Influence of long-term dietary administration of procymidone, a fungicide with anti-androgenic effects, or the phytoestrogen genistein to rats on the pituitary–gonadal axis and Leydig cell steroidogenesis

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Abstract

Procymidone is a fungicide with anti-androgenic properties, widely used to protect fruits from fungal infection. Thereby it contaminates fruit products prepared for human consumption. Genistein-containing soy products are increasingly used as food additives with health-promoting properties. Therefore we examined the effects of long-term dietary administration (3 months) of the anti-androgen procymidone (26·4 mg/animal per day) or the phytoestrogen genistein (21·1 mg/animal per day) to rats on the pituitary–gonadal axis in vivo, as well as on Leydig cell steroidogenesis and on spermatogenesis ex vivo. The procymidone-containing diet elevated serum levels of LH and testosterone and, furthermore, Leydig cells isolated from procymidone-treated animals displayed an enhanced capacity for producing testosterone in response to stimulation by hCG or dibutyryl cAMP, as well as elevated expression of steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage (P450 scc) and cytochrome P450 17α (P450c17). In contrast, the rate of DNA synthesis during stages VIII and IX of spermatogenesis in segments of seminiferous tubules isolated from genistein-treated rats was decreased without accompanying changes in the serum level of either LH or testosterone. Nonetheless, genistein did suppress the ex vivo steroidogenic response of Leydig cells to hCG or dibutyryl cAMP by down-regulating their expression of P450 scc. Considered together, our present findings demonstrate that long-term dietary administration of procymidone or genistein to rats exerts different effects on the pituitary–gonadal axis in vivo and on Leydig cell steroidogenesis ex vivo. Possibly as a result of disruption of hormonal feedback control due to its anti-androgenic action, procymidone activates this endocrine axis, thereby causing hypergonadotropic activation of testicular steroidogenesis. In contrast, genistein influences spermatogenesis and significantly inhibits Leydig cell steroidogenesis ex vivo without altering the serum level of either LH or testosterone.


Introduction

It is increasingly clear that certain industrial chemicals, pharmaceuticals, phytosterols and food supplements can mimic or antagonize the actions of endogenous hormones and in this manner adversely affect the endocrine and reproductive systems (Gray et al. 2001). Because of the crucial roles played by hormones in controlling development (Colborn et al. 1998), such endocrine disruption can exert profound effects. Environmental chemicals suspected of acting as endocrine disrupters can be divided into two major classes: anti-androgens, primarily pesticides, that are antagonists of the androgen receptor; and compounds with estrogen-like structures that act as agonists for estrogen receptors. Although anti-androgenic and estrogenic are two major classes of endocrine disruptor compounds, it should be noted that there are additional major groups of compounds such as dioxins, phthalates and thyroidal or anti-thyroidal endocrine disruptors that are as important as anti-androgenic or estrogenic compounds.

The pesticide procymidone is used as a fungicide and was shown to be present in fruit products prepared for human consumption (Ostby et al. 1999, Ohno et al. 2003). It is a typical anti-androgen, competitively inhibiting the binding of androgens to the human androgen receptor and thereby preventing androgen-induced gene expression (Ostby et al. 1999). This substance reduces anogenital distance and the size of accessory sex glands in male pups.
(Ostby et al. 1999), and, moreover, upon long-term administration induces hypergonadotropism and Leydig cell tumors in rats (Murakami et al. 1995).

In contrast, the phytoestrogen genistein has a structural similarity to 17β-estradiol (Price & Fenwick 1985) and binds to both α and β estrogen receptors with a moderate selectivity for the β isof orm (Manas et al. 2004). Humans are exposed to this phytoestrogen via their diet, where a major source is soy beans and related products (Price & Fenwick 1985). Thus it has been reported that serum concentrations of phytoestrogens are higher in Japanese than in Finnish men (Adlercreutz et al. 1993) and are also higher in infants who consume large amounts of food products derived from soy beans (Setchell et al. 1997).

In the present investigation we have examined whether long-term intake of a relatively high dietary level of procmidone or genistein by rats influences the functions of their pituitary–gonadal axis in vivo. Since this axis plays a central role in the regulation of reproduction and fertility, steroidogenesis by Leydig cells and the intensity of spermatogenesis were also studied ex vivo in these same rats.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s nutrient mixture F-12, modified Eagle’s medium (MEM), Hank’s balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺ and penicillin/streptomycin were all obtained from Gibco/BRL (Life Technologies, Paisley, Scotland, UK). BSA (fraction V), Percoll, Heps, collagenase type I, human chorionic gonadotropin (hCG) and dibutyryl cAMP ((Bu)₂cAMP) were obtained from Sigma Chemical Co., St Louis, MO, USA.

