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In Vivo and *In Vitro* Effects of Some Plant Hormones on Rat Erythrocyte Carbonic Anhydrase and Glucose-6-Phosphate Dehydrogenase Activities

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The present study was undertaken to determine *in vivo* and *in vitro* effects of some plant growth regulators on rat erythrocyte carbonic anhydrase (CA) and glucose-6-phosphate dehydrogenase (G6PD) activities. Both *in vivo* and *in vitro*, spermidine and kinetin did not affect enzymatic activities of CA and G6PD, whereas putrescine decreased these activities, and abscisic acid increased them. Since plants use such growth regulators, their effects should be considered on mammals consuming them since they may possess important biological effects.

Keywords: Spermidine; Kinetin; Putrescine; Abscisic acid; Carbonic anhydrase; Glucose-6-phosphate dehydrogenase; Rat

INTRODUCTION

Plant growth regulators (PGRs)—endogenous hormones of plants—are widely used chemicals, often included in the diet of all herbivorous and omnivorous animals.[1–5]

The amount of such substances introduced into the environment may soon exceed that of insecticides.[1] The effects of different PGRs on insects have been investigated, but reports on vertebrates are very limited.[6] In the literature, it has been reported that PGRs cause an increase in the number of splenic plaque-forming cells and circulating white blood cells, hematocrit values, and thymus weight in young deer mice.[7] El-Mofty and Sakr found that GA_3 (gibberellic acid, a plant growth hormone) induced liver neoplasm in Egyptian toads and they suggested that the tumours could be diagnosed as hepatocellular carcinomas.[8]

Some biogenic polyamines, such as putrescine and spermidine, act as plant growth regulators, and are essential for cell renewal and it was previously believed that every cell in the body is able to synthesise polyamines. However, it has been shown that, as in the case of essential amino acids, the diet can supply sufficient amounts of polyamines to support cell renewal and growth. The major sources of putrescine are fruits, cheese and non-green vegetables.[9] All foods contribute similar amounts of spermidine to the diet, although its level is generally higher in green vegetables. However, only a part of the polyamines supplied by the diet is available for use by the body. Polyamines are readily taken up from the gut lumen, probably by passive diffusion, and are partly metabolised during the process of absorption. More than 80% of the putrescine is converted to other polyamines and non-polyamine metabolites, mostly amino acids.[10] The effects of polyamines have not been studied on mammals.

The enzyme carbonic anhydrase (EC 4.2.1.1.) catalyses reversible hydration of CO_2 to HCO_3^- and H^+ and is present in nearly all organisms.[11] Fourteen distinct isoenzymes of carbonic anhydrase have been characterised in higher vertebrates.[11,12] The main physiological function of all carbonic anhydrase isoenzymes is to facilitate the interconversion of CO_2 and HCO_3^- so that they play key roles in diverse processes such as physiological pH control and gas balance, calcification, biosynthesis of lipids, ureaneogenesis (in animals), and photosynthesis (in plants).[11–13]

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) catalyses the first step of the pentose phosphate

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metabolic pathway and is a unique supplier of NADPH in red blood cells.[14] NADPH production is decreased in G6PD deficiency. The most important role of NADPH is reduction of glutathione. Reduced glutathione has a free thiol group and acts as an antioxidant. Normally, the ratio of reduced glutathione to oxidised glutathione is approximately 500. Reduced glutathione also plays a role in some detoxification reactions by reducing inorganic and organic peroxides.[15,16]

The present study was undertaken in order to determine *in vivo* and *in vitro* effects of some PGRs on rat erythrocyte CA and G6PD (since human and mouse G6PD enzymes show homology). It should be mentioned that literature reports concerning the effects of such compounds on these enzymes in higher animals are very limited.

MATERIALS AND METHODS

Materials

Sepharose 4B, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma–Aldrich Co. (Sigma–Aldrich, Taufkirchen, Germany). p-Aminobenzene sulfonamide and L-tyrosine were from E. Merck (Merck, Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either Sigma–Aldrich or Merck.

