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The in Vitro Effects of Dexamethasone on Sheep Lens Glucose-6-Phosphate Dehydrogenase

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Glucose-6-phosphate dehydrogenase (G6PD) enzyme was purified from sheep lenses, and the in vitro effects of dexamethasone on the enzyme's activity were investigated. Sheep lens glucose-6-phosphate dehydrogenase was purified 10,000-fold by using ammonium sulphate fractionation and 2′,5′-ADP-Sepharose 4B affinity gel chromatography for a yield of 83.8% and a specific activity of 7.6 EU/mg protein. Enzyme activity was determined by Beutler's method. Dexamethasone strongly inhibited the enzyme under in vitro conditions. The I_{50} value of the dexamethasone was 3.05 mM.

Key Words: Sheep eye lens, dexamethasone, glucose-6-phosphate dehydrogenase.

Introduction

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is a key enzyme that catalyses the oxidation of D-glucose 6-phosphate to D-glucose-O-lactone 6-phosphate in the presence of $NADP^+$[1,2]. It is well known that the dehydrogenase exists in the oxidative part of the pentose phosphate metabolic pathway. The major role of the pathway is to produce NADPH, which is used for the protection of cells against oxidative damage[3,4]. In addition, NADPH is widely used in biosynthesis and in drug detoxification[5−7]. Many enzymes play an important role in maintaining the

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integrity of the lens metabolism[8]. The pentose phosphate metabolic pathway functions in the lens to generate reduced equivalents (NADPH), which are used mainly for the maintenance of reduced glutathione (GSH) levels[8,9]. The mammalian lens contains an usually high concentration of GSH, the highest level being in the epithelium[10]. Lens G6PD activity has been investigated in some mammals such as bovine and rats[11,12].

G6PD deficiency is widespread throughout the world, especially along the Mediterranean coast[13]. This enzyme deficiency is found in all tissues, including the crystalline lens[14]. Genetic abnormality and age may cause deficiency of this enzyme[13]. A cataract is usually defined as any opacity in the lens. A number of studies have revealed a relationship between cataractogenesis and G6PD deficiency[15–18]. The possible causes, mechanisms and biochemical and biophysical changes occurring during cataractogenesis have been the subject of intense study for many years. It has also been suggested that etiological factors have important roles in causing cataract development, such as heredity, latitude, exposure to light and some drugs[13,19]. Numerous individual causes of cataract exist and often multiple factors act together. The association between corticosteroid use and posterior subcapsular cataract has been noted consistently[20,21]. The fall in nonprotein and protein –SH could be the first event in the well-known biochemical changes that occur in steroid-induced cataract. The mechanism underlying steroid-induced damage could be due to a conformational change of lens crystallins which results in an unmasking of –SH groups with a consequent increased susceptibility to oxidation[21,22].

Dexamethasone is commonly used in the treatment of some inflammatory eye diseases[23–26]. We aimed to investigate the in vitro effects of dexamethasone on lens G6PD activity and to identify a possible adverse effect such as inhibition of G6PD, which is suggested to be associated with cataract formation.

Materials and Methods

Materials

2′, 5′’-ADP-Sepharose 4B was obtained from Pharmacia. $NADP^+$, glucose-6-phosphate, protein assay reagent and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals of analytical grade were obtained from either Sigma or Merck.

In Vitro Studies

Preparation of Homogenate

The lenses were extracted at 4 °C from sheep obtained from a local slaughterhouse. The lenses were washed 3 times with 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM 2-mercaptoethanol (2-ME). The lenses were pulverised in liquid nitrogen (approximately –163 °C), transferred to 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM 2-ME, and centrifuged at 4 °C, 39,100 x g for 30 min. Supernatant was used in further studies[27].

Ammonium Sulphate Fractionation and Dialysis

The homogenate was subjected to precipitation with ammonium sulphate (between 10 and 90%). Ammonium sulphate was slowly added to the homogenate and constant stirring was used for complete dissolution. This mixture was centrifuged at 10,000 x g for 15 min and the precipitate was dissolved in 50 mM of phosphate

buffer (pH 7.0). Enzyme activity was determined both in the supernatant and in the precipitate for each respective precipitation step. The enzyme was observed to precipitate at the 0-30% precipitation step. The resultant solution was clear, and contained partially purified enzyme. This solution was dialysed against 4 °C in 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0) for 2 h with 2 changes of buffer[28].

