IN VIVO EFFECTS OF DIOSGENIN ON THE ACTIVITY OF SOME DRUG-METABOLIZING ENZYME SYSTEMS

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Abstract. This study investigated the in vivo effect of Diosgenin, isolated from Asparagus officinalis, on the total quantity of Cytochrome P450, the activity of some enzymes - Ethylmorphine N-demethylase (marker for CYP3A) and Aniline Hydroxylase (marker for CYP2E1), the quantity of malondialdehyde and reduced glutathione compared to Phenobarbital in rats. Diosgenin and Phenobarbital, administered alone, increased statistically significant, compared to control, the total amount of Cytochrome P450 – with 36 % and with 85 %; the activity of EMND with 58 % and with 184 %. Diosgenin didn’t influence the AH activity compared to Phenobarbital. Both Diosgenin and Phenobarbital had no statistically significant effect on GSH level. On the quantity of MDA, Diosgenin had no effect, while Phenobarbital increased it with 27 %. Our results showed that Diosgenin exhibited inducible effect similar to the effect of Phenobarbital on the activity of some drug-metabolizing enzyme systems.

Key Words: diosgenin, phenobarbital, Asparagus officinalis, aniline hydroxylase, ethylmorphine N-demethylase

Introduction

Diosgenin is a steroid sapogenin, which can be found in Wild Yam (Dioscoreaceae); Asparagus officinalis L. (Liliaceae); Tribulus terrestris L. (Zygophyllaceae), etc. Wild Yam was used by natives Americans as an expectorant and a remedy for intestinal spasm, biliary colic, rheumatic pain and a range of gynecological symptoms including dysmenorrheal, pelvic cramps and problems associated with menses, childbirth and menopause.

Diosgenin is widely used as a source for the half-synthesis of sex hormones, corticosteroids and contraceptive drug (13). Its anti-inflammatory effect is proved on experimental Indomethacin-induced intestinal inflammation, as well as its spasmyloytic action in biliary colics (17). Diosgenin is a well-known antihypercholesterolemic agent. Biliary cholesterol secretion is mediated by different transporters and is stimulated by cholesterol and non-cholesterol steroids – Cholate and Diosgenin. It was found that in older humans, Diosgenin reduced serum lipid peroxidation, lowered serum triglycerides, phospholipids, increased HDL levels (1).

In previous experiments we found that Diosgenin had excessive statistically significant antioxidant effect under conditions of oxidative stress, induced by some pathological and age-related factors. We suggested that the antioxidant effect of Diosgenin was related with the decrease of lipid peroxidation and storage of GSH (12).

It is known that orphan nuclear hormone receptors CAR (constitutive androstane receptor) and PXR (pregnane X receptor) take part in the regulation of hepatic drug metabolism. There are data that Diosgenin increased the expression of lipid-sensing nuclear receptor PXR (18). Phenobarbital (PB) and Phenytoin are the most well known activators of PXR and CAR (16). PXR has been shown to take part in the regulation of hepatic metabolism – especially biochemically and genetically activation of CYP3A genes.

Thus, the purpose of this work was to assess the in vivo possible effect of Diosgenin, isolated from Asparagus officinalis, on the activity of some drug-metabolizing enzyme systems, compared to Phenobarbital in rats.
Materials and Methods

Plant material, extraction and isolation procedures

Roots of Asparagus officinalis were collected in June 2003 in the northeast region of Bulgaria, locality Beljaev hill, nearby town of Beloslav. A voucher specimen was deposited in Herbarium of Sofia University (SO 97380).

Plant material was extracted repeatedly with 80 % MeOH then concentrated in vacuo. Residue was suspended in H₂O, and sequentially partitioned with Et₂O, CHCl₃, EtOAc and n-BuOH. Diosgenin was obtained after acid hydrolysis (5 % H₂SO₄ for 4h) of BuOH extract, followed by column chromatography on silica gel and preparative TLC. Compound was identified by physical and spectral data, comparing with literature (4) (Figure 1).

