Uncoupling and Inhibition of the Respiratory Chain in Rat Liver Mitochondria by Some Naturally Occurring Estrogens and Their Metabolites

Torbjörn J.-O. Lundh* and Björn O. Lundgren†

Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, P.B. 7024, S-750 07 Uppsala, Sweden

The effects on oxidative phosphorylation in rat mitochondria of some of the most common estrogenic isoflavones and zearalenone and their metabolites were assayed polarographically with an oxygen electrode. In general, the substances act as uncoupling agents. At 0–100 μM concentrations, biochanin A and formononetin exerted half to one-tenth the uncoupling activity of 2,4-dinitrophenol, while coumestrol and genistein caused lesser effects and daidzein and equol caused slight uncoupling. Zearalenone, α-zearalenol, and β-zearalenol exerted uncoupling about equal with that of genistein. The isoflavones with highest uncoupling were likewise most effective as inhibitors of the respiratory chain and inhibit the electron transport at the same site as rotenone. The potential effect in vivo is discussed.

INTRODUCTION

Naturally occurring estrogens in food plants are mainly divided in two classes, plant estrogens and the resorcyclic acid lactones or fungal estrogens [for a review see Price and Fenwick (1985)]. According to Lindner (1976), more than 40 plant species have been shown to contain substances active in biological assays for estrogenic activity. Of the identified compounds, isoflavones and coumestans are the most common.

The isoflavonoids formononetin, biochanin A, daidzein, and genistein are normally present in high concentrations, especially in legumes such as red clover (*Trifolium pratense*) and subterranean clover (*Trifolium subterraneum*) (Pettersson and Kiessling, 1984; Shutt, 1976). High concentrations of coumestrol have been found in lucern and soya products (Knuckles et al., 1976; Shutt et al., 1969).

High consumption of the plants mentioned above has been shown to cause reproductive disturbances, such as infertility in sheep, with serious economic problems, especially in Australia [for example, Bennetts et al. (1946) and Shutt (1976)].

Unlike the two previous groups of estrogens, the resorcyclic acid lactones are not intrinsic components of food plants but are secondary mold metabolites. The most common estrogen of this group is zearalenone, a product of the fungus *Fusarium*, which commonly infests cereal crops already in the field and has been implicated in hyperestrogenism in swine (Kurtz et al., 1969; Olsen, 1985) fed zearalenone-contaminated food.

Many plant flavonoids have widespread biological activities including inhibition of the mitochondrial ATPase (Bohmont et al., 1987), of NADH oxidase (Hodnick et al., 1987), and of succinoxidase (Hodnick et al., 1986). Steroids, such as hormonally active estrogens, have also been shown to inhibit the electron transfer (Stoppani et al., 1968). Furthermore, Stenlid (1970) reported that flavonoids, including some of the estrogenic isoflavones, inhibit the formation of ATP in plant mitochondria and suggested that the inhibition of ATP formation is combined with an uncoupling effect. The present work was therefore carried out to determine if the most common naturally occurring estrogens (isoflavones, coumestrol, and zearalenone) affect ATP formation in the mammalian mitochondria by uncoupling oxidative phosphorylation and/or inhibiting the respiratory chain.

MATERIALS AND METHODS

Animals. Male Spraque-Dawley rats weighing 200–250 g were purchased from A-Lab, Sollentuna, Sweden. The rats were housed at a room temperature of 23 °C and with a 12 h light/dark cycle; they were fed a standardized pellet food (Ewos, Södertälje, Sweden) and tap water ad libitum.

Preparation of Mitochondria and Activity Measurement. Livers were removed immediately after decapitation of unanaesthetized animals and were placed in an ice-cold buffer of sucrose (25 M), Tris-HCl (5 mM), and EDTA (1 mM), pH 7.4. The livers were homogenized in a Potter-Elvehjem homogenizer, and the mitochondria were isolated by differential centrifugation according to the procedure of Tottmar et al. (1973). The oxygen consumption was measured polarographically with a Clark oxygen electrode in a thermostat-controlled electrode chamber at 30 °C, described by Eastabrook (1967). The standard medium contained KCl (80 mM), KH_2PO_4 (5 mM), $MgCl_2$ (5 mM), Tris buffer (20 mM, pH 7.4), and 100 μL of mitochondrial suspension (10 w/v %) in a final volume of 3 mL. Substrates used were glutamate (5 mM) or succinate (5 mM). To measure the respiratory control ratio (the quotient of the respiratory rate with and without ADP present), ADP (0.3 mM) was added to the mitochondrial suspension after the addition of substrate and the test substance. The different additions are exemplified in Figure 1.