2′, 5′-ADP Sepharose 4B Affinity Chromatography

Two grams of dried 2′, 5′-ADP Sepharose 4B gel was used for a 10 mL column volume. The gel was washed with 400 mL distilled water to remove foreign bodies and any air in the swollen gel was eliminated. The gel was suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0) and then packed in a small column (1 x 10 cm) and equilibrated with the same buffer (50 mL). The gel was washed with 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0). The flow rates for washing and equilibration were adjusted by peristaltic pump 50 mL/h. A dialysed sample obtained previously was loaded on a 2′, 5′-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 mL/h. Then the column was sequentially washed with 20 mL 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), 25 mL of 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85) and 0.1 M KCl/0.1 M K-phosphate (pH 7.85) until the final absorbance was 0.05. Elution was carried out with 80 mM K-phosphate, 80 mM KCl, 0.5 mM $NADP^+$ and 10 mM EDTA (pH 7.85). Eluates were collected in 2 mL tubes and their activity was separately measured as EU/mL. Enzyme activity was measured in final fractions, and the activity-containing tubes were pooled. All of the procedures were performed at 4 °C[28,29].

Activity Determination

The in vitro enzyme activity was investigated as follows: 580 μL of distilled water, 20 μL of sheep lens G6PD enzyme sample, 100 μL of 2 mM $NADP^+$, 100 μL of 0.1 M $MgCl_2$, and 100 μL of 1 M Tris-HCl buffer (pH 8.0) containing 5 mM EDTA were added to the reference cuvette, and 20 μL of sheep lens G6PD enzyme sample, 100 μL of 2 mM $NADP^+$, 100 μL of 0.1 M $MgCl_2$, 100 μL of 1 M Tris-HCl buffer (pH 8.0) and 5 mM EDTA containing 100 μL of 6 mM glucose-6-phosphate (G6P) were added to the sample cuvette. The enzymatic activity was measured by Beutler's method[30]. NADPH produced in the reaction mixture was measured at 340 nm as absorbance (OD). One unit of enzyme (EU) activity was defined as the enzyme amount reducing 1 μ mol $NADP^+$ per 1 min at 25 °C, pH 8.0.

Protein Determination

Quantitative protein determination was done spectrophotometrically at 595 nm according to Bradford's method, with bovine serum albumin used as a standard[31].

SDS Polyacrylamide Gel Electrophoresis

The purified enzyme was subjected to SDS polyacryamide gel electrophoresis according to Laemmli's method[32]. It was carried out in 4% and 10% acrylamide concentrations for stacking and running gels containing 0.1% SDS, respectively. Protein standards, sheep erythrocyte G6PD, and sheep lens G6PD were applied to the gels (20 μg for each sample and standard). Gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without dye. The electrophoretic pattern was photographed.

In vitro studies by dexamethasone

In order to determine the effects of dexamethasone on G6PD, several concentrations of dexamethasone (0.38, 0.77, 1.54, 3.08, 4.38 mM), were added to separate tubes containing purified enzyme. The enzyme activity was measured in these tubes while taking the tubes containing no drug as a control (100% activity). The experiments were repeated 3 times. The I_{50} values were obtained after percent activity was plotted vs. dexamethasone concentration.

Results

The purification process of sheep lens G6PD is summarised in Table 1. The first step used was ammonium sulphate fractionation. At intervals of 0-60%, ammonium sulphate saturation was performed in homogenate and the enzyme in the lens was almost completely separated (99%) from 6-phosphogluconate dehydrogenase activity by subjecting the supernatant to 0-30% saturated ammonium sulphate fractionation and then the resulting pellet was dissolved with 50 mM phosphate buffer (pH 7.0). After ammonium sulphate fractionation, 2′, 5′-ADP Sepharose 4B affinity gel chromatography was followed. The elution profile of 2′, 5′-ADP Sepharose 4B affinity gel chromatography is shown in Figure 1. The procedure of Ninfali et al. [28] was followed to purify the enzyme from sheep lens; specific activity of 7.6 EU/mg protein and 10,000-fold was achieved with an 83.8% yield (Table).