Chemicals

In our experiments: KCl (Merck, Germany), K₂HPO₄ (Merck, Germany), NaH₂PO₄ (Merck, Germany), Sodium dithionite (Merck, Germany), Ethylmorphine (Sigma Aldrich), NADP (Sigma Aldrich), Glucoso-6-phosphate (Sigma Aldrich), Semicarbazide (Sigma Aldrich), ZnSO₄ (Merck, Germany), Ba(OH)₂ (Merck, Germany), Nicotinamide (Sigma Aldrich), MgCl₂ (Merck, Germany), Trichloroacetic acid (Merck, Germany), DTNB (5,5'-dithiobis(2-nitrobenzoic acid) (Sigma Aldrich), Na₂CO₃ (Merck, Germany), Thiobarbituric acid (Sigma Aldrich) were used.

Animals

In our experiments 4 months (young) male Wistar rats with body weight 180-200 g were used. Rats were housed in plexiglass cages in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided ad libitum. Rats were purchased from the National Breeding Center, Sofia, Bulgaria.

Animals were divided in 3 groups: 1 – control; 2 – treated with Diosgenin (50 mg/kg p.o./ 6 days) (2) and 3 – treated with Phenobarbital (75 mg/kg i.p./ 4 days) (5).

The experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University-Sofia, Bulgaria. The principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) and Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) were followed strictly throughout the experiment.

Isolation of rat liver microsomes

The rat liver microsomes were isolated by ultracentrifugation. Isolation was carried out by method of Guengerich and Martin (6). Liver was homogenate in 0.01 M Pottassium buffer + 1.15 % KCl (pH = 7.4). The initial homogenate was centrifuged at 9000xg for 30 min to pellet nuclei and mitochondria, and then supernatant was centrifuged at 105 000 x g for 60 min to obtain a microsome pellet. Microsomes were frozen in Potassium buffer + 1.15 % KCl (pH = 7.4).

Evaluation of Phase I of biotransformation

Determination of total amount of Cytochrome P450

The Cytochrome P450 content in rat liver microsomes was measured spectrophotometrically by the method of Omura and Sato (14).

It was determined by the measurement of absorption on the differentiated spectrum of microsomes reduced with dithionite and saturated with CO between 450 nm and 490 nm. Cytochrome P450 quantity was measured by molar extinction coefficient 91 mM⁻¹cm⁻¹ and was expressed as nmol/mg protein.

The following enzyme assays, which display some CYP isoforms, were used: ethylmorphine-N-demethylation (CYP3A) (15) and aniline hydroxylation (CYP2E1) (9).

Determination of ENMD activity

The activity of Ethylmorphine N-demethylase (EMND) was measured spectrophotometrically by the amount of produced formaldehyde during the demethylation of Ethylmorphine.

Incubations were carried out in open 25-ml Erlenmeyer flasks at 37°C in shaking water bath. Each reaction mixture consisted of: 1 ml microsomal suspension, containing 1 mg protein; 1ml Potassium buffer + 1.15 % KCl (pH = 7.4); 0.5 ml 58 mM Ethylmorphine and 0.5 ml NADPH-generating system.
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**(Determination of AH activity)**

The activity of Aniline hydroxylase (AH) was determined spectrophotometrically by the amount of produced p-aminophenol. Incubations were carried out in open 25-ml Erlenmeyer flasks at 37°C in shaking water bath.

Each reaction mixture consisted of: 1 ml microsomal suspension, containing 1 mg protein; 1 ml 7 mM Aniline and 2 ml NADPH-generating system (containing 248 nM NADP (S, 5 mM Glucoso-6-phosphate, 25 mM Nicotinamide and 13 mM MgCl$_2$). The reaction was started with NADPH-generating system and the incubation continued 20 min. The reaction was stopped with 2 ml 20 % Trichloroacetic acid.

The mixture was centrifuged at 4 000 rpm for 20 min. After the centrifugation, the reaction mixture contained: 2 ml of supernatant, 1 ml 10 % Na$_2$CO$_3$ and 2 ml 2 % Phenol; and was incubated for 30 min in 37°C. The amount of produced p-aminophenol was measured spectrophotometrically at 640 nm. The enzyme activity was expressed as nmol/min/mg protein.

Liver protein concentration was measured, using the method of Lowry et al. (11).

**(GSH depletion)**

The level of reduced glutathione (GSH) as sign of hepatotoxicity was measured using a spectrophotometric method described by Bai et al. (3).

