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Activities of xanthine oxidase, nitric oxide synthase, aromatase and level of cytochrome P450 1A1, 1A2 and 1B1 isoforms in rat upon parenteral genistein injections

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It has been established that genistein inhibits the xanthine oxidase and nitric oxide synthase activity in the rat liver, lung and brain and decreases the aromatase activity and the enzyme level in the rat ovaries and uteri. Genistein has been also established as the inductor of the rat liver cytochrome P450 1A1, 1A2 and 1B1 isoforms. It has been shown in vitro that genistein is both the xanthine oxidase allosteric inhibitor, decreasing the superoxide generation, and the aromatase competitive inhibitor.

Introduction. Reactive oxygen species as well as NO may induce such processes as cancerogenesis and apoptosis [1]. It is well-known that the apoptosis may be induced either by superoxide or NO, but not by peroxynitrite $ONOO^-$, the product of their condensation [1]. It was established that unsteroid estrogens, polychlorinated biphenyls and polychlorodibenzodioxins, are xanthine oxidase allosteric inhibitors that strongly decrease the superoxide production by this enzyme [2, 3]. Also these compounds being structurally related to steroids may inhibit the expression of inducible nitric oxide synthase genes [1]. On the other hand, polychlorinated biphenyls and polychlorodibenzodioxins are powerful inductors of CYP1A1, CYP1A2 and CYP1B1 expression, performing this through Ah-receptors [4].

In the last years it was established that fitoestrogens such as isoflavonoids were powerful antioxidants [5]. Different studies revealed the superoxide scavenging activity of isoflavonoids with estrogen activity, such as genistein, based on their reductive properties [5]. However, the complete mechanisms of isoflavonoids antioxidative activity are not so far investigated. Analysing the isoflavonoids structure quantitatively, we assumed that these compounds may be the allosteric xanthine oxidase inhibitors such as corticosteroids, polychlorinated biphenyls and polychlorodibenzodioxins. It is a well-known fact that xanthine oxidase produces free superoxide that induces the peroxidation processes [6]. So the antioxidative effect of some flavonoids may be based on their xanthine oxidase inhibiting activity. Structurally related to corticosteroids, fitoestrogens, namely genistein may inhibit the inducible nitric oxide synthase genes expression. Earlier it was established that some flavonoids possessed the aromatase inhibiting activity (the inhibition was isosteric) [7]. Aromatase (cytochrome P450 XIX A1) is the enzyme that turns testosterone into estradiol and androstendione into estrone [8, 9]. So fitoestrogens such as genistein may be the aromatase isosteric inhibitors and such as the estrogens may inhibit the expression of the aromatase gene. Genistein and some other isoflavons are structurally related to the polychlorinated biphenyl's and polychlorodibenzodioxins. So these compounds are probably may interact with the Ah-receptors and induce the CYP1A1, CYP1A2 and CYP1B1 expression.

The aim of the presented work was to study

xanthine oxidase, nitric oxide synthase and aromatase activities as well as cytochrome P450 1A1, 1A2 and 1B1 level in rat organism under conditions of parenteral genistein injections and the *in vitro* genistein effects on the xanthine oxidase, nitric oxide synthase and the aromatase activities.

Materials and Methods. The *in vivo* studied were performed on 10 Vistar female rats (age 3 months, weight 100 ± 20 g) from one generation were used for the investigations. Animals were divided into two groups. The first group (control, five rats) received 0.2 ml of the peach oil with intraperitoneum injections and the another group (five rats) received 500 μg of genistein per 1 kg of weight (genistein was dissolved in the peach oil) per 1 kg of weight with intraperitoneum injections. All injections were performed during three days with the interval in 24 hours. In two hours after the last injection animals were mortified and the liver, lung, brain, ovaries and uteri were immediately isolated and homogenized in the 0.25 M saccharose (1:5).

Measurement of xanthine oxidase activity. Xanthine oxidase activity was measured in the liver, lung and brain as we described earlier [10].

Nitric oxide synthase activity measurement. Nitric oxide synthase activity was measured in the liver, lung and brain in the reaction system that consisted of 0.1 ml of homogenate, 2.5 ml of 0.1 M tris-HCl buffer (pH 7.4), 0.3 ml of arginine (320 μM solution in the bidistilled water) and 0.1 ml of 1 mM NADPH + H^+ water solution by the method described in [11]. The products of the aerobic NO oxidation were also measured in the liver, lung and brain by the method, based on the Griess reaction [11].