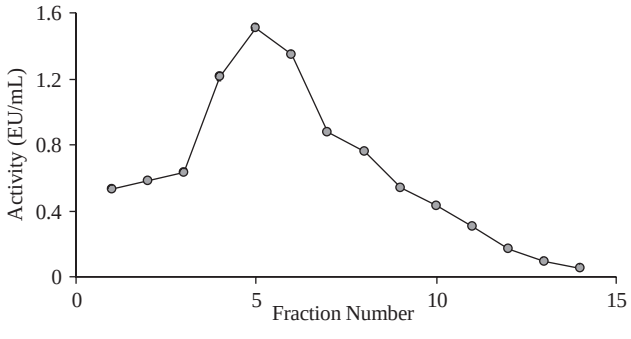

Figure 1. Activity-fraction number profile for elution of G6PD enzyme from 2′,5′-ADP-Sepharose 4B affinity chromatography.

Table. Purification scheme of glucose-6-phosphate dehydrogenase from sheep lens.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	0.012	57	15.6	889.2	0.68	0.00076	100	1
Ammonium sulphate precipitation (0-30%) saturation	0.12	5	7.16	35.8	0.6	0.017	88.2	22
2′,5′- ADP Sepharose 4B affinity chromatography	0.19	3	0.025	0.075	0.57	7.6	83.8	10,000

Figure 2 exhibits the SDS-PAGE performed for the purity and subunit molecular weight of the enzyme according to Laemmli's method[32]. The subunit molecular weight was 54,957 Da.

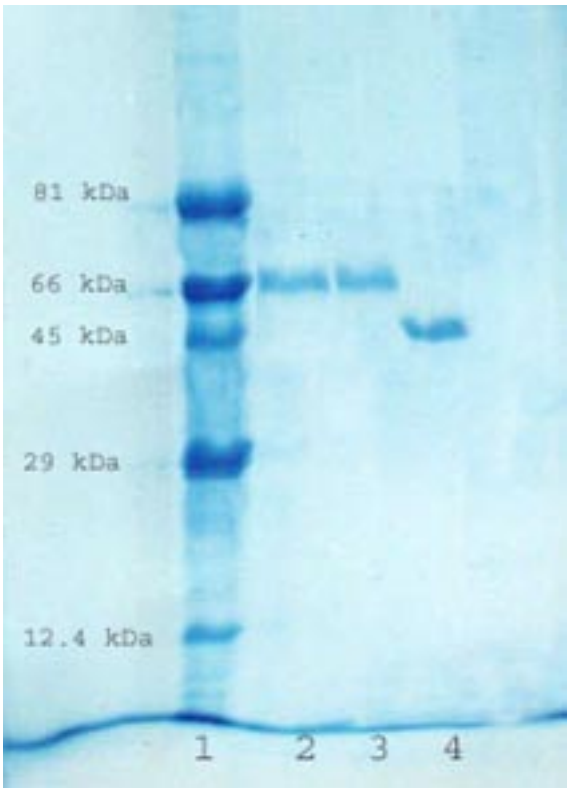

Figure 2. SDS–polyacrylamide gel electrophoresis of G6PD (Lane 1: Protein standards: Rabbit heart creatine phosphokinase (81 kDa), bovin serum albumin (66 kDa), chicken ovalbumin (45 kDa), and bovine carbonic anhydrase (29 kDa), horse heart cytochrome C (12.4 kDa); Lane 2 and 3: Sheep erythrocyte G6PD; Lane 4: Sheep lens G6PD).

Our study revealed that dexamethasone concentrations (between 0.38 and 4.38 mM) in vitro at low concentrations inhibited the G6PD activity of sheep lens. An activity % -[Dexamethasone] graph was drawn at 0.38, 0.77, 1.54, 3.08, 4.38 mM dexamethasone concentrations (Fig. 3). Dexamethasone concentration producing 50% inhibition (I_{50}) was calculated from an equation on the graph. The I_{50} value obtained from in vitro studies was 3.05 mM.

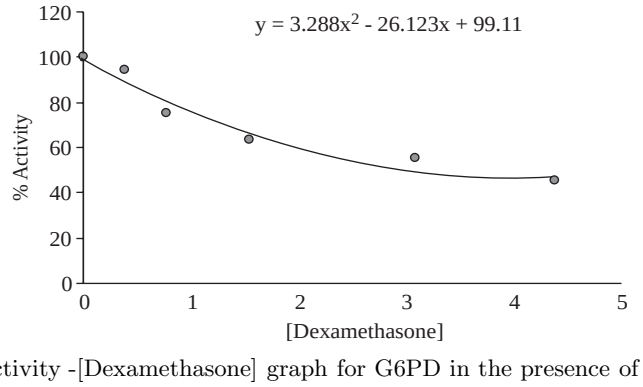

Figure 3. % Activity -[Dexamethasone] graph for G6PD in the presence of dexamethasone.

