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GLUCOSE 6-PHOSPHATE DEHYDROGENASE: *IN VITRO* AND *IN VIVO* EFFECTS OF DANTROLENE SODIUM

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Glucose 6-phosphate dehydrogenase: in vitro and in vivo effects of dantrolene sodium. Ş. BEYDEMIR, İ. GÜLÇIN, Ö.İ. KÜFREVIOĞLU, M. ÇIFTÇI. Pol. J. Pharmacol., 2003, 55, 787–792.

In our study, effects of dantrolene sodium on glucose 6-phosphate dehydrogenase (G6PD) were examined in the human erythrocytes *in vitro* and in rat erythrocytes *in vivo*. Human erythrocyte G6PD was purified using ammonium sulfate fractionation and 2',5'-ADP Sepharose 4B affinity chromatography. The enzyme activity was determined by Beutler's method. The overall purification procedures gave the human G6PD having the specific activity of 97.6 EU/mg of protein, which was purified 9760-fold with a yield of 39%. Dantrolene sodium inhibited the enzyme activity under *in vitro* conditions and the I_{50} value (drug concentration which produces 50% inhibition) of this drug was 0.91 mM. *In vivo* studies were performed in rats (Sprague-Dawley). Dantrolene sodium at 10 mg/kg inhibited the enzyme activity significantly ($p < 0.05$) 3 h after dosing. We conclude that dantrolene sodium showed inhibitory effect on G6PD activity both *in vitro* and *in vivo*.

Key words: *glucose 6-phosphate dehydrogenase, erythrocytes, human, dantrolene sodium*

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INTRODUCTION

Dantrolene sodium, a blocker of intracellular Ca^{2+} release from the sarcoplasmic reticulum, is a skeletal muscle relaxant [22]. It is used in the treatment of muscle spasticity, malignant hyperthermia and neuroleptic malignant syndrome and depresses excitation-contraction coupling in the muscle [8]. There is no effect of dantrolene sodium on neuromuscular transmission, and it does not affect the electrical properties of the skeletal muscle membrane [8]. In addition, it has been suggested that dantrolene sodium affects the membrane calcium channel of smooth muscle cells and inhibits calcium influx [19, 20].

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is the first key enzyme in the pentose phosphate metabolic pathway and is involved in the generation of NADPH, which is indispensable for biosynthesis of reduced glutathione (GSH) [13, 25]. GSH prevents hemoglobin denaturation, preserves the integrity of red blood cell membrane sulfhydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells [12, 23, 24].

G6PD deficiency is an X-chromosome-linked hereditary disorder. The enzyme deficiency is widespread throughout the world especially in Mediterranean costs [26]. The enzyme deficiency is found in all tissues, including the erythrocytes [17]. The patients with G6PD deficiency generally can respond to drugs or food with hemolytic crises or neonatal jaundice. Besides, genetic abnormality and age may cause deficiency of this enzyme [5]. There are a lot of kinds of G6PD deficiency, at least 400 clinical and biochemical variants have been described up until now [13].

Multitude of chemical materials and drugs are being used in therapies. However, we have found only a few literature reports related to changes in specific enzyme activities. Several papers have indicated that some decreases and increases were found in human erythrocyte carbonic anhydrase-I and II isozymes [2, 3]. In addition, Bülbül et al. investigated the inhibitory effects of sulfonamide derivatives, which are used in tumor treatment, in fish, on rainbow trout carbonic anhydrase activity [7]. Additionally, effects of metamizol and magnesium sulfate on G6PD from human erythrocytes have been examined [10].

In this report, the *in vitro* effect of dantrolene sodium on G6PD purified from human erythrocytes and the *in vivo* effect on the enzyme activity from rat erythrocytes were investigated.

MATERIALS and METHODS

Materials

Dantrolene sodium, $NADP^+$, glucose 6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma-Aldrich GmbH (Sternheim, Germany). 2',5'-ADP Sepharose 4B was provided by Pharmacia. All other chemicals used were of analytical grade and were purchased from either Sigma or Merck.

Preparation of the hemolysate and hemoglobin estimation

Fresh blood samples from healthy subject were collected to EDTA-containing tubes. The hemolysate was prepared according to the previous study [9]. Hemoglobin (Hb) concentration in hemolysate was determined by cyanmethemoglobin method [1, 11, 21]. All studies were performed at +4°C.

Ammonium sulfate fractionation and dialysis

The hemolysate was subjected to precipitation with ammonium sulfate. Ammonium sulfate fractionation was done according to the previous study [9]. The enzyme was observed to precipitate at 35–65% precipitation step. The resultant solution was clear, and contained partially purified enzyme. It was dialyzed at 4°C against 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0), for 2 h with two changes of buffer.