Animals

Male Sprague–Dawley rats were bred and raised in our own colony. Their mothers and the experimental animals themselves were maintained on a specially prepared soy-free potato protein-supplemented commercially available rat chow (Sniff; Soest, Germany). At the age of 3 months subgroups of animals were divided into three treatment groups so that there were no statistically significant differences among group body–weight means. Each treatment group contained 10 or 11 rats, which were given the same food to which genistein (1 g/kg) or procymidone (1·25 g/kg) was added. Animals remained on this food for 12 weeks while control animals received the soy-free basic diet. The food to which the substances were added as well as the control food was isocaloric to normal maintenance food and well tolerated by the animals, of which the mean body weight at the beginning of the experiments was 368 ± 11 g and at the end 457 ± 12 g for controls and genistein-fed rats and 436 ± 9 g for the procymidone-treated rats. Mean food intake of the animals was 21·1 g/animal per day regardless of whether test substances were added or not. Thus each animal ingested an average of 26·4 mg procymidone/day (60·6 mg/kg body weight) and 21·1 mg genistein/day (46·2 mg/kg body weight). These are doses in the lower range compared with those commonly used by others: see Gray et al. (1999) and more recently Kang et al. (2004) for procymidone and Li & Yu (2003) for genistein. Following this treatment, the animals were killed, their testes used for isolation of Leydig cells and serum samples collected and maintained at –80 °C for later analysis of testosterone and luteinizing hormone (LH) levels.

Isolation and culture of Leydig cells

Leydig cells were prepared from the testes of control and genistein- or procymidone-treated rats by collagenase treatment, as described earlier (Svechnikov et al. 2001). Briefly, decapsulated testes were incubated with collagenase (0·25 mg/ml) for 20 min at 37 °C, after which the mixture of interstitial cells thus obtained was collected by centrifugation at 300 g for 10 min, followed by washing in HBSS containing 0·1% (w/v) BSA. To isolate Leydig cells, this cell suspension was then loaded on top of a discontinuous Percoll gradient (consisting of layers with 20, 40, 60 and 90% Percoll in HBSS) and subsequently centrifuged at 800 g for 20 min. The third fraction from the top of the gradient, enriched in Leydig cells, was collected and these isolated Leydig cells washed twice in DMEM/F12 and thereafter resuspended in DMEM/F12 supplemented with 15 mM Heps (pH 7·4), 1 mg/ml BSA, 365 mg/l glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

The purity of these Leydig cells was 85%, as determined by histochemical staining for 3β-hydroxysteroid dehydrogenase (Payne et al. 1980), and the cell viability, as assessed by Trypan Blue exclusion, was consistently greater than 90%. Subsequently, 100 µl of a suspension of 1·5 × 10⁵ cells/ml was placed into each well of 96–well plates (Falcon, Franklin Lakes, NJ, USA), and cultured for 2 h and thereafter incubated with or without hCG (0·01–10 ng/ml) or (Bu)₂cAMP (0·01–1 µM) for an additional 3 h.

Analysis of hormone concentrations

Media collected from the cultures of Leydig cells were stored at –20 °C prior to determination of testosterone employing the Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA), according to the manufacturer’s instructions. Serum concentrations of testosterone were assayed by a coated tube assay (DSL, Sinsheim, Germany). Serum LH was determined by a RIA kindly provided by the National Hormone and Pituitary Program (Dr AF Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA).
Microdissection and staging of seminiferous tubules

Testes were decapsulated and subjected to microdissection in PBS containing no calcium or magnesium (Gibco/BRL) with the aid of a trans-illuminating stereomicroscope. The stages of spermatogenesis in the seminiferous epithelium were identified as described previously (Parvinen & Vanha-Perttula 1972, Wahab-Wahlgren et al. 2003). Stage I, containing type A4 spermatogonia in the S phase, stage V, containing type B spermatogonia in the S phase, stage VIIa, containing preleptotene spermatocytes with no DNA synthesis and stage VIII–IX, containing preleptotene spermatocytes in the S phase (Parvinen et al. 1991), were chosen for culture in vitro. 2 mm segments of seminiferous tubules in these four different stages, each in 10 µl PBS, were transferred to individual wells on 96-well plates (Falcon 3072 Microtest III; Becton Dickinson, Lincoln Park, NJ, USA) for subsequent culture.

