio-2-nitrobenzoic acid) was measured in a spectrophotometer at 412 nm. The amount of GSH was estimated by reference to the absorbance obtained with a standard GSH curve (Sigma, St. Louis, MO, USA) at concentrations of 0.1, 0.2, 0.5 and 1 nM/25 μl .

GSSG was evaluated by applying a modified version of the procedure described by Griffith (14), using 2-vinylpyridine to mask the sulphidryl group of the GSH. The solution of 5-sulphosalicylic acid supernatant (100 μl) was mixed with 2 μl of 2-vinylpyridine, and the pH adjusted to between 6 and 7 with triethanolamine. After incubation for 60 min, the samples were measured as described in the previous paragraph.

Table I. Mean value (95% CI) of the series of measurements (basal and at 30, 60, 90 and 120 min), for glycaemia, total glutathione, GSSG and GSH during the performance of the insulin-induced GH stimulation test.

	Results series (min. after starting)				
	Basal (95% CI)	30 (95% CI)	60 (95% CI)	90 (95% CI)	120 (95% CI)
Glucose (mmol/l)	5.05 (4.90 to 5.21)	3.18 ^c (2.90 to 3.47)	4.23 ^c (3.95 to 4.51)	4.56 ^b (4.29 to 4.83)	4.71 ^c (4.51 to 4.90)
Total glutathione (GSt) (μmol/l)	52.24 (38.8 to 65.7)	45.15 ^c (32.9 to 57.4)	41.73 ^c (30.0 to 53.4)	41.30 ^c (29.1 to 53.5)	41.76 ^c (30.2 to 53.4)
Oxidized glutathione (GSSG) (μmol/l)	37.20 (27.4 to 47.0)	29.02 ^c (21.0 to 37.0)	25.87 ^c (18.5 to 33.2)	25.33 ^c (17.7 to 33.0)	25.16 ^c (18.0 to 32.3)
Reduced glutathione (GSH) (μmol/l)	17.27 (13.0 to 21.6)	12.16 ^c (8.9 to 15.4)	11.35 ^c (8.1 to 14.6)	15.48 ^b (11.4 to 19.6)	17.37 (13.2 to 21.5)
GSH/GSSG ratio	0.63 (0.51 to 0.75)	0.53 ^a (0.43 to 0.63)	0.63 (0.36 to 0.90)	1.27 ^b (0.84 to 1.70)	1.46 ^b (0.87 to 2.05)

Level of significance over the basal reference value (a: $P < 0.05$, b: $P < 0.01$, c: $P < 0.001$) obtained at 30, 60, 90 and 120 min after the start of the insulin-induced GH stimulation test.

Statistical analysis

The results are expressed as the mean and the 95% CI of the mean. We compared the values obtained at different times during the performance of the test, using a technique based on comparison of the means for related variables (t-test paired). The association between the variables at the different times of the study was established by means of a linear regression analysis.

RESULTS

The insulin-induced hypoglycaemia test in short children induces non-severe hypoglycaemia of short duration that in our sample in no case fell below the level of 2.5 mmol/L. In response to the insulin-induced hypoglycaemia test, the secretion of GH is stimulated in healthy children. In our sample of 41 children, 19 presented GH deficit.

Insulin-induced hypoglycaemia provokes an initial reduction in the GSH/GSSG ratio, which falls to a minimum at 30 min after starting the test, although the values rise again at 60 min after beginning the test. At 30 min, the mean values of glycaemia had fallen from 5.05 mMol/L (95% CI: 4.90 to 5.21) to 3.18 mMol/L (2.90 to 3.47), with a subsequent increase to return to near-basal values (Table I). Fig. 1 shows that during the test, GSt and GSSG decreased significantly. At 90 min after the start of the test, GSH and the GSH/GSSG ratio had

risen significantly above the basal levels.

We did not find a statistically significant relation between glucose concentration and GSt at baseline. However, at 30, 60, 90 and 120 min we did observe a significant association in the regression analysis between the reduction of glucose after the start of the test (30 min) and the glutathione pool in plasma (Fig. 2). This seems to indicate that the modifications in the glucose concentration and in the glutathione pool have the insulin-induced hypoglycaemia test as a common cause. In our sample, the children with low levels of GSH at 30 min after starting the test maintained these low values at 60 and 120 min. We observed no statistically significant association between the glutathione pool and the GH values at the different moments of the test.

DISCUSSION

The present study shows that during the insulin-induced hypoglycaemia test, the pool of plasma glutathione diminishes, and that it affects both the oxidized and the reduced fractions in a surprising way. The decrease of the glutathione pool is associated with decreased levels of glucose in plasma after the stimulus with insulin.

The level of GSSG in plasma is a physiological indicator of the activity of the intracellular defence

