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The influence of a standardized soybean extract (Glycine max) on the expression level of cytochrome P450 genes *in vivo*

Wpływ standaryzowanego ekstraktu sojowego (Glycine max) na poziom ekspresji cytochromu P450 *in vivo*

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Abstract

Objective: *Soybean isoflavones are phytoestrogens that reduce menopausal symptoms and decrease the risk of certain chronic diseases, such as cancer and cardiovascular diseases. Despite the widespread use of soybean isoflavones as functional food and dietary supplements, data regarding the safety, as well as herb-drug interactions, remain scarce. The aim of the study was to investigate the influence of soybean extract on the expression levels of cytochrome P450 (CYP) genes using real-time PCR (RT-PCR).*

Materials and methods: *Male Wistar rats were fed a standardized soybean extract containing 37% isoflavones (100mg/kg) for 3 and 10 days. cDNA was synthesized from total RNA isolated from the liver using reverse transcription. The level of CYP genes expression was analyzed using RT-PCR method.*

Results: *Soybean extract administration resulted in a significant increase of CYP1A1 expression level compared with the control group (1.5-fold; $p<0.05$). An inductory effect was also observed for CYP2D1 by 32% ($p<0.01$) after 10 days of treatment. No statistically significant differences were noted for CYP1A2, CYP2C6 and CYP3A2. In case of CYP3A1, the mRNA level of this gene was reduced by almost 35% ($p<0.05$) both, after 3 and 10 days. CYP2D2 expression was also inhibited by the extract, but to a lesser degree when compared to CYP3A1. Moreover, insignificant decrease of CYP2E1 expression level by 25% ($p<0.01$) was observed after 3 days of treatment.*

Conclusions: *These findings suggest that soybean extract may change the expression of CYP enzymes involved in biotransformation of xenobiotics (drugs, procarcinogens).*

Key words: **cytochrome P450** / **soybean** / **interactions** / **rat model** / **expression level** /

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Streszczenie

Cel pracy: *Izoflawony sojowe są fitoestrogenami, które redukują objawy menopauzy i zmniejszają ryzyko wystąpienia wielu chorób chronicznych, takich jak nowotworowych czy sercowo-naczyniowych. Pomimo powszechnego stosowania izoflawonów sojowych jako żywności funkcjonalnej i suplementów diety, dane dotyczące bezpieczeństwa jak również występowania interakcji pomiędzy preparatem roślinnym a lekiem syntetycznym są bardzo ograniczone.*

Celem pracy było zbadanie wpływu ekstraktu sojowego na poziom ekspresji genów cytochromu P450 (CYP) wykorzystując technikę real-time PCR (RT-PCR).

Materiały i metody: *Szczury rasy Wistar traktowano standaryzowanym ekstraktem (100mg/kg) zawierającym 37% izoflawonów przez 3 i 10 dni. cDNA syntetyzowano z całkowitego RNA izolowanego z szczurzej wątroby stosując odwrotną transkrypcję. Poziom ekspresji genów CYP analizowano wykorzystując technikę RT-PCR.*

Wyniki: *Wykazaliśmy, że ekstrakt sojowy powodował znaczny wzrost poziomu ekspresji CYP1A1 w porównaniu do grupy kontrolnej (1,5-krotnie; p<0,05). Indukcyjny efekt obserwowano również dla CYP2D1 o 32% (p<0,01) po 10 dniach stosowania. Brak istotnych różnic zanotowano dla CYP1A2, CYP2C6 i CYP3A2. W przypadku CYP3A1, poziom mRNA tego genu był redukowany o 35% (p<0,05) zarówno po 3 i 10 dniach. Ekspresja CYP2D2 była również hamowana przez ekstrakt, ale w mniejszym stopniu w porównaniu do CYP3A1. Ponadto, nieznaczny spadek poziomu ekspresji CYP2E1 o 25% (p<0,01) obserwowano po 3 dniach stosowania.*

