Turkish Journal of Medical Sciences

Volume 34 | Number 5 Article 2

1-1-2004

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Turk J Med Sci
34 (2004) 295-299

EXPERIMENTAL / LABORATORY STUDIES

The Effects of Melatonin on 6-Phosphogluconate Dehydrogenase: An In Vitro and In Vivo Study

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Received: May 23, 2003

Abstract: Melatonin is known to influence a variety of biological processes including circadian rhythms, neuroendocrine, and cardiovascular and immune functions as well as thermoregulation. Melatonin is the chief secretory product of the pineal gland, although it is also produced in other organs. In this study, in vitro effects of melatonin on 6-phosphogluconate dehydrogenase from human erythrocytes and in vivo effects of melatonin on 6-phosphogluconate dehydrogenase from rat (Sprague-Dawley) erythrocytes were studied. Human erythrocyte 6-phosphogluconate dehydrogenase was purified in 3 steps, namely haemolysate preparation, ammonium sulphate fractionation (35-65%) and 2´,5´-ADP Sepharose-4B affinity gel chromatography. 6-Phosphogluconate dehydrogenase was purified with a recovery rate of 88.8%, 2254-fold. Enzyme activity was spectrophotometrically measured using Beutler's method. In addition, 6-phosphogluconate dehydrogenase activity was inhibited in vitro by melatonin. For the in vivo study, 16 adult male Sprague-Dawley rats weighing 200-250 g were used. The animals were divided into 2 equal groups of 8 animals each: the control and melatonin-treated groups. Both groups were kept under special conditions for 6 h. Melatonin at a 10 mg/kg pharmacological dosage also inhibited the enzyme significantly ($P < 0.05$) for 3 h invivo. However, enzyme activity increased to the normal level at 6 h.

Key Words: Erythrocytes, Melatonin, 6-Phosphogluconate dehydrogenase, In vivo, In vitro

Introduction

6-Phosphogluconate dehydrogenase (6-Phospho-D-gluconate: $NADP^+$ oxidoreductase, E.C.1.1.1.44; 6PGD) is an important enzyme of the pentose phosphate metabolic pathway. The enzyme catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 with a concomitant reduction of $NADP^+$ to NADPH. 6PGD was characterised from the standpoint of the kinetic and acid base chemical mechanisms (1-5). This reaction yields NADPH, which protects the cell against oxidative agents by producing reduced glutathione (6). NADPH is also a coenzyme participating in the synthesis of a number of biomolecules such as fatty acids, steroids, and some amino acids (7,8). In the case of NADPH deficiency, the concentration of reduced glutathione in living systems declines, resulting in cell death. For this reason, 6PGD can be defined as an antioxidant enzyme (9,10). Many drugs are known to affect several body enzymes in vivo (11-13). If any drug inhibits 6PGD, the decreased NADPH and GSH will cause cell damage, especially in older erythrocytes, resulting in severe health problems (13). It has been reported that Vitamin C stimulates 6PGD (14). However, no studies could be found on the in vitro and in vivo effects of various drugs on erythrocyte 6PGD.

Melatonin is a pineal-derived product. It is synthesised enzymatically from serotonin by the sequential action of serotonin N-acetyltransferase and hydroxyindole-O-methyltransferase. It is also produced in other organs and found in all body fluids after its release from the pineal gland (15). Melatonin is known to participate in many important physiological functions, including the control of seasonal reproduction, and also influences the immune system and circadian rhythms (16-19). It plays a key role in a number of disorders, such as seasonal depression (19). In addition, melatonin stimulates several antioxidant

enzymes such as superoxide dismutase and gluthatione peroxidase (20). Moreover, melatonin directly scavenges and also stimulates the activity of endogenous antioxidant enzymes such as glutathione peroxidase (21). However, there is no information about the effect of melatonin on 6PGD. In the present study, we investigated the effects of melatonin on red blood cell 6PGD activity.

Materials and Methods

Materials

6-Phosphogluconate (6PGA), nicotinamide adenine dinucleotide phosphate ($NADP^+$), melatonin, tris (hydroxymethyl)aminomethane (Tris) and the other chemicals were purchased from Sigma.

Preparation of the haemolysate and haemoglobin estimation

Fresh blood samples from the rats were placed into EDTA containing tubes and centrifuged (15 min, 2500 x g). Plasma and buffy coat (leucocytes) were removed. The package of red cells was washed 3 times with KCl (0.16 M) and haemolysate was prepared in 5 volumes of ice-cold water and then centrifuged (+4 °C, 10000 x g) for 30 min to remove the ghosts and intact cells. The haemoglobin (Hb) concentration in haemolysate was determined by the cyanmethaemoglobin method (22).

Activity determination

Enzymatic activity was measured using Beutler's method (23). In this spectrophotometric measurement, the reaction medium maintained at 25 °C contained 0.1 mM Tris-HCl (pH 8.0) with 0.5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$, and 0.6 mM 6PGD in a total volume of 1 ml. NADPH produced in the reaction mixture was measured at 340 nm. One unit of enzyme (EU) activity was defined as the enzyme amount reducing 1 µmol of $NADP^+$ per 1 min at 25 °C, pH 8.0.

Protein determination

Protein content in ammonium sulphate supernatant was quantified spectrophotometrically at 595 nm using Bradford's method (24). Bovine serum albumin was used as standard protein.

In vitro effect of melatonin on 6PGD

In order to determine the effects of melatonin on 6PGD, various concentrations of melatonin (0.86, 1.72, 2.58, 4.30, and 6.88 mM) were added to separate tubes containing purified enzyme. The enzyme activity was measured in these tubes taking the tubes containing no melatonin as the control. This value was taken as 100% activity.