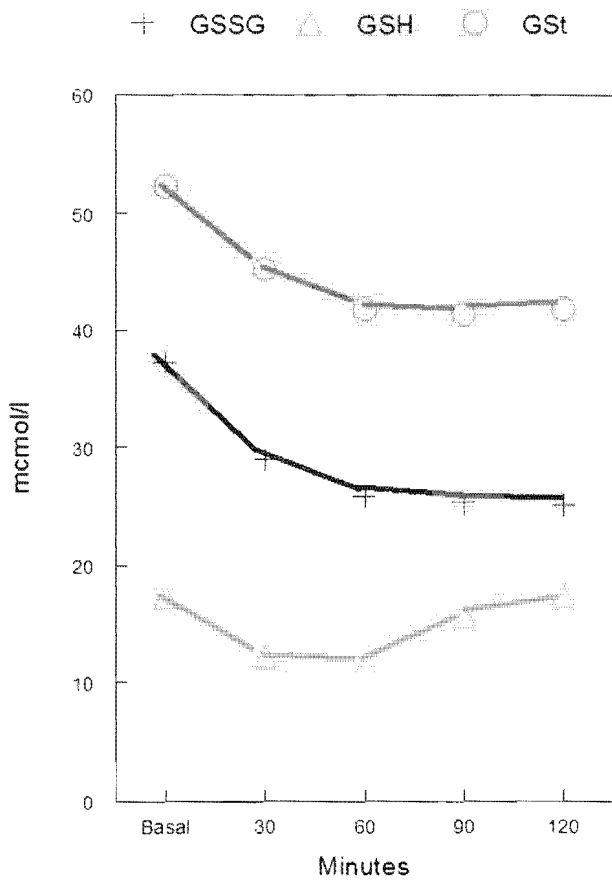


Fig. 1. Trend of mean values of GSSG, GSH and GST during the insulin-induced GH stimulation test.

system against reactive oxygen. The glutathione pool is a component of the cell's antioxidant defence mechanism, acting both as a substrate for glutathione peroxidase in the removal of hydrogen peroxide and directly as a free radical scavenger. Although the glutathione pool diminishes in its group after the test, the decrease in the GSH/GSSG ratio reflects a relative increase in GSSG, which could suggest a small increase in intracellular ROS after starting the test.

The glutathione crosses the cell membrane against a concentration gradient, from μmol levels in the plasma compartment to concentrations 100 times greater in the cytoplasm (15). Many glutathione transporters have been identified in the cell membrane, some of which are related to the transport of Na^+ while others manage only energy

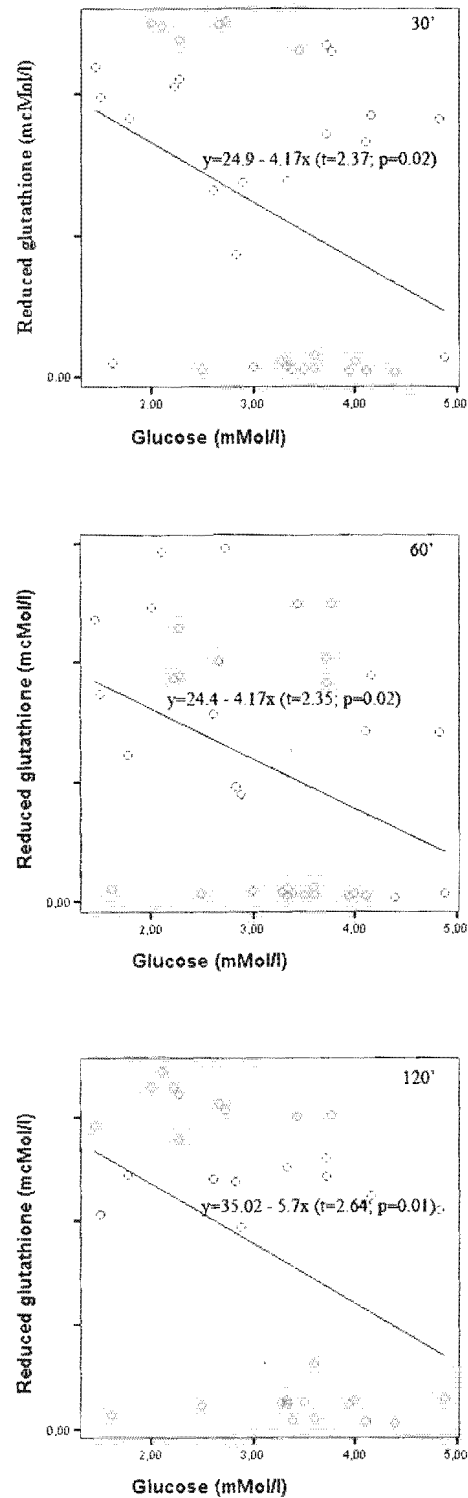


Fig. 2. Regression analysis between the levels of reduced glutathione at 30, 60 and 120 min with levels of glucose at 30 min after starting the insulin-induced GH stimulation test.

sources. During oxidation-reduction reactions, cells must maintain adequate levels of GSH relative to glutathione disulfide (GSSG); this can occur by either increasing cellular concentrations of GSH or by reducing GSSG through the catalytic action of GSSG reductase (15).

It has been shown (16) that exogenous GSH can provide significant protection from the toxicity induced by ROS or ATP depletion, and that this protection is obtained via the transport of the intact molecule of GSH into the cell. Moreover, hypoglycaemia provokes an inhibition of ATP synthesis, on which the synthesis of GSH partially depends, a process that is catalyzed by γ -glutamylcysteine synthetase and by GSH synthetase (17).

The administration of a high dose of insulin induces hypoglycaemia and a rise in plasma adrenaline, which affects the oxide reduction balance and leads to the formation of ROS. In these cases the secretion of adrenaline is proportional to the duration and severity of the hypoglycaemia (18), which, during the test of induced hypoglycaemia with insulin, is of short duration.

Jiang and Sato (19), observed that GSSG increased significantly with insulin-induced hypoglycaemia and that this state continued for more than 6 hrs after the hypoglycaemia was induced; in the present study, the decrease in the GSH/GSSG ratio was obtained at the expense of an increase in GSSG. The increases in GSH were of lesser importance and did not explicitly reflect the existence of statistically significant differences from baseline values.

The results derived from the present study lead us to conclude that the insulin-induced GH stimulation test produces a decrease in plasma levels of the glutathione pool, and that this decrease persists at least during the 2 hours following the start of the test.

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