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EFFECT OF DEHYDROEPIANDROSTERONE ON HUMAN ERYTHROCYTES REDOX METABOLISM: INHIBITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY *IN VIVO* AND *IN VITRO*

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Summary—In order to elucidate the role of G6PD inhibition by DHEA on erythrocyte redox metabolism, we measured: (1) G6PD activity in erythrocytes collected at different times after injection of synthetic ACTH in 13 normal subjects; (2) G6PD activity and GSH levels in erythrocytes incubated in the presence of different DHEA concentrations; (3) DHEA distribution and metabolic clearance in red cells, together with G6PD activity; (4) GSH regeneration after hydroperoxide oxidative stress in red cells preincubated in the presence of DHEA.

In vivo DHEA increase elicits a clear-cut inhibition of red cell G6PD activity. Decreasing DHEA goes in step with the recovery of enzyme activity. *In vitro* G6PD inhibition by DHEA reaches its maximum within 10–15 min (20–25% inhibition at 10^{-7} M DHEA concentration) and the recovery time is dose-dependent. More than 2/3 of DHEA is concentrated in the red cell after 5 min incubation. GSH levels change in step with G6PD activity. After oxidative stress by BHP, DHEA-treated cells fail to restore normal GSH concentrations.

These results show that DHEA inhibits human erythrocyte G6PD activity at concentrations usually observed after ACTH plasma increase and increases red cell sensitivity to oxidant agents. Moreover, it is possible that the high DHEA concentrations present in target tissues may interfere with metabolic pathways in which NADPH is the cofactor.

INTRODUCTION

In vitro inhibition by dehydroepiandrosterone (DHEA) of glucose-6-phosphate dehydrogenase (G6PD) from human placenta [1] and human erythrocytes [2] has been described. Free DHEA is an excellent non-competitive inhibitor of G6PD in human erythrocytes [3]. Its sulphate, on the other, proved ineffectual [4].

Under physiological conditions a distinct inverse relationship between DHEA levels and G6PD activity has been observed [5]: moreover, administration of DHEA sulphate to obese patients resulted in a temporary increase in their low plasma DHEA levels and, by this, in a simultaneous decrease in erythrocyte G6PD activity [6].

Circulating DHEA sulphate represents a large pool of at least 3.5 mg and is not subject to diurnal fluctuations [7]. Circadian rhythms in plasma levels of DHEA are observed [8], however, and rapid changes can also be elicited by stimulation of suppression of the adrenal glands.

The physiological concentration of DHEA in plasma was believed to be too low to regulate G6PD activity. Target tissues, on the other hand, may contain much higher levels [9].

In order to establish the DHEA concentrations required to inhibit G6PD activity *in vivo* and *in vitro*, and the result of G6PD inhibition on erythrocyte redox metabolism, we measured: (1) G6PD activity in erythrocytes collected at different times after injection of synthetic ACTH. ACTH stimulation was used to increase plasma DHEA because, like all adrenal steroids, except pregnenolone, it has little effect on G6PD activity [2, 10]; (2) G6PD activity and GSH levels in erythrocytes incubated in the presence of different DHEA concentrations; (3) DHEA distribution and metabolic clearance in red cells together with G6PD activity; (4) GSH regeneration after hydroperoxide oxidative stress in red cells preincubated in the presence of DHEA.

EXPERIMENTAL

In vivo *studies*

Five healthy men and 5 healthy women in the luteal phase, aged 20–40 years, received 250 μg synthetic ACTH i.v. after an overnight fast. Blood samples were collected before and after 60, 90, 120 and 180 min after the injection. Plasma for DHEA determination was separated and stored at −20°C. Three additional ACTH tests were performed: in

Abbreviations: G6PD: glucose-6-phosphate dehydrogenase; DHEA: dehydroepiandrosterone; GSH: reduced glutathione; BHP: *t*-butyl-hydroperoxide.

these cases erythrocytes were separated from plasma at different times, washed twice and lysed by freezing and thawing, then stored at −20°C together with plasma for DHEA determination. In both sets of experiments, DHEA was measured by radioimmunoassay: it was extracted with ethyl–ether (1:10, v/v) and freed from cross-reacting steroids by celite-ethylene glycol minicolumn chromatography [11]. The DHEA fraction was assayed against a standard curve with a range of 15–1000 pg per tube, using an antiserum raised against DHEA-3-hemisuccinate-BSA-conjugate (BioMèrieux, Charbonnières-les-Bains, France). Duplicate determinations showed a variation coefficient less than 13% over the range used in the assay. Haemolysates were prepared from freshly drawn blood and G6PD activity was measured according to the ICSH recommendation [12] and expressed as I.U. (μmol/min)/g hemoglobin.