Wnioski: *Wyniki badania sugerują, że ekstrakt sojowy może zmieniać ekspresję enzymów CYP związanych z biotransformacją ksenobiotyków (leków, prokarcynogenów).*

Słowa kluczowe: **cytochrom P450** / **soja** / **interakcje** / **model szczurzy** / / **poziom ekspresji** /

Introduction

Soybean (*Glycine max*) is one of the most commonly used dietary supplements and functional foods. It contains isoflavones, mainly genistein and daidzein, which are considered the best dietary sources of phytoestrogens. Numerous data from experimental studies confirm beneficial effects of soybean isoflavones on human health. They reduce menopause symptoms and may lower the risk of certain chronic diseases, such as cancer or cardiovascular diseases [1, 2, 3].

Epidemiologic research suggests that Asian populations, following traditional diet with high levels of soybean products, have significantly lower incidence of breast, colon and prostate cancers [4]. Many studies have reported that the estrogenic effect of soybean isoflavones plays a role in the prevention of osteoporosis caused by estrogen deficiency in postmenopausal women and ovariectomized animals [5, 6, 7].

Moreover, human studies of soybean consumption provide compelling evidence for its positive effect on improved lipid profiles, including reduction in low-density lipid (LDL) and triglycerides and an increase in high-density lipid (HDL) levels [8,9]. In addition, potential chemoprotective mechanism is claimed to be the effect of soybean consumption on procarcinogen activation and carcinogen metabolism [10]. Increased metabolism of ingested procarcinogens will lead to faster clearance and reduction of their carcinogenicity [11].

Modulation of the cytochrome P450 (CYP) activity in hepatic microsomes is of great importance as these enzymes are involved in the metabolism of a variety of xenobiotics (drugs, carcinogens) and food components, as well as the synthesis of endogenous compounds such as steroids [12]. The CYP2C, CYP2D and CYP3A subfamilies are the most active CYPs for drug metabolism, especially the CYP3A4 isoform that is responsible for metabolism of about 50% of clinically used drugs [13].

Moreover, the CYP3A subfamily, besides drug metabolism, plays an important endogenous role in the degradation of bile acids and in the metabolism of estrogens [14, 15]. It is also implicated in activation of procarcinogens such as aflatoxins B1 and food-derived heterocyclic amines. High CYP3A4 activity is suggested to be a risk factor for breast cancer [16,17,18]. The CYP1A, CYP2A and CYP2E subfamilies metabolize many protoxins and procarcinogens to their ultimate reactive metabolites [19]. However, CYP1A1 is the major cytochrome P450 responsible for carcinogen activation. Several studies suggest that metabolism of foreign compounds by these enzymes does not always lead to their detoxification. In some cases, the oxidized or reduced products may initiate chemical carcinogenesis and drug toxicity [20].

Diet, especially rich in flavonoids, can also have influence on drug metabolism. Flavonoids, including isoflavones, have been claimed to have beneficial effects related to their antioxidative activity, but some of them may also affect the activity of CYP and enhance the activation of carcinogens or influence the metabolism of drugs [21]. Differences in the rate of metabolism of drugs can have its source in herb-drug interactions. Furthermore, detailed studies on CYP-flavonoid interactions concerning prevention of food carcinogen activation are much needed as data about the influence of soybean isoflavones on CYP are scarce. Therefore, the profile of mRNA abundance of CYP enzymes provides vital information about possible interactions between herbal medicines and synthetic drugs.

Objective

In the following study, we have examined the effect of standardized soybean extract on gene expression of major CYPs on rat model. Human CYP1A1/2, CYP2E1 known as conserved through the species, are represented in rats by their counterparts CYP1A1/2 and CYP2E1, whereas human CYP3A4/5, CYP2D6 and CYP2C9 isozymes correspond to the rats CYP3A1/2, CYP2D2 and CYP2C6 isozymes.

