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Effect of melatonin on enzyme activities of glutathione reductase from human erythrocytes in vitro and from rat erythrocytes in vivo

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Abstract

The in vivo and in vitro effects of melatonin on enzyme activity of glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) were investigated in this study. Glutathione reductase was purified from human erythrocytes 5.823-fold with a yield of 24% by ammonium sulfate fractionation, affinity chromatography on 2′,5′-ADP Sepharose 4B and gel filtration chromatography on Sephadex G-200. Enzyme activity was determined by the Calberg and Mannervik method using a spectrophotometer at 340 nm.

For in vitro experiments, the enzyme activity increased at 0.02 mM and decreased at 0.08 mM melatonin concentration and reached a plateau above 0.08 mM. Melatonin was administered 10 mg/kg intraperitoneally (ip) and had a stimulatory effect on the enzyme. In vivo studies were performed for melatonin in Sprague–Dawley rats and time-dependent effects were demonstrated. Glutathione reductase activity in erythrocytes was increased more by melatonin at 1 and 3 h. These results indicate that pharmacological levels of melatonin increased enzyme activity in erythrocytes. They also indicate that melatonin may be pharmacologically useful in patients with a deficiency of the enzyme in red blood cells causing hemolytic anemia.

Keywords: Glutathione reductase; Melatonin; Erythrocyte

1. Introduction

Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, EC 1.8.1.7; GR) catalyzes the GSSG to two molecules of GSH, which is a tripeptide (g-L-glutamylcysteinylglycine), as shown below:

$$GSSG + NADPH + H^+ \overset{\text{Glutathione reductase}}{\rightleftharpoons} 2GSH + NADP^+$$

The reaction requires the presence of NADPH. In the erythrocytes, the hexose monophosphate pathway is the only source of NADPH. The enzymes of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase catalyze the reduction of $NADP^+$ to NADPH in this pathway (Lehninger et al., 2000).

Glutathione reductase enables several vital functions of the cell, such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis, by maintaining a high ratio of GSH/GSSG (Schirmer et al., 1989). GSH maintains the thiol redox potential in cells by keeping sulfhydryl groups of intracellular proteins in the reduced form (Cotgreave and Gerdes, 1998), and it is also a reaction partner for the detoxification of exogenous compounds and a storage and transport form of cysteine (Meister and Anderson, 1983; Cooper and Kristal, 1997). Decreased glutathione levels have also been reported in several diseases, such as acquired immune deficiency syndrome (Akerlund et al., 1997), Parkinson's disease (Jenner and Olanow, 1998), and diabetes (Yoshida et al., 1995; Vijayalingam et al., 1996). It has been reported that a high GSSG concentration inhibits a number of enzyme systems including protein synthesis (Deneke and Fanburg, 1989).

Melatonin (*N*-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland and retina, is found in all body fluids after its release from the pineal gland. The synthesis of this pineal

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secretory product is under rhythmic control and it has been shown to bind to specific membrane receptors (Brzezinski, 1997). It regulates visual, circadian, cardiovascular and neuroendocrine functions through activation of high-affinity cell membrane G protein-coupled receptors (Brzezinski, 1997; Masana and Dubocovich, 2001). The predominant signaling pathway for melatonin receptors is through inhibition of cAMP formation via a pertussis sensitive G-protein (Reiter et al., 1997a). Melatonin has been found to be a highly effective direct scavenger of reactive radicals and intermediates hydrogen peroxide, singlet oxygen, nitric oxide, peroxynitrite anion and peroxynitrous acid, being the scavenger of the lipid peroxyl radical under debate (Reiter et al., 2000, 2001a,b). Acting as an indirect antioxidant, melatonin induces superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose 6-phosphate dehydrogenase (Reiter, 1998), and inhibits γ-glutamylcysteine synthase and nitric oxide synthase (Reiter et al., 2001a). Owing to its lipophilic characteristic (Costa et al., 1995), which allows it to cross the blood–brain barrier, its highly efficient free radical scavenging properties and its capacity of inducing antioxidative enzymes, melatonin has been proposed to be an endogenous protective agent against brain oxidative damage (Reiter, 1995, 1997, 1998).

2. Materials and methods

2.1. Materials

2′,5′-ADP Sepharose 4B was purchased from Pharmacia. Melatonin, Sephadex G-200, GSSG, NADPH, and protein assay reagent and chemicals for electrophoresis were purchased Sigma Chem. Co. All other chemicals used were analytical grade and purchased from either Sigma or Merck.