Purification of G6PD

The hemolysate solution obtained previously was loaded on the 2',5'-ADP Sepharose 4B affinity column, and the flow rate was adjusted to 20 ml/h. Then, the column was sequentially washed with 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate, (pH 6.0) and 25 ml 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The washing with 0.1 M KCl + 0.1 M K-phosphate, (pH 7.85) was continued until the final absorbance difference became 0.05. Finally, the enzyme was eluted with the solution of 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in the final fractions,

and the activity-containing tubes were pooled. In the resultant solution, the protein was determined. During all procedures, the temperature was kept at +4°C [2].

Determination of G6PD activity

The enzyme activity was measured by Beutler's method [1]. One enzyme unit (EU) was defined as the enzyme amount reducing 1 μmol $NADP^+$ per 1 min.

Protein determination

During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to the Bradford method, using bovine serum albumin as the standard [6].

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure [16] with 3% and 8% acrylamide concentrations for running and stacking gel, respectively. The gel solution was supplemented with 10% SDS.

In vitro dantrolene sodium effect

In order to determine the effects of dantrolene sodium on G6PD, five different concentrations of dantrolene sodium (0.17, 0.43, 0.6, 0.86, and 1.3 mM) were added to separate tubes containing purified enzyme. The enzyme activity was measured in these tubes, taking the tubes containing no drug as control (100% activity). The I_{50} values were obtained after activity in % was plotted *vs.* drug concentration. Drug concentration that produced 50% inhibition (I_{50}) was calculated from the graphs.

In vivo dantrolene sodium effects

Eight adult Sprague-Dawley rats (200–250 g) were selected for intraperitoneal administration of dantrolene sodium (10 $mg.kg^{-1}$). Blood samples (0.5 ml) were taken from each rat prior to dantrolene sodium administration as well as at 1, 3 and 6 h thereafter. They were collected into test tubes containing EDTA (2 mM) and centrifuged at 2500 × g for 15 min at +4°C. The erythrocyte pellet was washed three times with cold 0.16 M KCl and the supernatant was discarded. One volume of erythrocyte pellet was suspended in five volumes of ice water to give an erythrocyte hemolysate. G6PD activity was determined with Beutler's method [1]. Data were expressed as the mean ± SD (standard deviation). Statistical analysis comprised significance testing of the difference between means (control vs. test) using a two-tailed Student's *t*-test at the levels: 0.05, 0.01, and 0.001.

RESULTS

The purification process of human erythrocyte G6PD is summarized in Table 1. The first step used was ammonium sulfate fractionation. At the intervals of 35–65% ammonium sulfate saturation were made in hemolysate and the enzyme was almost completely separated (99%) from 6-phosphogluconate dehydrogenase activity by subjecting the supernatant to 0–35% saturated ammonium sulfate fractionation and then resulting pellet was dissolved with 50 mM phosphate buffer (pH 7.0). After ammonium sulfate fractionation, 2',5'-ADP Sepharose 4B affinity gel chromatography was performed. The elution profile of 2',5'-ADP Sepharose 4B affinity gel chromatography is shown in Fi-

Table 1. Purification scheme of G6PD from human erythrocytes by 2',5'-ADP Sepharose 4B affinity gel chromatography

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	0.42	75	41.6	3120	31.5	0.010	100	1
Ammonium sulfate precipitation (35–65)%	1.13	15	0.93	13.95	16.95	1.215	53.8	121.5
2,5-ADP Sepharose 4B affinity chromatography	4.1	3	0.042	0.126	12.3	97.6	39	9760

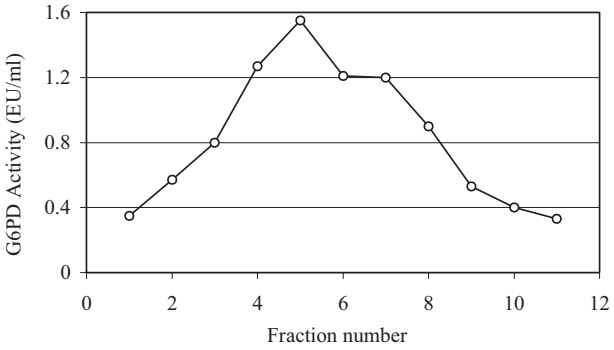

Fig. 1. Elution profile of G6DD activity from 2',5'-ADP Sepharose 4B affinity gel chromatography

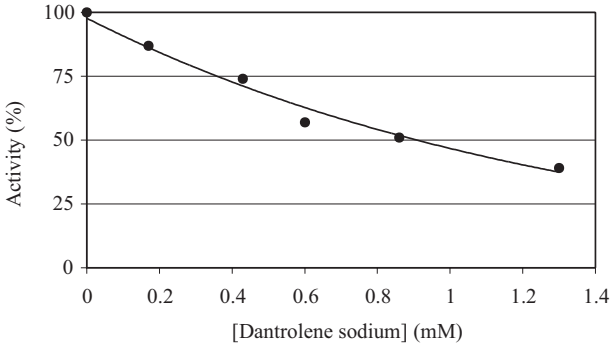

Fig. 3. Effect of dantrolene sodium concentration on human G6PD activity

Fig. 2. SDS-polyacrylamide gel electrophoresis of G6PD purified by affinity gel. (Lane 1: G6PD; Lane 2: yeast alcohol dehydrogenase)

gure 1. The procedure of Ninfali et al. [21] was followed to purify the enzyme from human erythrocytes. Specific activity of the preparation was 97.6 EU/mg of protein and 9760-fold purification was achieved with 39% yield (Tab. 1).