In vitro *studies*

Blood from normal donors with heparin as anticoagulant was used within 4 h. Blood was separated into cells and plasma: plasma and leukocytes were removed by aspiration and filtration through cellulose. Cells were washed three times with isotonic phosphate saline buffer (pH 7.4) containing 5.5 mM glucose and resuspended in the same buffer to hematocrit of 40%. The red cell suspension was brought to the temperature of incubation (37°C) and DHEA and/or *t*-butyl-hydroperoxide (BHP) were added as specified in the Results; the final mixtures were removed at appropriate time intervals for the determination of G6PD activity. Parallel GSH determinations were carried out by the 5-5′-dithiobis-(2-nitrobenzoic acid) method [13]. In order to investigate DHEA distribution together with G6PD activity, washed erythrocytes were suspended in isotonic phosphate saline buffer (pH 7.4), containing 5.5 mM glucose (hematocrit 40%, 37°C). DHEA was added to the suspension at final concentrations corresponding to 0.022, 0.086, 0.35 and 3.5×10^{-6} M solutions. After incubation for 5 min at 37°C, samples were taken for G6PD determination, the suspension was centrifuged at 3000 *g* (0°C), the supernatant was removed and stored at −20°C for DHEA determination. The cells were washed twice with cold buffer; after the removal of the last supernatant, the erythrocytes were lysed by freezing and thawing and stored at −20°C for DHEA determination. BHP was purchased from Koch-Light, Colnbrook, Bucks, U.K.; DHEA, all the other fine chemicals and the enzymes were purchased from Sigma Chemical Co., St Louis, MO, USA.

RESULTS

Plasma DHEA increases after ACTH (basal level: 7.3 ± 2.8 pmol/ml). The mean maximum increase of plasma DHEA is observed after 2 h (Fig. 1). The measure of red cell DHEA, performed in three subjects, shows a positive correlation ($P < 0.001$) between intracellular and plasma DHEA (Table 1). The DHEA increase elicits a clear-cut inhibition of red cell G6PD activity (basal levels: 9.8 ± 0.3 I.U./g Hb): maximum enzyme inhibition is observed 120 min after ACTH injection. In the 3 subjects in which red cell DHEA is measured, enzyme inhibition peaks together with intracellular DHEA increase in 2 cases. No change in G6PD activity is noted in the third subject, who shows a poor DHEA response to ACTH. Decreasing intracellular DHEA goes in step with the recovery of enzyme activity.

The dose-dependence of *in vitro* G6PD inhibition by DHEA is shown in Fig. 2. Maximum inhibition is reached within 10–15 min at any dose. At a DHEA concentration of 0.022×10^{-6} M a 11% inhibition is observed after 5 min incubation; at 0.086×10^{-6} M DHEA inhibition is 20% after the same period. At 0.35×10^{-6} M DHEA, inhibition reaches 30% after 15 min incubation. Higher concentrations (3.5×10^{-6} M) do not enhance inhibition. Complete recovery is observed within 30 min at lower concentrations, within 120 min at 0.35×10^{-6} M: no recovery takes place at higher concentrations (3.5×10^{-6} M).

Figure 3 shows that G6PD impairment results in a sudden, dose-dependent decrease in red cell GSH. Moreover, GSH levels change in step with G6PD activity: complete recovery is obtained at lower DHEA concentrations, while the recovery is partial with higher concentrations.

Table 2 shows DHEA distribution between the medium and the erythrocytes after 5 min incubation at different starting concentrations of the steroid (0.022, 0.086, 0.35 and 3.5×10^{-6} M). DHEA fraction inside the red cells is very similar in all conditions and represents about 68% of total DHEA. As far as the metabolic clearance is concerned, it appears that up to 50% DHEA is metabolized by the red cell in 5 min. At an intracellular concentration of

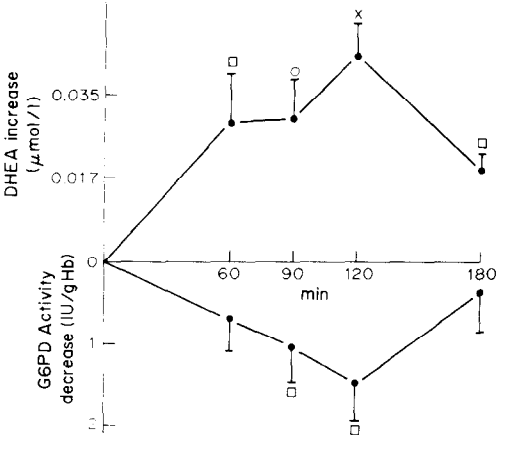

Fig. 1. Plasma DHEA increase compared to basal level (0.007 ± 0.0028 μM) and red cell G6PD activity decrease (basal level: 9.8 ± 0.3 I.U./g Hb) in ACTH-injected subjects. Mean values ± SE (□ $P < 0.05$; ○ $P < 0.02$; × $P < 0.01$).