Thymidine incorporation

The segments of seminiferous tubules were incubated in 100 µl α-modified Eagle’s minimum essential medium (α-MEM; ICN Biomedicals, Aurora, OH, USA) supplemented with 1-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and α-thioglycerol (7.5 × 10⁻⁵ M), at 34 °C for 24 h. Following 20 h of this incubation, the segments were labelled with 0.5 µCi [³H]thymidine (5-0 Ci/mmol; Amersham Biosciences, Little Chalfont, Bucks, UK) for the remaining 4 h and thereafter maintained frozen in 100 µl α-MEM containing 10% DMSO and 10% fetal calf serum until use. After thawing, the segments were harvested onto glass-fibre filters (Skatron, Lier, Norway) using a Skatron Instruments Cell Harvester. Each sample was then punched out and transferred to vials containing Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA, USA) for determination of the radioactivity incorporated as a measure of DNA synthesis, employing a Beckman LS500CE scintillation spectrometer (Beckman Coulter, Bucks, UK).

Western blot analysis

The effects of genistien and procymidone on the levels of expression of cytochrome P450 side-chain cleavage (P450 scC), cytochrome P450 17α (P450c17) and steroidalogenic acute regulatory protein (StAR) by Leydig cells were examined by PAGE/Western blotting as described earlier (Svechnikov et al. 2003). Briefly, the cells were washed twice with PBS and then lysed and sonicated in a buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol and 10% glycerol. Subsequently, the fraction thus solubilized was separated by deeris by centrifugation at 10 000 g for 6 min and the proteins present in the resulting supernatants (30 µg protein from each sample) separated by electrophoresis on 12.5% SDS/polyacrylamide gels and thereafter transferred electrophoretically to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences), using 25 mM Tris/HCl and 185 mM glycine (pH 8.3) containing 20% methanol. The PVDF membrane was stained with 5% Ponceau S for 5 min to control equal transferring of proteins to the membrane. Only membranes with equal levels of proteins from all lanes were processed further.

Following this transfer, the PVDF membrane was incubated in a blocking buffer (Tris-buffered saline (TBS) containing 5% non-fat dry milk) for 1 h and then subjected to three washes with TBS containing 0.1% Tween. Polyclonal antisera directed against StAR (kindly provided by Dr DM Stocco; Clark et al. 1994) and P450c17 (a gift from Dr DB Hales; Hales 1992) were used for incubation at 1:3000 and 1:2000, respectively, in TBS containing 5% non-fat dry milk overnight at 4 °C. P450 scC antibody (goat polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:1000 under the same conditions as described for StAR and P450c17. After washing, membranes were incubated with donkey anti-rabbit or rabbit anti-goat IgG secondary antibodies conjugated with horseradish peroxidase (Amersham Biosciences) and the bands detected by incubation with ECL Plus Western blotting agent (Amersham Biosciences) and subsequent exposure to Hyperfilm ECL (Amersham Biosciences). Finally, the ECL Hyperfilms were scanned with an HP Scan Jet 5100C using HP Precision scan software (Hewlett-Packard Sverige AB, Kista, Sweden) and the extent of antibody binding quantified using NIH Image 1.57 software.

Statistical analyses

Differences between the various values were analysed for statistical significance by one-way analysis of variance (ANOVA), with supplementation by Student–Newman–Keuls t-test. P<0.05 was considered as statistically significant.

Results

Effects of dietary genistien or procymidone on the levels of LH and testosterone in rat serum

Dietary administration of procymidone to rats for 12 weeks resulted in 3- and 6-fold increases in their serum concentrations of LH and testosterone, respectively; whereas similar treatment with genistien was without significant effect on these parameters (Fig. 1).

Effects of dietary genistien or procymidone on hCG- or (Bu)₂cAMP-stimulated steroidogenesis by rat Leydig cells ex vivo

As depicted in Fig. 2, testosterone production by Leydig cells isolated from procymidone-treated rats was more
potently stimulated by hCG and cAMP than was the corresponding production by cells isolated from control animals receiving a soy-free diet. In contrast, Leydig cells prepared from genistein-treated rats almost completely lost their responsiveness to hCG and (Bu)\(_2\)cAMP.

**Effects of genistein or procymidone on the levels of expression of StAR, P450scc and P450c17 by Leydig cells**

To determine whether the significant alterations in Leydig cell steroidogenesis *ex vivo* observed in rats administered either genistein or procymidone, as well as the increase in the plasma concentration of testosterone observed in procymidine–treated animals, were correlated with changes in the expression of the key steroidogenic enzymes, the levels of these proteins were measured by Western blotting. Leydig cells isolated from procymidone–treated animals expressed 1·8-fold more StAR protein (Fig. 3A) and a 4·5-fold higher level of P450c17 (Fig. 3C) than did cells from control rats; in contrast, genistein was without effect on the expression of these proteins. Furthermore, whereas the expression of P450scc by Leydig cells isolated from genistein–treated rats was suppressed 4-fold compared with control cells, dietary administration of procymidone significantly increased this expression (Fig. 3B).