Materials and methods

Study design

Standardized soybean extract containing 37% isoflavones expressed as genistein was obtained from Pierre Fabre Sante, France. The experiment with male Wistar rats (200-250) was performed in accordance with Polish government regulations (Decree on Animal Protection 01.21.2005, Dz. U. No 33; 289). The study has been approved by Local Ethics Committee of the Use of Laboratory Animals in Poznan (No 43/2005). The rats were housed in plastic cages in the Department of Pharmacology, University of Medical Sciences in Poznan. The animals were kept in a climate-controlled room with 12-h light/dark cycle and allowed access to commercial rat chow and tap water *ad libitum*. The rats were randomly divided into four groups, A to D (n=10). Group A was fed the standardized soybean extract (100 mg/kg p.o., once a day, for 3 days) and group B constituted the control group and was fed standard diet. Group C was given the same extract as group A (100 mg/kg p.o., once a day) but for the duration of 10 days, whereas group D served as controls for group C. Sixteen hours after the last administration the rats were decapitated. Samples of liver were immediately frozen in liquid nitrogen and stored at -80ºC.

Total RNA isolation and cDNA synthesis

RNA was isolated from the rat livers using TriPure Isolation Reagent (Roche, Germany) according to the manufacturer's protocol. The RNA pellets were washed with 70% ethanol and dissolved in DEPC water. RNA samples were stored at -80°C. The concentrations and the purity of RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Eppendorf, USA). cDNA was synthesized from 2 µg of total RNA in a total volume of 20 µl using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). The obtained transcripts were stored at -20°C or used directly for the real-time quantitative PCR (RT-PCR).

Real-time PCR

The level of gene expression in liver tissues was analyzed by real-time quantitative PCR (RT-PCR). The primers used for CYP1A1/2, CYP2D1/2, CYP3A1/2, CYP2E1, CYP2C6 and GAPDH amplifications and RT-PCR conditions were described by Mrozikiewicz et al. [22]. All oligonucleotide sequences were synthesized by TIB Molbiol (Poland). Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis and melting curve analysis. RT-PCR was carried out using a LightCycler TM Instrument (Roche, Germany) and a LightCycler DNA Master SYBR Green I kit (Roche, Germany) according to the instructions of the manufacturer. GAPDH was used as a housekeeping gene for normalization. RT-PCR was carried out in a reaction volume of 10µl using HotStart Tag DNA polymerase. The quantitative PCR was monitored by measuring the increase in fluorescence by the binding of SYBR Green I dye to the generated double-stranded cDNA. Complementary DNA was quantified by comparison of the number of cycles required for amplification of unknown samples with those of series of cDNA standard dilutions. The data were evaluated with the Roche LightCycler Run 5.32 software.

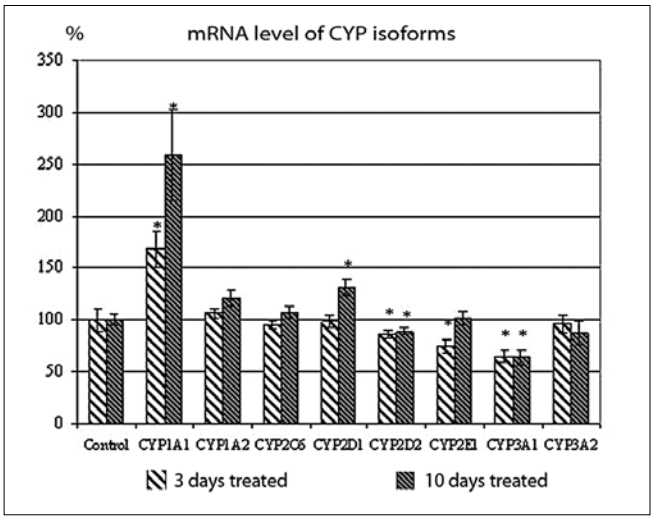

Figure 1. The influence of soybean extract on the expression level of CYP genes in the rat liver after 3 and 10 days of treatment. The control group were defined as 100%. Data were presented as mean ±SEM of 10 rats in each group. *p<0.05 as compared with the control group.