Table 1. DHEA concentrations (pmol/ml) in erythrocytes and in plasma, and red cell G6PD activity (I.U./g hemoglobin) after injection of 250 μg synthetic ACTH

Time (min)		0	60	90	120	180
Subject 1	Red cell DHEA	6.10	14.00	22.50	28.00	13.30
	Plasma DHEA	34.00	78.00	82.00	82.00	68.00
	G6PD activity	8.30	7.89	7.22	6.37	8.48
Subject 2	Red cell DHEA	3.10	5.50	13.30	8.90	5.10
	Plasma DHEA	10.20	38.90	49.50	41.00	39.00
	G6PD activity	9.40	8.00	7.65	7.99	10.50
Subject 3	Red cell DHEA	5.50	3.40	3.70	5.50	3.80
	Plasma DHEA	28.70	24.20	31.40	37.60	13.30
	G6PD activity	9.42	8.52	9.04	8.74	9.40

17 pmol/ml RC, G6PD activity decreases by 13.5% at 56 pmol/ml RC a 25% inhibition is observed. Higher intracellular concentrations (390 and 2800 pmol/ml RC) do not further increase enzyme inhibition.

Addition of a concentration of BHP equimolar with GSH quantitatively oxidizes GSH. In the presence of glucose, control erythrocytes regenerate all their GSH in about 30 min. DHEA-treated cells fail to regain the normal GSH level. In this case 3.5×10^{-6} M DHEA resulted in 37% inhibition of G6PD activity (Fig. 4).

DISCUSSION

Our results show that DHEA inhibits human erythrocyte G6PD activity at the plasma concentrations usually observed after ACTH, both *in vivo* and *in vitro*.

Inside the red cell, DHEA is converted to androstenediol by 17-β-hydroxysteroid dehydrogenase. This NADPH-dependent reaction is probably responsible for both the disappearance of inhibition and the concomitant regeneration of GSH. Steroids with a free 17-oxo group are reduced by 17β-hydroxysteroid dehydrogenase in intact and lysed human erythrocytes, and up to 50% of DHEA is converted to androstenediol by intact human erythrocytes [14]. In our *in vitro* experimental conditions (no plasma proteins in the buffer medium) the uptake of DHEA by intact red cells is very rapid: this uptake can be considered as a partition between a lipid-rich membrane phase (red cell) and an aqueous buffer phase (medium). As far as the percent distribution of DHEA in both phases is concerned, more than 2/3 of the compound is concentrated in the red cells. When the DHEA concentration in the suspension is 0.086×10^{-6} M ($\sim 10^{-7}$ M), the red cell concentration is 56 pmol/ml RC and G6PD activity decreases by 25%. The very fast DHEA metabolic clearance rate can be an additional explanation for rapid G6PD activity and GSH levels recovery.

G6PD inhibition leads to a decrease in intracellular GSH levels. This level is a sensitive index of hexose monophosphate shunt activity, since an adequate supply of GSH is assured via the NADPH-dependent glutathione reductase, and NADPH is regenerated in the G6PD and 6-phosphogluconate dehydrogenase reactions of the shunt [15, 16].

G6PD catalyzes the first reaction and plays an essential regulatory role in the shunt. Under physiological conditions, G6PD activity is mainly regulated by $NADP^+$ (with which the enzyme interacts cooperatively) and NADPH (a product inhibitor): G6PD, in turn, is the major erythrocyte controller of $NADP^+$, NADPH levels and, through the glutatione

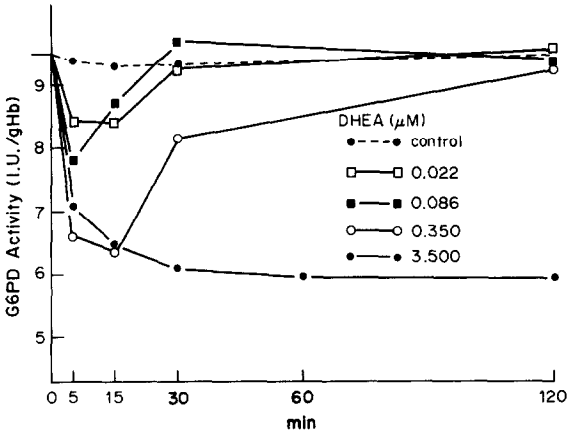

Fig. 2. Dose-dependence of human red cell G6PD activity *in vitro* inhibition by DHEA. Representative experiment from 4 with similar results.