Liver was homogenate with 5 % TCA. The homogenate was centrifuged on 4 000 rpm for 20 min. The reaction mixture contained: 1 ml supernatant, 1 ml 0.1 M Potassium buffer (pH = 7.4) and 1 ml DTNB (5,5’-dithiobis(2-nitrobenzoic acid)) solution. Absorbance was measured at 412 nm and expressed as nmol/g liver.

**(Lipid peroxidation)**

The level of malondialdehyde (MDA) was assayed as an index of lipid peroxidation. The amount of MDA was measured using a spectrophotometrical method described by Bai et al. (3).

Liver was homogenate with 0.1 M Potassium buffer (pH = 7.4). The reaction mixture contained: 1 ml liver homogenate, 1 ml 25 % TCA and 1 ml 0.67 % Thiobarbituric acid. Absorbance was measured at 535 nm. Malondialdehyde quantity was measured by molar extinction coefficient 155 mM$^{-1}$cm$^{-1}$ and was expressed as mmol/g liver.

**Statistical analysis**

Statistical analysis was performed using statistical programme ‘MEDCALC’. Results are expressed as mean ± SEM for eight rats in each group. The significance of the data was assessed using the non-parametric Mann–Whitney test. Values of P ≤ 0.05 were considered statistically significant.

**Results and Discussion**

Diosgenin, administered alone, increased statistically significant the total quantity of Cytochrome P450 – with 36 %, the activity of EMND with 58 %, compared to control and had no effect on AH activity; while Phenobarbital increased statistically significant the total quantity of Cytochrome P450 – with 85 %, the activity of EMND – with 85 % and the activity of AH – with 120 %, compared to control (Table 1).

Diosgenin, administered alone, had no statistically significant effect on GSH and MDA level, compared to control. Phenobarbital statistically significant increased the level of MDA with 27 %, and had no effect on GSH level (Table 2).

Yu et al. found that biliary cholesterol secretion was mediated by the ATP-binding cassette (ABC) transporters and was stimulated by cholesterol and non-cholesterol steroids – Cholate and Diosgenin (18). At the same time, Diosgenin increased the expression of several Pregnane X Receptor (PXR) target genes and reduced its choleretic effect by ~ 70 % in PXR knock-out mice (18). Koster et al. suggested that Diosgenin or some of its metabolites induced CYP3A, by influence the PXR activity in mice (10). Phenobarbital is a well known activator of CAR (constitutive androstane receptor) and to a small extent PXR. It is known that PXR takes part in the mechanism of CYP3A induction (7).

Our results showed that in liver microsomes, isolated from rats after multiple treatments with Diosgenin and Phenobarbital, both compounds statistically significant increased the quantity of Cytochrome P450 and the activity of EMND. Diosgenin didn’t influence the AH activity compared to Phenobarbital.
Our results demonstrated that the Diosgenin’s mechanism of increasing the activity of CYP3A is possibly similar to those of Phenobarbital, and connected with the influence of PXR activity.

Handschin and Meyer presumed that drug-mediated activation of PXR was potentially beneficial in cholestasis. The steroid structure of Diosgenin and our established data for increasing the activity of CYP3A, suggested its possible role in the regulation of lipid homeostasis and explained its antihypercholesterolemic effect (8).

**Conclusion**

Diosgenin, isolated from *Asparagus officinalis*, administered alone, increased statistically significant, compared to the control, the total quantity of Cytochrome P450 and the activity of EMND (marker for CYP3A) and had no effect on AH activity (marker for CYP2E1), level of GSH and MDA.

<table>
<thead>
<tr>
<th>Group</th>
<th>EMND, nmol/min/mg protein</th>
<th>AH, nmol/min/mg protein</th>
<th>Cytochrome P450, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.365 ± 0.03</td>
<td>0.121 ± 0.05</td>
<td>0.158 ± 0.05</td>
</tr>
<tr>
<td>50 mg/kg diosgenin</td>
<td>0.575 ± 0.02 **</td>
<td>0.130 ± 0.05</td>
<td>0.215 ± 0.05 ***</td>
</tr>
<tr>
<td>75 mg/kg phenobarbital</td>
<td>1.037 ± 0.04 ***</td>
<td>0.266 ± 0.04 *</td>
<td>0.293 ± 0.04 ***</td>
</tr>
</tbody>
</table>

Table 1. Effect of diosgenin and phenobarbital on enzyme activity of EMND and AH and the Quantity of Cytochrome P450 in rat liver microsomes

**References**


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