Statistical analysis

The mRNA content for studied genes was expressed as mean ± SEM. The experimental data were analyzed using the SPSS 17.0 for Windows program. Mean values were compared by one-way ANOVA test. The value of $p<0.05$ was considered statistically significant.

Results

In this study, we determined the influence of standardized soybean extract on the expression levels of CYP genes involved in xenobiotics biotransformation. As shown in Figure 1, statistically significant difference was observed in the inductive capability of soybean extract on CYP1A1 activity, both after 3 and 10 days of treatment. This extract resulted in a significant increase of CYP1A1 expression level, over 0.6-fold ($p<0.01$) and 1.5-fold ($p<0.01$), respectively. An inductory effect of the extract was also observed for CYP2D1 by 32% ($p<0.01$) after 10 days of treatment. No statistically significant differences were observed for CYP1A2, CYP2C6 and CYP3A2 compared with the control group. In contrast to CYP1A1, the level of CYP3A1 mRNA was reduced by almost 35% ($p<0.05$), both after 3 and 10 days of treatment. In addition, CYP2D2 expression was also inhibited by the extract, but to a lesser degree in comparison with CYP3A1. The ethanolic extract caused a small decrease of CYP2D2 mRNA level, 14% ($p<0.05$) and 11% ($p<0.05$), respectively. Moreover, an insignificant decrease of CYP2E1 expression level by 25% ($p=0.005$) after 3 days of treatment was noted. However, no inhibition for this gene was observed after 10 days of extract application.

Discussion

Several studies have reported that flavonoids can modify CYP activity in different ways and cause various related biological effects, either beneficial or disadvantageous to human health. Moreover, flavonoids may change plasma concentration of pharmaceutical drugs resulting in overdose or loss of their

therapeutic effect. *In vitro* and *in vivo* studies have explored the possible influence of soybean isoflavones on a variety of substrates and CYP enzymes. These compounds can modulate CYP activity, especially CYP3A4 that oxidizes a diversity of substrates, including various drugs, steroids, carcinogens as well as natural macrolide products [23,24]. Hence, further studies are needed to determine how and by which enzymes foreign chemicals are metabolized. This knowledge allows predictions of whether the compounds derived from medical plants may cause herb-drug interactions or be susceptible to market interindividual variations in metabolism.

Our findings from the study showed the influence of a standardized soybean extract containing 37% isoflavones on the mRNA transcription level of the major isozymes of CYPs. It was indicated that soybean extract in rats caused significant induction of CYP1A1 (homolog to human CYP1A1) responsible for carcinogen activation. In this case, it is postulated that induction of CYP1A1 may reduce the carcinogenicity of foreign compounds because increased metabolism of ingested procarcinogens will lead to faster clearance and reduction of their carcinogenicity. However, such induction of CYP1A1 may also potentially result in an increase in the toxicity and carcinogenicity of procarcinogens. Therefore, it is difficult to explain the mechanism of CYP enzymes action in the metabolism of procarcinogens and development of carcinogenesis.

In contrast to our results, Ronis et al. demonstrated that consumption of soybean protein isolate significantly reduces induction of CYP1A1 and CYP1A2 mRNA compared to rats fed diet containing casein. They also focused on the possibility of interactions among soybean, polycyclic aromatic hydrocarbons (PAH) and other dietary components, that are known CYP1A inducers and the fact that diet may contribute significantly to interindividual differences in hepatic CYP1A1 and CYP1A2 expression [25].