The aromatase activity and quantity measurement. The aromatase activity was measured in the rat ovaries and uteri as we described earlier [12]. The aromatase level was quantified with the help of disk-electrophoresis. The microsomes were isolated from the described organs by the differential centrifugation. The microsomal proteins were eliminated with the sodium cholate [13] and divided by disk-electrophoresis [14]. The electrophoregrams were put at the reaction system (10 ml) that consisted of 0.1 M tris-HCl buffer (pH 7.4), 1 mM aqueous solution of NADPH + H^+ and 6 mM 17-methyltestosterone solution in 0.01 M HCl (1:25:1) and incubated during 1 hour. In 1 hour 2 ml of 0.2 % blue tetrazolium solution in 7 % NaOH was added to the reaction systems. After 24 h incubation the electrophoregrames were isolated and the blue sticks formed by the aromatase bound 17-methyltestosterone were eluted by 7 % CH_3COOH. The aromatase quantity was measured by differential spectrophotometry [15].

Quantification of cytochromes P450 1A1, 1A1 and 1B1. The cytochromes P450 1A1, 1A1 and 1B1 in rat liver were measured as we described earlier [14].

Study of the genistein effect on the xanthine oxidase activity. These investigations were performed on the highly purified homogeneous xanthine oxidase preparation obtained from rat liver by the affinity precipitation on xanthine—agarose after previous purification (purificational degree — 1100 times, the enzyme outlet — 73.5 %) [16]. Then the xanthine oxidase activity was measured as we described earlier [10] but the reaction systems included also 0.1—1.0 μM of genistein. A probable genistein binding with xanthine oxidase was studied as follows. The quantities of free genistein were measured in the reaction systems after 15 min incubation (t = 40 °C) by the described method [3, 14] before and after HCl destruction [2, 3] of probable genistein—xanthine oxidase interactions. Also the quantities of free genistein in the reaction systems without xanthine oxidase were measured. The binding was determined as the number of genistein molecules, bound with one molecule of xanthine oxidase.

The genistein effect on the superoxide generation by xanthine oxidase was also studied according to the method described [17].

Study of genistein effect on the nitric oxide synthase activity. These studies were performed on rat liver homogenates. The nitric oxide synthase activity was measured in the reaction systems that also included 0.1—1.0 μM of genistein as we described.

Study of genistein effect on the aromatase activity. These investigations were performed on highly purified rat uterine aromatase. The aromatase was purified by the affinity precipitation on 17-methyltestosterone—agarose after previous purification, that included the material homogenization, microsomes isolation, microsomal proteins elimination by sodium cholate and aromatase precipitation by the acetone. The aromatase activity was studied in the reaction systems that also included 25—500 μM of genistein.

Statistics. The results were statistically validated according to the Student's *t*-criterion. The results were considered as significant at $p < 0.05$.

Results and Discussion. The xanthine oxidase activity in liver, lung and brain of genistein injected rats decreased in comparison with the control group as well as the nitric oxide synthase activity and the level of the nitric oxide aerobic oxidation products (table 1). Both the aromatase activity and level decreased in the rat ovaries and uteri compared to the control group (table 2). The level of the cytochrome P450 1A1, 1A2 and 1B1 isoforms in the liver of

genistein injected animals strongly increased in comparison with the control group (table 1).

As we established earlier unsteroid estrogens, polychlorinated biphenyls and polychlorodibenzodioxins, are xanthine oxidase allosteric inhibitors. So it was suggested that genistein as the compound that structurally related to the described unsteroid estrogens may be xanthine oxidase allosteric inhibitor. In the case of inhibition of the nitric oxide synthase activity, genistein may influence the nitric oxide synthase directly or regulate the expression of the inducible NO-synthase genes. The aromatase inhibition may be caused by the inhibition of its gene expression. But the direct enzyme inhibition may also take place because, as we indicated, it was established that some flavonoids are the aromatase isosteric inhibitors [7]. The level of cytochrome P450 1A1, 1A2 and 1B1 is controlled only by the Ah-receptors [4]. It is well-known that these isoforms are inducible by the polychlorinated aromatic compounds [4]. According to the quantitative analysis of the structure-activity relationship [18] the orbital structures of the polychlorinated aromatic unsteroid estrogens and genistein are display major relationships. So genistein interacts with Ah-receptors as their well-known