In vivo effect of melatonin on 6PGD

Sixteen adult male Sprague-Dawley rats weighing 200-250 g were used for the experiment. The animals were divided into 2 equal groups of 8 animals each: the control and melatonin-treated groups. Both groups were kept under special conditions (in a windowless room, at a temperature of 22 °C, with a light on for 14 h) for 6 h. Before intramuscular melatonin injection, the control blood samples (0.5 ml of whole blood with EDTA) were obtained from the animals; a pharmacological dosage of melatonin (10 mg/kg) was injected into the group. Blood samples were taken from each group 1, 3, and 6 h after injection. Haemolysates were prepared from all blood samples as described above. Haemoglobin levels and 6PGD activity were then determined.

Statistical analysis

Results are presented as mean ±SD. All parameters were analysed using one-way variance analysis. The least significant difference multiple range test was used to compare the mean values. Acceptable significance was recorded when P values were <0.05. Statistical analysis was performed using SPSS (version 9.0, Chicago, IL, USA).

Results and Discussion

As can be seen in Table 1, in the first step, the haemolysate sample was precipitated using ammonium sulphate (35-65%). Impurities were thus significantly eliminated. Then 2´,5´-ADP Sepharose 4B column chromatography was performed. The enzyme 6PGD of the rat erythrocytes was purified 2254-fold by this method.

It is well known that many chemicals have adverse effects on the organism when used for therapeutic or

Table 1. Purification scheme of 6-phosphogluconate dehydrogenase from human erythrocytes.

Steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Haemolysate	0.021	25	10.22	255.5	0.4	0.0015	100	1
Ammonium sulphate precipitation (35-65%)	0.50	12	0.275	3.30	0.312	0.094	48	62.7
2′,5′-ADP Sepharose 4B chromatography	0.071	5	0.021	0.105	0.355	3.38	88.8	2254

other purposes (25). These effects may be dramatic and systematic (26). A good example of this is how in 1926 pamaquine used for malaria treatment caused severe adverse effects in patients within a few days, resulting in black urination, hyperbilirubinaemia, a dramatic decrease in blood Hb levels, and finally death in cases of severe G6PD deficiency (27). Similarly, acetazolamide inhibits carbonic anhydrase (CA), giving rise to severe diuresis (28).

Melatonin reduced the activity of cytochrome c oxidase, the enzyme respiratory chain (29). In addition, this hormone reduced the lipase esterase and alkaline phosphatase activity of the liver in male mink (30). However, there is no information about the effect of melatonin on human erythrocyte 6PGD.

Figure shows the in vitro effects of the melatonin on the enzyme activity. All concentrations inhibited enzyme activity in vitro. Five different melatonin concentrations were used to determine the effects on human 6PGD activity. As can be seen in Figure, it was evident from the in vitro studies that the 6PGD was inhibited up to 6.88 x 10^{-5} M by melatonin. For the in vivo studies, 20 units from adult (200-250 g) Sprague-Dawley rats were selected. The 6PGD activity of the control group was 188 ± 19.3 EU /gHb. The melatonin injection was performed on the control group intramuscularly. After the melatonin injection, the enzyme activities of the groups were measured at 1, 3 and 6 h. The corresponding activities were 120 ± 13.8, 106 ± 12.5 and 193 ± 16.1, respectively, and the greatest inhibition was found 3 h after injection (Table 2).

As be can be seen in Table 2, the in vivo studies showed that melatonin inhibited enzyme activity by 36.2% at 1 h ($P < 0.05$) and by 43.6% at 3 h ($P <$

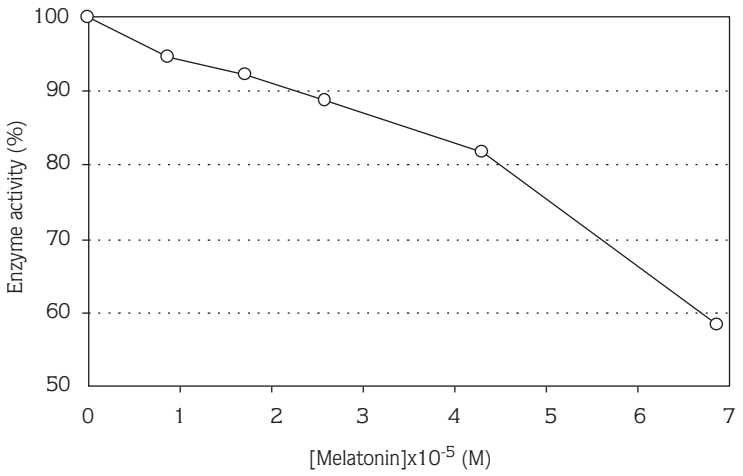

Figure. Activity (%) vs. melatonin concentration plot of 6PGD.

Table 2. In vivo effects of melatonin (10 mg/kg body weight) on rat red blood cell 6-phosphogluconate dehydrogenase activity (n = 8).

Time after administration (h)	6PGD activity (EU/gHb)
0	188 ± 19.3
1	120 ± 13.8
3	106 ± 12.5
6	193 ± 16.1

0.05). Six hours after melatonin treatment, a slight activation appeared, but this value was not statistically significant ($P > 0.05$).

In conclusion, we demonstrated that melatonin reduced activity under in vitro and in vivo conditions on human and rat erythrocyte 6-phosphogluconate dehydrogenase, respectively.

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