Animal Studies

Adult (200–250 g) Spraque Dawley rats raised for 2 months were separated into four groups, each group containing 10 animals. Firstly for control experiment, 0.5 mL blood sample from each rat was placed into test tubes containing EDTA. Then either 3.917 mg/kg putrescine, 6.452 mg/kg spermidine, 1.91 mg/kg kinetin or 2.346 mg/kg abscisic acid (ABA) were injected intraperitoneally into each rat group.[17]

Blood samples (0.5 mL) were taken from each rat at 1, 3 and 5 h after injection, centrifuged at 2500 × g for 15 min and the erythrocyte pellet washed with 0.16 M KCl three times and the supernatant discarded.[18,19] The erythrocyte pellet was haemolysed in five volume of ice water at 4°C. These haemolysates were used for measurements of CA and G6PD activity.

Purification of Rat Erythrocyte Carbonic Anhydrase by Affinity Chromatography

Erythrocytes were purified from fresh rat blood (10 ml). Following low-speed centrifugation (1,500 rpm for 15 min.) (MSE, MISTRAL 2000) and removal of plasma and buffy coat, the red cells were isolated, washed twice with 0.9% w/v NaCl, and haemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation (20,000 rpm for 30 min.) (Heraeus Sepatech, Suprafuge 22) at 4°C and the pH of the haemolysate adjusted to pH 8.7 with solid Tris. The pH-adjusted haemolysate was then subjected to affinity chromatography [*Chromatography System:* chromatography column: 1.36 × 30 cm (Sigma Chemical Company), bed volume: 25 ml; peristaltic pump (Pharmacia Chemical Company), and fraction collector (AO Instrument Company, U.S.A.)] at 4°C for the purification of rat CA.[20]

An aliquot (10 ml) of pH-adjusted rat erythrocyte haemolysate was applied to the Sepharose 4B-L-tyrosine-sulfanylamide affinity column pre-equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The rat carbonic anhydrase were eluted with 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6) (flow rate: 20 ml h^{-1}, fraction volume: 4 ml). The absorbency at 280 nm was used to monitor protein elution. CO_2-hydratase activities in the eluates were determined and the active fractions were collected.[21,22]

In Vitro and *In Vivo* Total CA Activity

Preparation of the haemolysate, ammonium sulphate precipitation, dialysis, preparation of affinity gel, application of enzyme solution to affinity column and enzyme elution were done according to Arslan *et al.*[20] The control of enzyme purity, using Laemmli's procedure,[23] was carried out using SDS-PAGE. Pure enzyme activity was 2492 EU/ml. In order to determine effects of plant growth regulators on CA activity, purified enzyme eluates were used with putrescine, spermidine, kinetin and abscisic acid.

The haemolysates obtained from rats injected with putrescine, spermidine, kinetin or abscisic acid were used for *in vivo* CA activity.

The activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[21] CO_2-Hydratase activity was calculated $(t_0 - t_c/t_c)$ where t_0 and t_c are the times (seconds) for pH change (from 10 to 7.4 colour change from red to yellow) of the non-enzymatic and the enzymatic reactions, respectively.

Purification of G6PD by Affinity Chromatography

The dialyzed sample was loaded onto a 2′, 5′ ADP Sepharose 4B affinity column and the gel was washed with 25 mL of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), then 25 mL of 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85), and finally with 0.1 M KCl/0.1 M K-phosphate (pH 7.85).

TABLE I *In vivo* effects of putrescine, spermidine, kinetin and abscisic acid on rat erythrocyte CA activities

	EU/g Hb			
Time (h)	Putrescine	Spermidine	Kinetin	Abscisic acid
t_0 Control	4036 ± 75.38	4023 ± 78.02	4096 ± 105	4083 ± 76.37
1	1400 ± 71*	4036 ± 170.39	4116 ± 76.37	7311 ± 104*
3	1554 ± 35*	4023 ± 117.29	3991 ± 107.50	7274 ± 210*
5	3975 ± 175	4035 ± 64.69	3924 ± 62.16	4130 ± 230

Results are expressed as the mean ± S.D., n = 10. Differences between means (test vs. control) were analysed by Student's *t*-test. *$p < 0.001$. EU, enzyme unit. Dose: 3.917 mg/kg putrescine, 6.452 mg/kg spermidine, 1.91 mg/kg kinetin and 2.346 mg/kg abscisic acid (ABA).