Moreover, our study showed that a standardized soybean extract significantly reduced the mRNA abundance of CYP3A1 (homolog to human CYP3A4) in the rat liver microsomes. It is suggested that inhibition of CYP3A1 by soybean extract may lead to decreased metabolism of clinically used drugs with therapeutic and toxicological consequences as isoflavones from soybean may change plasma concentration of pharmaceutical drugs, resulting in overdose or loss of their therapeutic effects. Furthermore, our data also showed an insignificant decrease of CYP2E1 expression level after 3 days of treatment. These results indicate that dietary isoflavones did not cause the inhibition of CYP2E1 (homolog to human CYP2E1) after prolonged application of this extract. In case of other CYPs, lack of influence of standardized soybean extract on the mRNA abundance was observed.

According to Laurenzana et al., genistein administrated resulted in significant decrease of CYP3A and CYP2C expression level in male rats [26]. In addition, both genistein and equol caused inhibition of CYP1A and CYP2E1 in human and mouse microsomes [27]. In other study, it was also shown that hydrolysed soybean extract in human liver microsomes caused inhibition of CYP1A2, CYP2A6, CYP2D6, CYP2C9 as well as CYP3A4, whereas only some of these enzymes were insignificantly inhibited by unhydrolysed soybean extract [28].

In contrast to the presented studies, induction of CYP3A was observed in weanling rats after treatment of soybean protein isolate [29]. Li and Shay also demonstrated CYP3A4 induction in hepatoma cells *in vitro* after administration of isoflavone extracts, genistein and equol [30]. In addition, it was claimed that genistein is a CYP3A substrate and it is common for CYP substrates to induce their own metabolism [31]. However, it is unclear whether isoflavones are the bioactive soybean component responsible for CYP3A induction involved in the activation of dietary procarcinogens and endogenous estrogens. Hence, it is suggested that elevated CYP3A activity may also increase cancer risk [29].

Other studies have shown that dietary isoflavone had no inducible affect on the hepatic mRNA abundance of the major isozymes of CYP. Helsby et al. reported that genistein and equol did not affect the protein content and activity of CYP1A1/2 CYP2E1 and CYP3A1 in mice [32]. Furthermore, in a later study they presented that the soybean isoflavone and its metabolites significantly reduced the protein content and activity of CYP1A2. However, they suggested that the decrease of CYP1A2 activity was not such to explain the chemopreventative influence of soybean isoflavone [27].

Kishida et al. reported that genistein and daidzein did not increase the CYP content of the liver microsomes of mice [33]. They also demonstrated lack of an inducible effect of a fermented soybean extract on the mRNA level of hepatic CYP isozymes in rats. Regardless, an insignificant decrease of the hepatic mRNA abundance of CYP1A2 and CYP3A2 was observed, although there were no significant differences among the groups [34].

Moreover, epidemiological studies suggest that high dietary intake of soybean products may lower appearance of certain chronic diseases in populations [35,36]. However, the mechanism underlying the effects of soybean consumption on CYP450 activity remains unclear since changes in the expression level of these enzymes may result from the soybean protein itself or from associated phytochemical components such as isoflavones, phytosterols and saponins. Consequently, soybean consumption may result in wide interindividual variability in the activity of CYP enzymes.

Conclusions

These results of our study showed that decrease of CYP3A1 (human CYP3A4) mRNA expression may cause a rise in plasma drug levels, leading to an undesirable pharmacological effect and the incidence of overdose. Furthermore, it is suggested that the increase of CYP1A1 (human CYP1A1) expression level may not only reduce the carcinogenicity of foreign compounds by increased metabolism of ingested procarcinogens, but also may activate some compounds to their carcinogenic metabolite that may initiate chemical carcinogenesis. We postulate that a significant increase of CYP1A1 expression level in our study may result from either the phytochemical profile of the soybean extract as well as from concentration of ethanol used in extraction process or other mechanism of action of compounds present in the extract.

Generally, the nature of the herbal preparation used in these studies and the duration of administration can also play an important role in terms of the outcomes of the different studies. Therefore, further studies are needed to determine the soybean extract effects on other CYP isozymes and how the results of these *in vivo* investigations can be extrapolated to *in vitro* situations.

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