Test Substances. The effects of the following substances on mitochondrial respiration were investigated: the phytoestrogens daidzein (4',7-dihydroxyisoflavone), genistein (4',5,7-trihydroxyisoflavone), and formononetin (7-hydroxy-4'-methoxyisoflavone), which was obtained from K&K Rare and Fine Chemicals, Plainview, NY; biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) from Sigma Chemical Co., St. Louis, MO; coumestrol (7,12-dihydroxycoumestan) from Eastman Kodak Co., Rochester, NY; and the formononetin metabolite equol (7,4'-dihydroxyisoflavan), which was donated by H. Adlercreutz, Helsinki, Finland. The mycotoxin, zearalenone, was a gift from Commercial Solvents Corp., and the metabolites α-zearalenol and β-zearalenol were produced by reduction of zearalenone in our laboratory. The well-known uncoupling agent 2,4-dinitrophenol (DNP) and the inhibitor rotenone were obtained from Sigma. The chemicals were of the highest purity commercially available. The isoflavones and the mycotoxins were also determined by HPLC techniques to >95% purity.

The test compounds were dissolved in dimethyl sulfoxide (DMSO), except for rotenone and 2,4-dinitrophenol, which were

† Present address: Department of Biology, Pharmacia Ophthalmics, S-751 82 Uppsala, Sweden.

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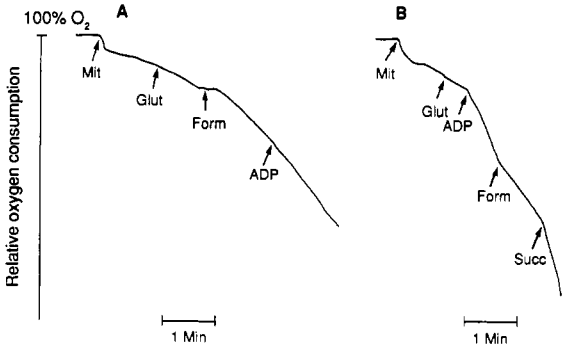

Figure 1. Typical polarographic tracing from rat mitochondria showing (A) uncoupling and (B) inhibiting effects of the plant estrogen formononetin. Experimental conditions are as described under Materials and Methods. Additions: Mit, mitochondrial suspension; Glut, glutamate (5 mM); Form, formononetin (100 μM); ADP, adenosine diphosphate (0.3 mM); and Succ, succinate (5 mM).

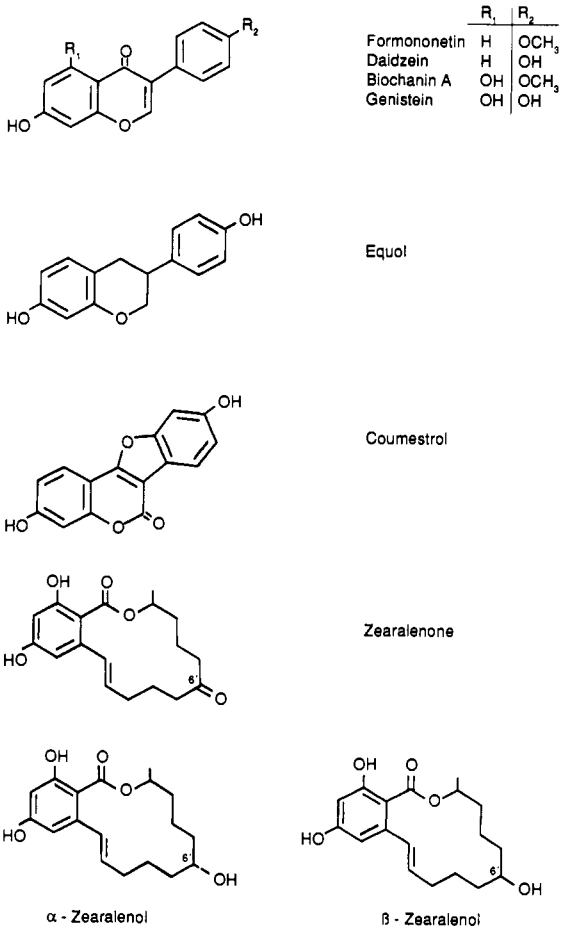

Figure 2. Chemical formulas of the plant estrogens, the mycotoxin zearalenone, and their major metabolites.

dissolved in methanol. The final concentration of the solvents did not exceed 0.8%. The chemical formulas of the plant estrogens, mycotoxin, and their metabolites are shown in Figure 2.