2.2. Preparation of the hemolysate

Fresh human blood was collected into Eppendorf tubes with 0.1 M sodium citrate, 0.16 M glucose, 0.016 M sodium phosphate, and 2.59 mM adenine for anticoagulation. It was centrifuged at 2500 g for 15 min and the plasma and leukocyte coat were removed. The erythrocytes were washed three times with 0.16 M KCl solution including 1 mM ethylene diamine tetra acetic acid; the samples were centrifuged at 2500 g each time and the supernatants were removed. The washed erythrocytes were hemolysed with five volumes of ice-cold distilled water containing 2.7 mM ethylene diamine tetra acetic acid and 0.7 mM β-mercaptoethanol, and centrifuged at 4 °C at 20,000 g for 30 min to remove residual intact cells and membranes (Beutler, 1984).

2.3. Ammonium sulfate fractionation and dialysis

In order to precipitate the enzyme, the hemolysate was brought to 30–70% with solid $(NH_4)_2SO_4$. Ammonium sulfate was added slowly to the hemolysate and stirred until dissolved completely. The mixture was centrifuged at 5000 g for 15 min. Precipitate was dissolved in 50 mM of phosphate buffer including 1 mM ethylene diamine tetra acetic acid, pH 7.0 and dialyzed at 4 °C in the same buffer for 2 h with two changes of buffer (Erat et al., 2005).

2.4. 2′,5′-ADP Sepharose 4B affinity chromatography

Two grams of dry 2′,5′-ADP Sepharose 4B were resuspended in 0.1 M potassium acetate/0.1 M potassium phosphate buffer, pH 6.0, and were packed into a small column (1 × 10 cm). The gel was equilibrated with the same buffer by means of a peristaltic pump. The flow rate was adjusted to 20 ml/h. The dialyzed couple obtained from ammonium sulfate precipitation was loaded onto the column, and washed with 25 ml 0.1 M potassium acetate + 0.1 M potassium phosphate, pH 6, and 25 ml 0.1 M potassium acetate + 0.1 M potassium phosphate, pH 7.85. Then washing continued with 50 mM of potassium phosphate including 1 mM ethylene diamine tetra acetic acid, pH 7.0, until the final absorbance difference became 0 at 280 nm. Proteins bound on the gel were eluted with a gradient of 0 to 0.5 mM GSH and 0 to 1 mM NADPH in 50 mM K-phosphate, containing 1 mM ethylene diamine tetra acetic acid, pH 7.0. Active fractions were collected and dialyzed with 50 mM of K-phosphate including 1 mM ethylene diamine tetra acetic acid, pH 7.0, at 4 °C (Erat et al., 2005).

2.5. Sephadex G-200 gel filtration chromatography

Five grams of dry Sephadex G-200 were incubated in distilled water at 90 °C for 5 h. After removal of the air in the gel, it was loaded onto a column (2 × 50 cm). Flow rate was adjusted to 15 ml/h by means of a peristaltic pump. The column was equilibrated with 50 mM Tris–HCl, 50 mM KCl buffer, and pH 7.5, until the final absorbance difference became zero at 280 nm and the pH value became equal to that of the equilibration buffer. The dialyzed sample from the affinity chromatography column was mixed with 5% glycerol. The final sample was loaded onto the column and 2 ml of each elution was collected in Eppendorf tubes. The absorbance values were determined at 280 and 340 nm in each fraction. Active fractions

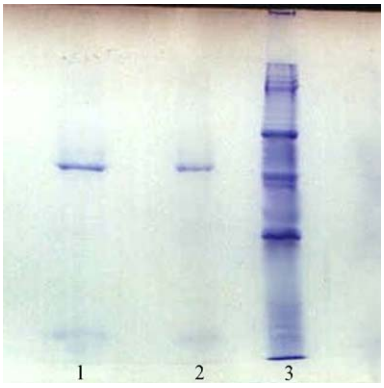

Fig. 1. SDS-PAGE photograph: Lanes 1 and 2: Human erythrocyte glutathione reductase. Lane 3: Standard proteins: *E. coli* β-galactosidase (116 kDa), bovine albumin (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa).

were lyophilized and stored at −85 °C for use in the determination of the in vitro effect of melatonin.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

To determine the enzyme purity, Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The acrylamide concentrations of the stacking and separating gels were 3% and 10%, respectively, and 1% sodium dodecyl sulfate was also added to the gel solution. The gel was stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water. Then the gel was washed with many changes of the same solvent without dye. Cleared protein bands were photographed (Fig. 1).

2.7. Activity assay

Glutathione reductase activity was determined as described by Carlberg and Mannervik (1985) at 25 °C. The assay system contained 40 mM Tris–HCl buffer, pH 8.0, including 0.8 mM ethylene diamine tetra acetic acid, 1 mM GSSG and 0.1 mM NADPH. The activity was measured by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH at 25 °C. One enzyme unit is defined as the oxidation of one μmol NADPH per minute under the assay conditions.