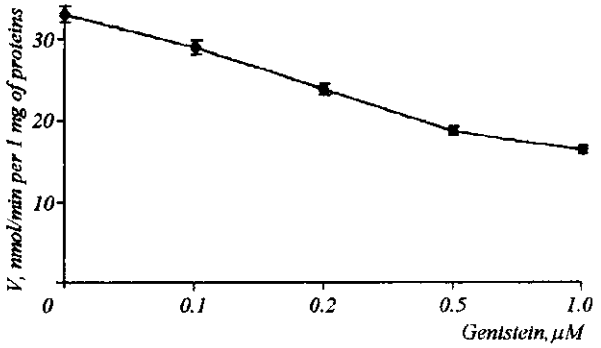

Fig. 1. Dependence of the xanthine oxidase activity on the genistein concentrations in the reaction system

Table 1
The xanthine oxidase and nitric oxide synthase activities, NO aerobic oxidation products and cytochrome P450 1A1, 1A2 and 1B1 level in the liver, lung and brain of intact and genistein injected rats (n = 5)

The group of rats	Organs	Xanthine oxidase activity, nmol/min per 1 mg of proteins	Nitric oxide synthase activity, nmol/min per 1 mg of proteins	NO aerobic oxidation products, nmol per 1 mg of proteins	Cytochrome P450 1A1, 1A2 and 1B1 level, nmol per 1 mg of protein
Control +	Liver	4.45±0.35	0.74±0.06	7.5±0.1	1.43±0.025
+ Genistein	Liver	1.24±0.06*	0.20±0.03	6.9±0.05*	3.18±0.028*
Control +	Lung	0.73±0.02	3.00±0.21	9.7±0.01	—
+ Genistein	Lung	0.375±0.045*	1.96±0.15*	7.2±0.2*	—
Control +	Brain	1.00±0.025	2.8±0.4	4.9±0.1	—
+ Genistein	Brain	0.11±0.01*	0.50±0.05*	2.2±0.2*	—

*p < 0.05 in comparison with intact group.

Table 2
The aromatase activity and quantity in the ovaries and uteri of intact and genistein injected rats (n = 5)

The group of rats	Organs	The aromatase activity, nmol/min per 1 mg of proteins	The aromatase quantity, nmol per 1 mg of proteins
Control +	Ovaries	1.4±0.028	5.12±0.2
+ Genistein	Ovaries	1.05±0.012*	3.6±0.12*
Control +	Uterus	18.22±0.64	0.37±0.03
+ Genistein	Uterus	9±2*	0.2±0.01*

*p < 0.05 in comparison with intact group.

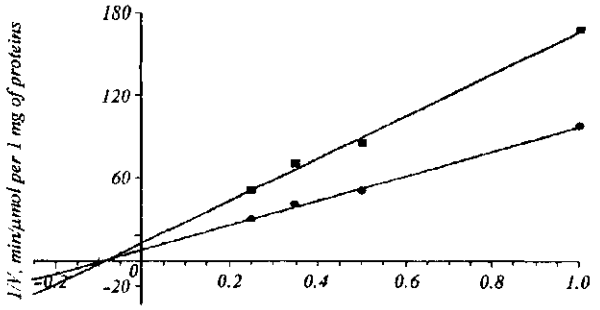

Fig. 2. Reverse dependence of the xanthine oxidase activity on the substrare concentration in reaction systems in the Lineweaver's and Burk's coordinates

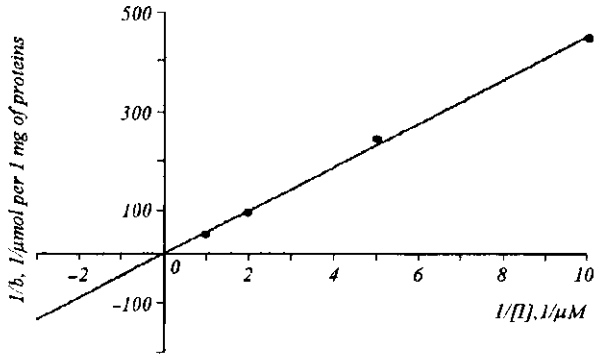

Fig 3. Reverse dependence of genistein binding to xanthine oxidase on its concentration in the reaction system ([*I*] — the inhibitor concentration)

ligands and induces the CYP1A1, CYP1A2 and CYP1B1 genes expression.

It was shown *in vitro* that genistein is a powerful xanthine oxidase inhibitor (fig. 1) and according to the kinetic studies the inhibition is not competitive (fig. 2). After incubation of the reaction systems icluding genistein of any concentration the amount of free genistein was less than injected. But after HCl destruction in all cases the free genistein level became equal to that of injected. In the reaction systems without xanthine oxidase the amount of free genistein was the same as injected. These results allow to conclude that genistein binds to xanthine oxidase. The dependence of the bound genistein quantity on its concentration in the reaction systems was reflected in the reverse coordinates (fig. 3). The dissociation constants (K_d) and B_{max} were: calculated as K_d = = 10 μM, B_{max} = 101 molecules per 1 enzyme molecule.