Washing continued until absorbance of the eluate was reduced to 0.05 at 280 nm. Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85) solution at 20 mL/h flow rate. Eluates were collected in 2 mL tubes and the G6PD activity of each tube determined. Active fractions were collected. All of the procedures were performed at 4°C.[18,24,25]

In Vitro and *In Vivo* G6PD Activity

Preparation of the hemolysate, ammonium sulphate precipitation and dialysis were done according to Ninfali *et al.*,[18] except that 0.5 mM $NADP^+$ was used instead of 0.2 mM. Preparation of the affinity gel and application of the enzyme solution to the affinity column were done according to Muto *et al.*,[26] and washing was done according to Morelli *et al.*[25] The control of enzyme purity, using Laemmli's procedure,[23] was carried out using SDS-PAGE. Pure enzyme activity was 2.8 EU/ml. In order to determine effects of plant growth regulators on G6PD activity, purified enzyme eluates were used with putrescine, spermidine, kinetin and abscisic acid.

The hemolysates obtained from rats injected with putrescine, spermidine, kinetin or abscisic acid were used for *in vivo* G6PD activity.

The enzymatic activity was measured by Beutler's method.[14] One enzyme unit was defined as the enzyme amount reducing 1 μ mole $NADP^+$ per 1 min.

RESULTS AND DISCUSSION

Many chemicals at relatively low dosage affect the metabolism of biota by altering normal enzyme activity.[23] The effects can be dramatic and systemic.[27] CA and G6PD are important enzymes for body metabolism because CA regulates pH in most tissues and G6PD catalyses the first step of the pentose phosphate metabolic pathway. Therefore, the effects of some plant hormones on rat erythrocyte CA and G6PD were investigated.

For the *in vivo* studies, after the injection of plant growth regulators, CA and G6PD activity values were determined at 1, 3 and 5 h postadministration, as shown in Tables I and II, respectively. When control and putrescine values for CA and G6PD were compared, putrescine decreased the activities of CA and G6PD and the maximal inhibitions on CA and G6PD activities were found at 1 h after injection. Similarly in the *in vitro* studies, all putrescine concentrations decreased CA and G6PD activities (Figures 1 and 2).

At both the *in vivo* and the *in vitro* studies, CA and G6PD activities with spermidine and kinetin at 1, 3, 5 h and control activities were the same indicating no effect of these two hormones on CA and G6PD activities both *in vivo* (Tables I and II) and *in vitro* (data not shown because difference between data is not important statistically).

CA and G6PD activities in the presence of abscisic acid were determined at 1, 3 and 5 h, and the results are shown in Tables I and II. When control and abscisic acid values for CA and G6PD were compared, abscisic acid increased CA and G6PD activities and the maximal activations on CA and G6PD were found 1 h after injection. Similarly in the *in vitro* studies, all abscisic acid concentrations increased CA and G6PD activities (Figures 3 and 4).

TABLE II *In vivo* effects of putrescine, spermidine, kinetin and abscisic acid on rat erythrocyte G6PD activities

	EU/g Hb			
Time (h)	Putrescine	Spermidine	Kinetin	Abscisic acid
t_0 Control	7.04 ± 0.50	6.72 ± 0.80	5.52 ± 0.21	7 ± 0.52
1	2.83 ± 0.40*	6.96 ± 0.57	5.59 ± 0.20	8.58 ± 0.27*
3	5.97 ± 0.32*	6.94 ± 0.62	5.71 ± 0.24	8.68 ± 0.19*
5	7.05 ± 0.19	6.63 ± 0.56	5.63 ± 0.14	7.02 ± 0.30

Results are expressed as the mean ± S.D., n = 10. Differences between means (test vs. control) were analysed by Student's *t*-test. *$p < 0.001$. EU, enzyme unit. Dose: 3.917 mg/kg putrescine, 6.452 mg/kg spermidine, 1.91 mg/kg kinetin and 2.346 mg/kg abscisic acid (ABA).