2.8. In vitro effect of melatonin

In order to determine the in vitro effect of melatonin, activities were assayed at different melatonin concentrations. For this, 800 μl of 50 mM Tris–HCl buffer including 1 mM ethylene diamine tetra acetic acid, pH 8.0, 70 μl of distilled water, 50 μl of 2 mM NADPH, 50 μl of 20 mM GSSG, and 30 μl human pure glutathione reductase enzyme sample were added to the cuvette. The blind cuvette included all the other solutions without an enzyme sample. Then melatonin was added to the incubation mixtures to form 0.00, 0.01, 0.02, 0.04, 0.08, 0.12, 0.16, 0.2, 0.3, and 0.4 mM melatonin concentrations. Activities were determined at 25 °C by spectrophotometer. The data obtained were analyzed by Students *t*-test, and are presented as means±SD (0.56±0.01, 0.571±0.02 ($p>0.05$), 0.78±0.02 ($p<0.05$), 0.541±0.002 ($p>0.05$), 0.468±0.03 ($p<0.05$), 0.539±0.01 ($p>0.05$), 0.548±0.02 ($p>0.05$), 0.549±0.02 ($p>0.05$), 0.529±0.02 ($p>0.05$), and 0.529±0.02 ($p>0.05$), respectively). Measurement without melatonin was regarded as the control. The results are shown in Fig. 2.

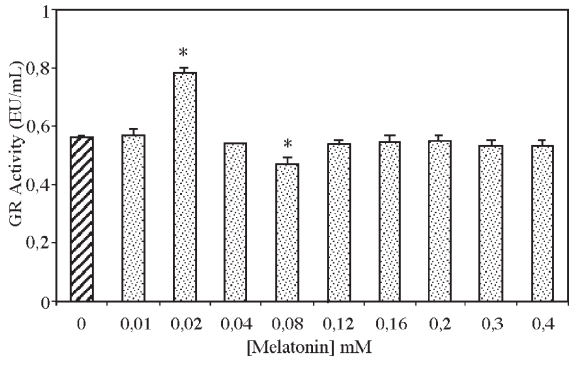

Fig. 2. In vitro effects of melatonin on human erythrocyte glutathione reductase enzyme activity (significant, *[Melatonin] versus control, significant (*$p<0.05$, $n=3$).

Table 1
Effect of melatonin on rat erythrocyte glutathione reductase enzyme activity in vivo ($n=6$)

Time (h)	$\bar{X}$±SD EU/(g Hb)$^{-1}$	p
Control	5.468±0.756	–
1	8.033±0.403	<0.01
3	6.983±0.172	<0.01
6	5.566±0.463	>0.05

2.9. In vivo effect of melatonin

Six male Sprague–Dawley rats (200±20 g) were used for the in vivo studies. The animals were housed individually and were fed standard laboratory chow and water before the experiment. The animal rooms were windowless with automatic temperature (22±1 °C) and lighting controls (14 h light/10 h dark cycles). For the control, a 0.5-ml blood sample was taken from the tail vein before the drug was administered. Then 10 mg/kg melatonin was administered to the rats intraperitoneally. After melatonin administration, 0.5 ml blood samples were taken at 1, 3 and 6 h. All of the blood samples were added to test tubes with ethylene diamine tetra acetic acid. Hemolysate was prepared as mentioned in the in vitro studies. Activity measurement and other in vivo studies were also carried out at 25 °C. The data obtained were analyzed by Students *t*-test, and are presented as mean±SD.

3. Results

Glutathione reductase activity increased up to a 0.02 mM ($p<0.05$) concentration of melatonin compared to the control; however, melatonin inhibited ($p<0.05$) the enzyme activity at 0.08 mM and activity reached a plateau below 0.12 mM of melatonin (Fig. 2).

After these experiments, the effects of melatonin were also studied in vivo (Table 1). The activity of the control group, which did not contain any melatonin, was 5.468±0.756 EU/g Hb. After the melatonin injection the activities were 8.033±0.403 ($p<0.01$), 6.983±0.172 ($p<0.01$), and 5.566±0.463 ($p>0.05$) EU/g Hb 1, 3 and 6 h. Thus, the findings of the in vivo studies related to melatonin supported the results obtained in vitro, indicating a strong increase in enzyme activity after melatonin treatment.

4. Discussion

Oxidative stress, referring to the unusual presence of molecules with a high potency to abstract electrons from biomolecules, has an important role in the pathogenesis of various diseases (Serban et al., 1996; Ruiz et al., 1999).