Therefore, genistein inhibits xanthine oxidase through binding to the enzyme. According to the quantity of genistein and its electron structure it may form a steady complex with the amino acids in the xanthine oxidase allosteric center — one histidine, one serine, two tyrosine and two phenylalanine residues [2]. We have also established that xanthine oxidase dependent superoxide production strongly decreases in the reaction systems with genistein (fig. 4). The inhibiting effect of xanthine oxidase dependent superoxide formation may be the main base of the genistein antioxidant activity.

It has been established that genistein does not change the nitric oxide synthase activity *in vitro*. So the most reasonable explanation may be that genistein like many steroids and structurally related compounds [1] is the inhibitor of the inducible nitric oxide synthase genes expression. The aromatase inhibiting activity of genistein has been also revealed *in vitro* (fig. 5). The inhibition is competitive according to the kinetic studies (fig. 6). Therefore genistein is the direct inhibitor of aromatase as well as the inhibitor of its gene expression.

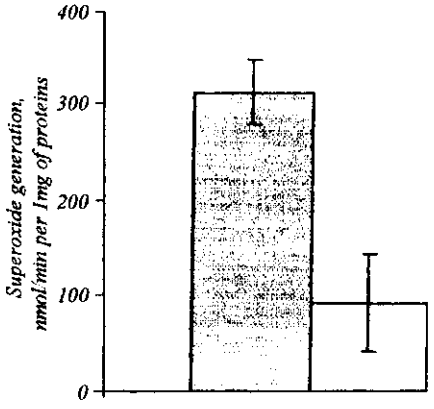

Fig. 4. The rate of superoxide generation by xanthine oxidase without genistein (black colour) and in the presence of 1.0 μM genistein

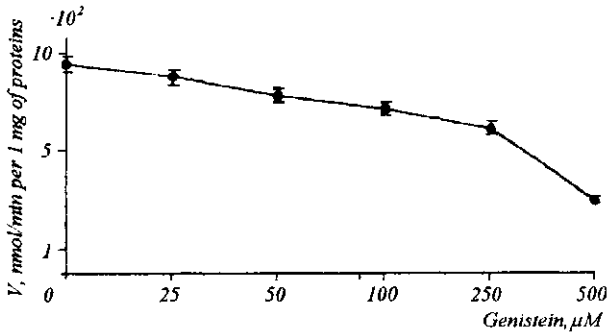

Fig. 5. Dependence of the aromatase activity on the genistein concentration in the reaction systems

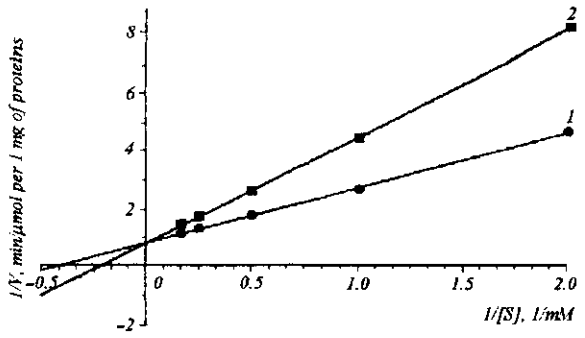

Fig. 6. Reverse dependence of the aromatase activity on the substrate concentration in reaction systems in the Lineweaver's and Burk's coordinates — without genistein (*1*) and in the presence of 100 μM genistein (*2*) ([*S*] — substrate concentration)

The results presented demonstrate the novel data concerning the antioxidative activity and inhibiting effect of genistein on nitric oxide and estrogens formation. It was also established genistein is the powerful cytochrome P450 1A1, 1A2 and 1B1 inductor.

В. В. Сумбаєв, І. М. Ясинська

Активність ксантиноксидази, синтази оксиду азоту та ароматази, а також вміст ізоформ 1А1, 1А2 та 1В1 цитохрому Р450 в організмі щурів при парентеральному введенні геністеїну

Резюме

Показано, що геністеїн пригнічує активність ксантиноксидази, синтази оксиду азоту в печінці, легенях та головному мозку щурів, а також знижує активність ароматази в яєчниках та матці щурів in vivo. Доведено, що він є індуктором ізоформ 1А1, 1А2 та 1В1 цитохрому Р450. У дослідах in vitro з'ясовано, що геністеїн алостерично пригнічує ксантиноксидазу, знижуючи утворення супероксиду за її участю, та конкурентно — ароматазу.