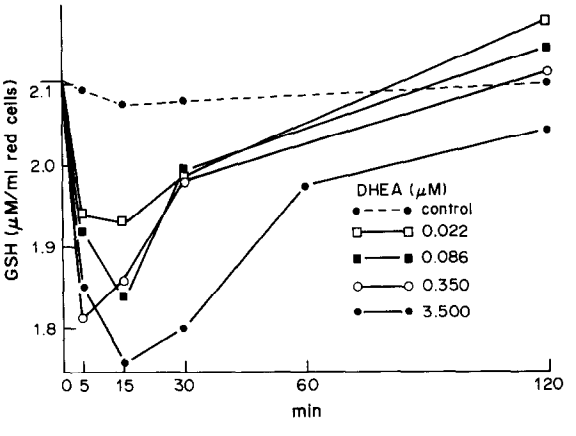

Fig. 3. Dose-dependence of GSH level decrease in human red cells incubated in the presence of DHEA. Representative experiment from 4 with similar results.

Table 2. DHEA distribution and metabolic clearance in red cells (RC) and related G6PD inhibition

Time 0	After 5 min incubation at 37°C, hematocrit 40%					
pmol DHEA/ml red cell suspension	pmol DHEA/ml red cell suspension*	pmol DHEA/ml red cells	pmol DHEA/ml supernatant	% DHEA in the red cells*	% DHEA metabolized*	% G6PD inhibition
22	9.8	17	5	70	56	13.5
86	34.8	56	20	65	59	25.0
350	210.0	390	90	74	40	27.5
3500	1750.0	2800	1050	64	50	25.0

*Calculated values.
Values are means of two experiments.

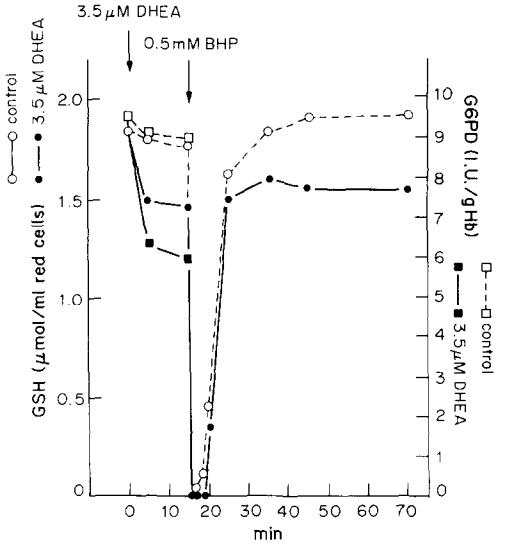

Fig. 4. Oxidation by BHP and regeneration of GSH in control and in DHEA-treated human red cells. G6PD activity inhibition in DHEA-treated cells is shown (−37% compared to the basal level). Representative experiment from 4 with similar results.

reductase, of GSH. Low GSH and NADPH levels are observed in G6PD-deficient erythrocytes [15, 16]: a very similar metabolic pattern occurs in erythrocytes after G6PD inhibition by DHEA.

When DHEA increases, G6PD inhibition and hexose monophosphate shunt impairment are present both in steady state conditions and after oxidative stress, as the BHP experiment demonstrates. BHP rapidly oxidizes erythrocyte GSH through the GSH-peroxidase [17]: depletion of GSH from normal cells cannot be attained in the presence of glucose, while DHEA-treated erythrocytes are unable to restore normal GSH concentration in the face of BHP oxidative challenge. This is also a distinctive feature of G6PD-deficient red cells [17] and indicates increased red cell sensitivity to oxidant agents. Membrane and cytoskeletal protein–SH groups are protected from oxidation by GSH in erythrocytes. Cross-linking of membrane and cytoskeleton proteins has been demonstrated *in vitro* through formation of intermolecular disulphide bonds in cells depleted of GSH [18]: these show decreased membrane deformability and survival.

Since DHEA can reach 10^{-7} M in human peripheral plasma after ACTH stimulation (corresponding to about 20–25% *in vitro* G6PD inhibition), it seems likely that it takes part in regulation of G6PD under physiological conditions. The fall in erythrocyte G6PD activity and GSH level after ACTH may be of importance in many clinical conditions: it is reasonable to suppose that in G6PD-deficient subjects, adrenal hypersecretion, whatever its cause (stress, fever . . .), may result in haemolytic crisis [19].

Moreover, it is possible that the high DHEA concentrations present in target tissues, such as the placenta and the breast, may interfere with the metabolic pathways in which NADPH is the cofactor (mainly mixed function oxidases).

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