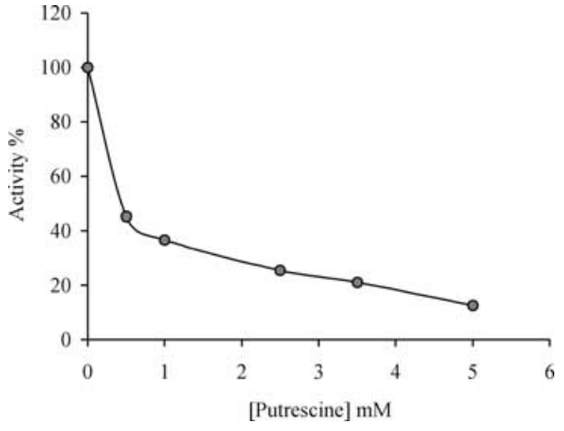

FIGURE 1 *In vitro* effects of putrescine on rat erythrocyte CA activities in the assay buffer. Data points are the mean of four replicate measurements.

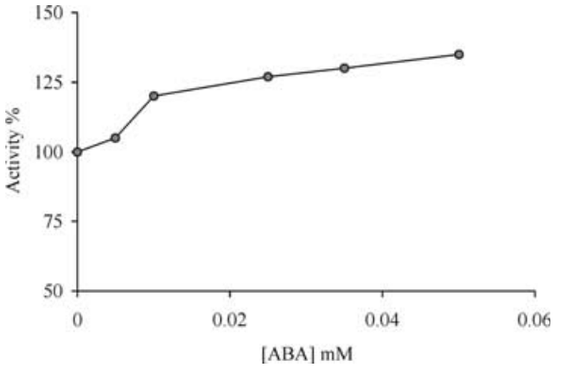

FIGURE 3 *In vitro* effects of abscisic acid on rat erythrocyte CA activities in the assay buffer. Data points are the mean of four replicate measurements.

In the present study, both *in vivo* and *in vitro* CA activity is increased by abscisic acid, decreased by putrescine and is unaffected by spermidine and kinetin. In another study, it was found that plant hormones (abscisic acid and gibberellic acid) increased CA activity, but kinetin had no effect.[28] Similarly, some researchers have reported that naphthalene acetic acid, abscisic acid, 2,3,5-triiodobenzoic acid and gibberellic acid were effective both on bovine and human CA II at different levels.[29] Our present results support these studies. It has been reported that activity levels of CA isoenzymes in human erythrocytes vary considerably under certain pathological and physiological conditions[30] and changes in CA activity have been associated with metabolic diseases like diabetes mellitus and hypertension.[31,32] It has been known for some time that inhibition of CA impairs proton secretion into the proximal tubule lumen and thereby decreases bicarbonate re-absorption[30] which at the same time decreases the rate of acidification of urine so producing alkaline urine and eventually metabolic acidosis.[33] CA inhibitors are widely used drugs for the treatment or prevention of a variety of diseases such as glaucoma,[34] epilepsy,[35] gastric and duodenal ulcers[36] or acid-base disequilibria[37] among others.

In contrast to inhibitors, activators of this enzyme (for which at least 14 different isozymes have been isolated up to now in higher vertebrates)[38] have been much less investigated. Only recently the X-ray crystallographic structures of the first adducts of the physiologically relevant isozyme II (hCA II) with the activators histamine[39] and phenylalanine (in this case a tertiary complex, in which azide is also bound to the Zn(II) ion)[40] have been reported by Supuran and Scozzafava's group. Furthermore, few other QSAR[41–43] or synthetic chemistry[43–45] studies have been reported in the field of CA activators, although some of these compounds might be used in the treatment of the CA deficiency syndrome, a genetic disease of bone, brain and kidney affecting a large enough number of patients.[45] In this