RESULTS

Uncoupling of Oxidative Phosphorylation. The results of the uncoupling experiments are shown in Table I. The oxygen consumption rate when all required compounds are present, except the test substances, is taken as 100% state 4 respiration (resting state of respiration with no ADP added) for the substrate used. One of the most potent uncoupling plant estrogens, biochanin A, increased the oxygen consumption markedly with a maximum effect at 50 μM. Compared with biochanin A, the other plant estrogens formononetin, genistein, and coumestrol showed a smaller increase in respiration, and the highest uncoupling effect of these substances was at the highest concentration tested (100 μM). Daidzein had only a slight uncoupling effect and equol almost no effect at all, even at the highest concentrations.

The mycotoxin zearalenone was more effective as an uncoupler than the metabolites α- and β-zearalenol and was comparable to genistein.

However, none of the estrogenic substances studied was as active as the well-known uncoupler 2,4-dinitrophenol (DNP), except biochanin A, which was almost as active as DNP at a concentration of 50 μM (Table I). Much higher concentrations of the estrogens investigated were needed to increase the state 4 respiration compared with DNP.

Inhibition of the Respiratory Chain. The inhibition

Table I. Uncoupling of the Oxidative Phosphorylation in Rat Liver Mitochondria by Estrogenic Isoflavones, the Mycotoxin Zearalenone, and Their Metabolites[a]

substance	concn, μM	basal respiration,[b] %	respiratory control ratio[c]
formononetin	0	100	5.8
	10	143	5.4
	50	200	2.6
	100	338	1.1
daidzein	0	100	5.8
	50	105	5.1
	100	134	4.2
equol	0	100	5.8
	100	104	3.4
coumestrol	0	100	5.4
	10	125	3.5
	50	176	1.3
	100	227	1.0
genistein	0	100	5.4
	10	100	5.5
	50	219	1.9
	100	395	1.0
biochanin A	0	100	5.4
	10	126	4.1
	20	295	1.8
	50	589	1.0
	100	410	1.0
zearalenone	0	100	5.2
	8.5	80	5.3
	42.5	180	2.3
	85.0	410	1.0
α-zearalenol	0	100	5.2
	8.5	80	5.3
	42.5	135	3.4
	85.0	240	1.5
β-zearalenol	0	100	5.2
	8.5	100	5.0
	42.5	180	2.9
	85.0	320	1.0
2,4-dinitrophenol	0	100	6.2
	1	122	5.3
	5	233	2.5
	10	424	1.6
	20	584	1.3
	50	663	1.1
	100	763	1.0

[a] The results represent mean values of duplicates and triplicates with a mean variation of approximately 10%. For experimental details see Materials and Methods. [b] State 4 respiration, the respiration with no ADP added. [c] Oxygen consumption with ADP divided by oxygen consumption without ADP.

Table II. Inhibition of the Respiratory Chain in Rat Liver Mitochondria by Estrogenic Isoflavones and Their Metabolites[a]

substance	concn, μM	inhibition,[b] %
formononetin	50	26
	100	42
daidzein	50	7
	100	18
equol	50	17
	100	29
coumestrol	50	31
	100	53
genistein	50	20
	100	37
biochanin A	10	20
	20	27
	50	40
	100	80

[a] The results represent mean values of duplicates with a mean variation of approximately 10%. For experimental details see Materials and Methods. [b] The degree of inhibition is calculated as 100% oxygen consumption (only substrate with ADP) minus the percentages of oxygen consumption with the plant estrogens present.

studies were performed only with the plant estrogens. The oxygen consumption rate with a mitochondrial suspension and glutamate in the presence of ADP was postulated as 0% inhibition. The most effective inhibitor was biochanin A, followed by coumestrol, formononetin, and genistein (Table II). Equol and daidzein were the two weakest inhibitors of the respiratory chain.

To localize the inhibition sites of the plant estrogens, succinate was also used as a substrate. However, no visible inhibition by the plant estrogens was detected with succinate as substrate, which suggests that the investigated estrogens act as inhibitors at the same place as rotenone (site I) in the respiratory chain.