Discussion

Many chemicals when administered at relatively low doses affect the metabolism by altering normal enzyme activity, particularly through the inhibition of a specific enzyme. The effects can be dramatic and systemic[33]. For example, Naveh and Marshall investigated the anti-inflammatory effect of melanocyte-stimulating hormone (MSH), which is a known anti-inflammatory agent, after ocular surgery[34]. Additionally, halothane was found to inhibit red blood cell and liver G6PD enzyme activities significantly in the first week and to activate this enzyme in the second week of anesthesia compared with a control group[35]. In another study, G6PD enzyme activity was inhibited by melatonin in human erythrocytes in vitro and in rat erythrocytes in vivo[36]. G6PD seems to be utilised in the defence mechanism against the free radicals generated in the metabolism of some drugs. Enzyme deficiency has been shown in some tissues, including the lens[15]. G6PD deficiency is still the most common of all clinically significant enzyme defects in living system biology as a whole[15]. G6PD deficiency plays an important biochemical role in the metabolism of the lens[18], because the reaction catalysed by G6PD in the lens generates reduced equivalents (NADPH), which are used mainly for the maintenance of reduced GSH levels[4,10]. One possible function of GSH in the lens is to maintain the thiol (-SH) groups of proteins in a reduced state, thus preventing the formation of high molecular weight protein aggregates[10]. Furthermore, GSH may also prevent oxidative damage to the lens[10]. Orzalesi et al. have reported G6PD deficiency to be a predisposing factor for the development of a cataract[37]. In addition, in a study carried out on the erythrocyte and lens, G6PD in senile and presenile cataracts was examined and it was found that G6PD deficiency might be a cataractous pathogenetic factor for presenile cataract[17].

It has also been suggested that certain etiological factors have important roles in cataract development, such as heredity, latitude, exposure to light and some drugs such as corticosteroids[13,19]. Corticosteroids have been used to control inflammatory and immunological diseases of the eye. Corticosteroids cause several adverse reactions, including posterior subcapsular cataract formation[20−22]. It is known that oxidative stress occurs after the administration of some drugs in red blood cells and other tissues[38−40]. Dexamethasone is a corticosteroid which is commonly used in the treatment of some inflammatory eye diseases[23−26].

In our study, we investigated the in vitro effects of dexamethasone on sheep lens G6PD activity. For this purpose, sheep lens G6PD was purified by ammonium sulphate fractionation (0-30%) and 2′, 5′-ADP-Sepharose 4B affinity gel chromatography (Table and Figure 1) and its purity confirmed by SDS-PAGE (Figure 2). After overall purification, the G6PD enzyme was obtained with a recovery of 83.8% and a specific activity of 7.6 U/mg proteins. This enzyme was also purified 10,000-fold (Table). In 1999, Ulusu et al. reported purifying the G6PD enzyme with a yield of 13.7% , with a specific activity of 2.64 EU/mg proteins, and 19,700-fold using 2′, 5′-ADP-Sepharose 4B affinity gel chromatography and DEAE-Sepharose ion exchange column chromatography from bovine lens[11]. In addition, the enzyme was purified with a specific activity of 2.64 EU/mg proteins and 9150-fold from chicken erythrocytes by Yılmaz et al.[1]

As seen in gel photographs, sheep erythrocyte G6PD and sheep lens G6PD have different subunit molecular weights (66,880 Da and 54,957 Da, respectively). The subunit molecular weight of sheep lens G6PD was similar to bovine lens G6PD subunit molecular weight (69 kDa)[11]. Figure 3 shows that dexamethasone administration decreased enzyme activity. The I_{50} value was 3.05 mM.

In conclusion, dexamethasone, which inhibits G6PD activity in the lens, may be a contributing factor in cataract development. If the administration of dexamethasone to a patient with G6PD deficiency is

required, routine ophthalmic examinations should be performed. Because sheep lens G6PD may be quite different from that of the human enzyme, the penetration and dose/effect ratio under clinical settings are unknown. Further postmortem studies are needed to evaluate the enzyme activity in human lenses as well.

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