В. В. Сумбаев, И. М. Ясинская

Активность ксантиноксидазы, синтазы оксида азота и ароматазы, а также содержание изоформ 1А1, 1А2 и 1В1 цитохрома Р450 в организме крыс при парентеральном введении генистеина

Резюме

Установлено, что генистеин снижает активность ксантиноксидазы и синтазы оксида азота в печени, легких и головном мозге крыс, а также угнетает активность ароматазы в яичниках и матке крыс, уменьшая количество фермента in vivo. В опытах in vitro показано, что генистеин аллостерически ингибирует ксантиноксидазу, тормозя образование ею супероксида, и конкурентно — ароматазу.

REFERENCES

1. *Bruene B., Sandau K., von Knethen A.* Apoptotic cell death and nitric oxide: activating and antagonistic transducing pathways // Biokhimia (Moscow).—1998.—63.—P. 966—975.
2. *Sumbayev V. V.* The effects of corticosteroids, DDT and 4,9-dichlorodibenzodioxin on rat liver xanthine oxidase activity. The interactions between xanthine oxidase and liver cytochrome P 450 // Biokhimia (Moscow).—2000.—65.—P. 1146—1150.
3. *Sumbayev V. V.* Calculation of the amino acid structure of xanthine oxidase allosteric center // Amino Acids.—1999.—17.—P. 65—66.
4. *Koblyakov V. A.* Cytochrome P450 superfamily inductors as the cancerogenesis promoters // Biokhimia (Moscow)—1998.—63.—P. 1043—1058.
5. *Cos P., Ying L., Calomme M., Hu J. P., Cimanga K., Van Poel B., Pieters L., Vlietinck A. J., Vanden Berghe D.* Structure-activity relationships and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers // J. Nat. Prod.—1998.—61.—P. 71—76.
6. *Radi R., Tan S., Proclanov E.* Inhibition of xanthine oxidase by uric acid and its influence on superoxide radical production // Biochim. et biophys. acta.—1992.—122.—P. 178—182.
7. *Kellis J. T., Vickery L. E.* Inhibition of human estrogen synthetase (aromatase) by flavones // Science.—1984.—225.—P. 1032—1034.
8. *Korzekwa K. R., Trager W. F., Smith S. J., Osawa Y., Gillette J. R.* Theoretical studies on the mechanism of conversion of androgens to estrogens by aromatase // Biochemistry.—1991.—30.—P. 6155—6162.
9. *Brodie A., Lu Q., Long B.* Aromatase and its inhibitors // J. Steroid Biochem. Mol. Biol.—1999.—69.—P. 205—210.
10. *Sumbayev V. V., Rozanov A. Ya.* The investigation of regulation of the rat liver xanthine oxidase activity by reductors-antioxidants *in vitro* // Ukr. Biokhim. Zhurn. (Kyiv).—1998.—70, N 6.—P. 47—52.
11. *Hevel S. M., White K. A., Marletta M. A.* Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein // J. Biol. Chem.—1991.—266.—P. 22789—22791.
12. *Yasinska I. M.* Glutamate and aspartate as spacers for aromatase purification by affinity precipitation // Amino Acids.—1999.—17.—P. 66—67.
13. *James Margaret O.* Isolation of cytochrome P450 from hepatopancreas microsomes of the spiny lobster, *Panulirus argus*, and determination of catalytic activity with NADPH cytochrome P450 reductase from vertebrate liver // Arch. Biochem. and Biophys.—1990.—282.—P. 8—17.
14. *Sumbayev V. V.* Decomposition of 4,9-dichlorodibenzodioxin in the rat liver microsomes // Ukr. Biokhim. Zhurn. (Kyiv).—2000.—72, N 2.—P. 91—93.
15. *Omura T., Sato M.* The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties // J. Biol. Chem.—1964.—239.—P. 2379—2385.
16. *Pat.* N 22683 A 6C 12N 9/02. The method for xanthine oxidase purification / V. V. Sumbayev (1998) // Publ. 4/7/1998, Bull. N 3 UA.
17. *Elferink J. G. R.* Measurement of the metabolic burst in human neutrophils: a comparison between cytochrome *c* and nitroblue tetrazolium reduction // Res. Commun. Chem. Pathol. Pharmacol.—1984.—43.—P. 339—342.
18. *Voskresensky O. N., Sumbayev V. V.* Prediction of steroid compounds metabolism on the base of natural system of organic compounds // XV Int. Symp. on Med. Chem.: Book of Abstracts.—Edinburgh, 1998.—P. 208.

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