Undesirable biological effects of these highly reactive molecules are antagonized by enzymatic and nonenzymatic antioxidant defense systems. Many enzymes, such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, glutathione *S*-transferase, aldoketoreductase and DNA repair enzymes, provide an enzymatic antioxidant defense system. Nonenzymatic antioxidant defense systems include many different agents like vitamins (such as A, E and C), transferrin, lactoferrin, ceruloplasmin, uric acid, taurine, glutathione, cysteamine and cysteine. Glutathione metabolism is one of the most important protective systems in cells (Knapen et al., 1999).

There are many chemicals and drugs known to have adverse or beneficial effects on human enzyme and metabolic events. The effects can be dramatic and systemic. Inhibition of some important enzymes plays a key role in metabolic pathways. For example, some enzymes have been affected in some metabolic diseases such as diabetes mellitus (Christensen et al., 1982; Gupta et al., 1997).

Melatonin, the most important secretory product of the pineal gland, was recently found to be a free radical scavenger and antioxidant. Melatonin has been found to protect cells, tissues and organs against oxidative damage induced by a variety of free radical generating agents and processes, e.g., carcinogen safrole, lipopolysaccharide, kainic acid, Fenton reagents, L-cysteine, excessive exercise, glutathione depletion, carbon tetrachloride, ischemia–reperfusion, amyloid β protein, and ionizing radiation (Reiter, 1997b).

The radical-scavenging potency of melatonin is much greater than that of the most important endogenous radical scavenger, glutathione, and the classical hydroxyl radical scavenger, mannitol (Poeggeler et al., 1993). Melatonin has also been reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism, i.e. the activities of superoxide dismutase, glutathione reductase, glutathione peroxidase, glucose 6-phosphate dehydrogenase, and nitric oxide synthase (Melchiorri et al., 1997; Reiter et al., 1996; Ciftci et al., 2001). In addition, melatonin stimulates mRNA levels for superoxide dismutase and glutathione peroxidase (Pozo et al., 1994). Increased levels of the mRNAs for Mn-superoxide dismutase, CuZn-superoxide dismutase and glutathione peroxidase were also detected in rat cerebral cortex after the administration of physiological and pharmacological concentrations of melatonin (Kotler et al., 1998). However, it is well known that melatonin is a highly lipophilic compound, and, therefore, melatonin's function as a free radical scavenger and antioxidant is likely to be assisted by the ease with which it crosses morphophysiological barriers, e.g., the blood–brain barrier, and enters cells and subcellular compartments (Reiter et al., 1994). The hepatic metabolite of melatonin, 6-hydroxymelatonin sulfate, may also be a free radical scavenger, as suggested by Pierrefiche and Laborit (1995) since it was shown to be capable of antagonizing lipid peroxidation.

Glutathione peroxidase, one of the most important enzymes of glutathione metabolism, is a major antioxidative defense mechanism in the central nervous system. Pharmacological levels of melatonin have been reported to stimulate the activity of glutathione peroxidase in the brain (Barlow-Walden et al., 1995). Melatonin also has a similar stimulatory effect on glutathione peroxidase activity in the rat stomach and liver (Melchiorri et al., 1997; SeverYnek et al., 1996). Another significant observation related to the enzymatic antioxidative defense system is that reported by Pierrefiche and Laborit (1995), who demonstrated that melatonin stimulates the activity of glucose 6-phosphate dehydrogenase in both the liver and the brain. The enzyme resupplies the cell with NADPH, which is required for generating reduced glutathione from oxidized glutathione via the enzyme glutathione reductase. Stimulation of these enzymes by melatonin could have reduced damage by increasing the detoxification of radicals and reactive oxygen intermediates to non-toxic products by increasing the concentration of GSH.

A 10 mg/kg dosage of melatonin (molecular weight: 232.3 g/mol) given to humans (700 mg for 70 kg) iv results in a concentration of approximately 0.6 mM (Yeleswaram et al., 1997). This concentration is equal to the plateau in Fig. 2. Humans need less than 0.08 mM melatonin blood concentrations to increase glutathione reductase activity. A melatonin dose of 10 mg/kg given ip to rats was sufficient to increase glutathione reductase activity for at least 3 h after the injection. Thus, melatonin can increase glutathione reductase activity and may thereby provide antioxidant protection, not only in vitro, but also in vivo. In addition, melatonin inhibited glutathione reductase enzyme at 0.08 mM concentration in vitro significantly. It is also possible that it can cause noncompetitive inhibition by binding other sites affecting the three-dimensional structure of the enzyme at high substrate concentrations (Lehninger et al., 2000).

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