Figure 2 exhibits the SDS-PAGE carried out to test purity of the enzyme according to Laemmli's method.

Figure 3 shows the *in vitro* effects of the dantrolene sodium on the enzyme activity. Activity% vs. dantrolene sodium graph was drawn at 0.17, 0.43, 0.6, 0.86, and 1.3 mM dantrolene sodium concentrations (Fig. 3). I_{50} value was estimated at 0.91 mM (Tab. 2). The results of *in vivo* effects of the drug are presented in Table 3. In dantrolene sodium-treated group, the control enzyme activity was 4173 ± 621.5 EU (gHb^{-1}), while the respective values determined 1, 3 and 6 h after dantrolene sodium treatment were 3443 ± 502.5 ($p < 0.05$), 3285 ± 242.8 ($p < 0.005$), and 3340 ± 606.5 EU (gHb^{-1}) ($p < 0.05$). The greatest inhibition was found 3 h after the injection.

Table 2. Effect of dantrolene sodium (10 mg/kg *ip*) on rat G6PD activity

Time after administration (h)	G6PD activity (EU/gHb)[1,2]
0	4173 ± 621.5
1	3443 ± 502.5*
3	3285 ± 242.8**
6	3340 ± 606.5*

[1] Results are expressed as the means ± SD, $n = 8$; [2]* $p < 0.05$, ** $p < 0.005$ vs. control by Student's *t*-test (control value is enzyme activity before dantrolene administration; 0 h)

DISCUSSION

Dantrolene sodium, a peripherally acting skeletal muscle relaxant, is used in the treatment of muscle spasticity, malignant hyperthermia and neuroleptic malignant syndromes and depresses excitation-contraction coupling in the muscle fibre by inhibiting calcium release from the sarcoplasmic reticulum [18].

G6PD deficiency is an X-chromosome-linked hereditary disorder. The deficiency is a widespread condition affecting more than 100 million people in the world [15]. Oxidative stress occurs in erythrocyte cells and other tissues after treatment with some drugs. This situation can cause membrane destruction. As a result of this, chronic hemolysis in erythrocytes occurs.

Many chemicals at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme [15]. For example, in previous study, amikacin sulfate, ampicillin and netilmicin sulfate had inhibitory effects on rat erythrocyte G6PD activity. On the other hand, metamizole showed activatory effects on the enzyme [9]. Beydemir et al. reported the effects of some antibiotics on the activity of human erythrocyte carbonic anhydrase *in vitro* and rat erythrocyte carbonic anhydrase *in vivo* [4]. In addition, while gentamicin sulfate and vancomycin hydrochloride were found to inhibit sheep lens G6PD activity, sodium cefazolin and ceftriaxone were observed to activate this enzyme [5].

In another study, we determined the effect of dantrolene sodium on carbonic anhydrase from erythrocyte [14]. It was found that dantrolene sodium had inhibitory effect on the carbonic anhydrase activity at the concentration of 10 mg/kg as *in vivo*. However, there is no information about the effect of dantrolene sodium on the other enzymes.

In this study, human erythrocyte G6PD was purified 9760-fold by using ammonium sulfate fractionation (35–65%) and 2′,5′-ADP Sepharose 4B affinity chromatography (Tab. 1). SDS-PAGE confirmed the purity of the enzyme. As seen in the gel photograph, a high-purity enzyme was obtained with this method (Fig. 2).

The range of dantrolene sodium concentrations used was considered adequate to show the enzyme inhibition. In this regard, it was evident from *in vitro* studies that the G6PD was inhibited by dantrolene sodium at 0.17–1.3 mM concentrations (Fig. 3). The IC_{50} value was 0.91 mM for G6PD (R^2: 0.9786). In *in vivo* study, maximum inhibition of rat erythrocyte G6PD activity was observed at 10 $mg.kg^{-1}$ of dantrolene sodium within 3 h ($p < 0.005$; Tab. 2) after drug administration and inhibition still persisted after 3 h ($p < 0.05$; Tab. 2). Our data show that there is a relationship between *in vivo* and *in vitro* effects of dantrolene sodium on G6PD activity.

In conclusion, dantrolene sodium showed *in vitro* and *in vivo* inhibitory effects on erythrocyte G6PD activity. The use of dantrolene in a patient with G6PD deficiency can cause serious side effects and worsen health of this patient. For this reason, dantrolene sodium must be carefully used and its dosages should be very well ordered to decrease the side effects.

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