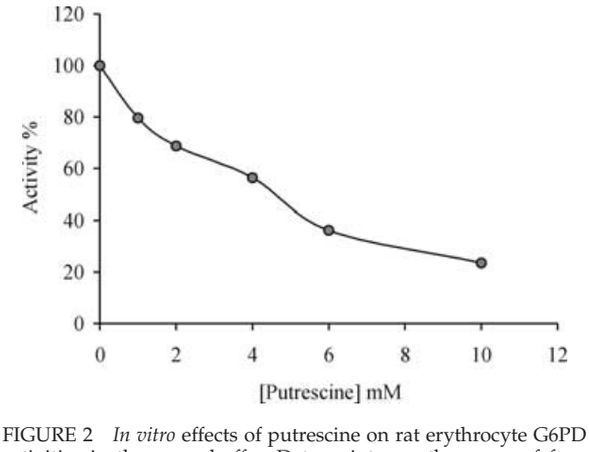

FIGURE 2 *In vitro* effects of putrescine on rat erythrocyte G6PD activities in the assay buffer. Data points are the mean of four replicate measurements.

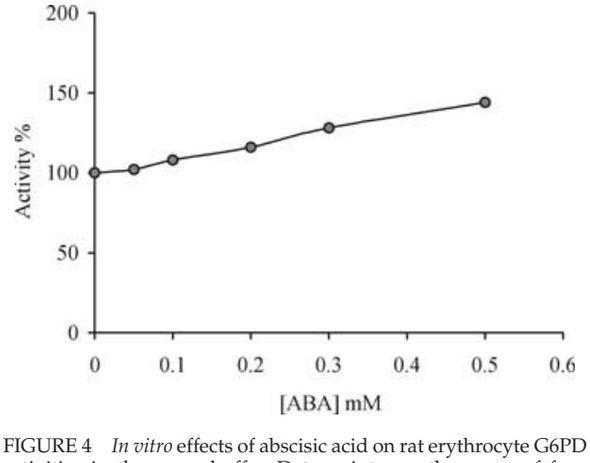

FIGURE 4 *In vitro* effects of abscisic acid on rat erythrocyte G6PD activities in the assay buffer. Data points are the mean of four replicate measurements.

condition, a certain CA isozyme gene (generally CA II, I or IV) is either not expressed, or its protein product is unstable due to deleterious mutations, and the corresponding CA isozyme is absent in the blood, kidney or lung of such patients.

In the present study, both *in vivo* and *in vitro* G6PD activity is increased by abscisic acid, decreased by putrescine and not affected by spermidine and kinetin. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is the key enzyme, which catalyzes the first step of the pentose phosphate metabolic pathway.[19] This enzyme was purified for the first time from human erythrocytes by Yoshida in 1965.[46] The pentose phosphate metabolic pathway is a unique source of NADPH in erythrocytes and synthesis of NADPH decreases in G6PD deficiency.[47] A major role of NADPH in erythrocytes is regeneration of reduced glutathione, which prevents hemoglobin denaturation, preserves the integrity of the red blood cell membrane sulfhydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells.[48,49] Decrease in G6PD results in NADPH and reduced glutathione deficiency in erythrocytes with scarcity of reduced glutathione in erythrocyte causing early haemolysis in the spleen.[50]

The most common red blood cell enzyme defect the world wide is G6PD deficiency.[49] G6PD deficiency is an X-linked trait; it is fully expressed in males and homozygous females and is variably expressed in heterozygous females.[51] More than 150 million males suffer from G6PD deficiency in the world and at least 400 variants have been described.[33] In normal erythrocytes, G6PD activity decreases with aging. In mild variants of this disease, the erythrocyte G6PD level is lower than in the normal person; in some variants even young erythrocytes do not endure stress. Glucose 6-phosphate dehydrogenase deficiency disorder is also named as primaquine sensitivity or favism.[49,52,53] G6PD deficiency is frequently seen in African, Mediterranean, Middle Eastern and Far Eastern nations and their lineages with a frequency ranging from 5%–40%.[49,51,54]

In summary, our results show that ABA increased CA and G6PD activity in rat erythrocytes. These findings indicate that ABA may be pharmacologically useful in patients where there is a deficiency of the enzymes in red blood cells. However putrescine has a significant inhibitory effect on CA and G6PD activity both *in vivo* and *in vitro*. Since effects of plant hormones on rat G6PD activity have not been previously reported, these results on G6PD activities are of interest for further researches. Overall, since plant growth regulators are used on plants, their effects on mammals eating the plants should be more widely taken into consideration.

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