DISCUSSION

Many plants contain more or less poisonous substances that protect them against different injuries caused by mammalian animals as well as other organisms, such as fungi, bacteria, and viruses. The estrogens investigated in this study are probably best known as substances causing reproductive disturbances in livestock (Bennetts et al., 1946), but these compounds, as well as rotenone, belong to the flavonoid family, which is known to have a wide range of effects on biological systems (Middleton, 1984).

Like many other phenolic substances, the investigated plant estrogens and the mycotoxins act as uncouplers in liver mitochondria. Among the tested plant estrogens, biochanin A, formononetin, genistein, and coumestrol show the highest uncoupling activity (Table I). Formononetin and biochanin A are often found in very high concentrations in some agriculturally important pasture legumes, e.g., red clover (0.5–5% of dry matter). The amount of genistein is relatively low in red clover, but large amounts have been detected in subterranean clover (in Australia) (Beck, 1964; Francis et al., 1967). Coumestrol and daidzein, on the other hand, are often found in soybean and alfalfa (Knuckles et al., 1976; Verdeal and Ryan, 1979), while equol is the major metabolite found in urine and blood plasma from ruminants fed pasture containing formononetin and daidzein (Braden et al., 1971; Lundh et al., 1990).

The fact that these substances, which are produced in high concentrations by the plants, also show the highest uncoupling and inhibition effect may probably be one mechanism by which plants protect themselves against microbial and insect attacks. Most data on the biological properties of isoflavonoid phytoalexins concern fungi, but bacteriostatic effects have also been shown [for a review see Bailey and Mansfield (1982); Smith and Banks, 1986].

The mycotoxin zearalenone and its metabolites α- and β-zearalenol are known to cause estrogenic disturbances in different animal species (Allen et al., 1981; Kurtz et al., 1969; Speers et al., 1971). The uncoupling effect of zearalenone and its metabolites shown in this study is, however, not unexpected as many mycotoxins, like the flavonoids, show a variety of effects on the metabolic processes in animal cells (Kiessling, 1986).

The substances studied by us have been shown to affect the mitochondrial respiration by uncoupling as well as inhibition of the respiratory chain. However, it is difficult to draw conclusions from these results as to their effects on respiratory chains in vivo. The comparatively low uncoupling activity by zearalenone and its metabolites may have little uncoupling effect in vivo because zearalenone is found in rather low concentrations in infested grain (30 ppm) in Sweden (Olsen, 1985). Furthermore, Olsen et al. (1985) show that zearalenone is metabolized very rapidly in pigs and the metabolite α-zearalenol was extensively found as glucuronid conjugates (more than 99%) in blood and urine. The plant estrogens, on the other hand, are often present in very high amounts in silage, and a daily intake of about 25–50 g of formononetin and biochanin A is not unusual for dairy cattle in Sweden. However, in ruminants, formononetin and biochanin A are mainly demethylated by the rumen microorganisms to daidzein and genistein, respectively. Genistein is further metabolized to *p*-ethylphenol and a phenolic acid, whereas daidzein is reduced to equol (Cox et al., 1984). Equol is the substance responsible for estrogenic disturbances in sheep grazing estrogenic pasture (Shutt and Braden, 1968) and is the major metabolite found in blood plasma (200–600 μg/100 mL) from cattle and sheep (Braden et al., 1971; Lundh et al., 1990). However, equol shows the lowest effect on the respiratory chain of all substances investigated in this study. Furthermore, equol and its parent compounds (formononetin and daidzein) as well as biochanin A and genistein are mainly found (more than 90%) conjugated with glucuronic acid in blood plasma from cattle and sheep (Braden et al., 1971; Lundh et al., 1990), which probably minimizes the uncoupling and inhibition effect on the respiratory chain in systemic organs in vivo. On the other hand, they may affect the organism locally in the intestinal areas where the absorption takes place. In ruminants, this takes place especially in the rumen and, to a lesser extent, in the other compartments of the complex stomach. However, in monogastric animals, the plant estrogens could probably cause problems not only in the intestinal organ but also in other organs depending on how the substances are metabolized and if they are conjugated already in the digestive tract or in the liver.

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Registry No. Daidzein, 486-66-8; genistein, 446-72-0; formononetin, 485-72-3; biochanin A, 491-80-5; coumestrol, 479-13-0; equol, 531-95-3; zearalenone, 17924-92-4; α-zearalenol, 36455-72-8; β-zearalenol, 71030-11-0.