**Effects of genistein or procymidone on spermatogenesis**

To characterize the effects of dietary administration of genistein or procymidone on testicular function in greater detail, the influence of such treatment on spermatogenesis was also examined. Genistein, but not procymidone, markedly reduced meiotic DNA synthesis during stages VIII and IX (Fig. 4), whereas neither compound exerted any effect on the other stages investigated (results not shown).

**Discussion**

The present investigation demonstrates that long-term dietary administration of the phytoestrogen genistein or of procymidone, a fungicide with anti-androgen action, to rats exerts different effects on their pituitary–gonadal axis.
In another experiment with an identical treatment regime serum concentrations of genistein were found to be 8 µM, concentrations at which uterine weight is slightly increased as a sign of estrogenic effect (results not shown). Concentrations of between 1 and 10 µg/ml (corresponding to high-nanomolar or low-micromolar concentrations) were reported in women under commercial soy isoflavone supplementation (Setchell et al. 2001). It can be speculated that the soy intake of some males is similar to that of females. Similarly, low-micromolar concentrations of genistein were found in infants who consumed large amounts of food products derived from soy beans (Setchell et al. 1997). Therefore, the effects to be discussed below may be of specific concern for male fertility. Procymidone concentrations in the serum of our experimental animals are unknown. It is known, however, that high-milligram quantities of procymidone were found in unhusked rice (1·4–2·3 mg/kg), tomatoes (about 5 mg/kg), grapes and wine (US Environmental Protection Agency (EPA) Prevention, Pesticides and Toxic Substances. EPA Office of Pesticide Programs, 1995. Publication number: EPA 735-R-95-001), suggesting that an intake of significant quantities of this compound in humans is possible. For these reasons the dose of procymidone used in the present experiments may also be of concern for male fertility in the human. A sign of bioavailability of the orally administered procymidone is activation of the pituitary-gonadal axis, which resulted in the hypergonadotropic activation of testicular steroidogenesis reflected in the findings that Leydig cells isolated from rats treated with this compound displayed an enhanced capacity to produce testosterone in response to stimulation by hCG or (Bu)2cAMP, as well as elevated expression of several key steroidogenic enzymes, i.e. StAR, P450 sc and P450c17. In contrast, treatment with genistein influenced spermatogenesis without altering the serum levels of LH or testosterone. At the same time, genistein suppressed the steroidogenic response of Leydig cells to hCG and (Bu)2cAMP by down-regulating the expression of P450 sc.

**Figure 3** Effects of dietary administration of genistein or procymidone treatment on the levels of StAR, P450 sc and P450c17 expression by Leydig cells. Samples of whole lysates (30 µg protein) of Leydig cells isolated from genistein- or procymidone-treated rats were analyzed by Western blotting using specific antisera for these proteins. Each enzyme-specific band was quantitated in relative densitometric units by scanning with NIH Image software. Means ± S.E.M from three independent Leydig cell preparations are presented. *P<0·05, **P<0·01 compared with the corresponding control.

**Figure 4** Inhibition of ex vivo DNA synthesis during stages VIII and IX of spermatogenesis in rat seminiferous tubules by genistein but not procymidone. Seminiferous tubules in stages VIII and IX isolated from treated rats were cultured at 34 °C for 20 h alone and then for 4 h in the presence of [3H]thymidine, after which the incorporation of radioactivity into DNA was quantified as described in the Materials and Methods section. The data presented are means ± S.E.M c.p.m. from six segments microdissected separately in each of three independent experiments. *P<0·05 compared with the control value.
The fungicide procymidone exerts its anti-androgenic activity by binding directly to the androgen receptor in the prostate (Hosokawa et al. 1993b). This finding led to the proposal that procymidone might inhibit the negative-feedback effect of androgen on the hypothalamus and/or the pituitary, thereby resulting in hypergonadotropism. This proposal is supported by the results reported here, as well as by several other observations that long-term dietary administration of procymidone to rats elevates serum levels of testosterone and LH and activates Leydig cell function (Hosokawa et al. 1993a, Murakami et al. 1995). Increased secretion of LH by the pituitary of procymidone–treated rats activates steroidogenesis by the Leydig cells and elevates the plasma testosterone level. LH plays a pivotal role in regulating the expression of steroidogenic enzymes in Leydig cells, as demonstrated by the findings of Chase and co-workers (1992), who reported that infusion of exogenous LH into rats for 5 days increased the activities and levels of the P450 scc and P450c17 enzymes in their Leydig cells. Interestingly, long-term procymidone-induced hypergonadotropism and hyperstimulation of Leydig cells has been shown to result in interstitial cell tumors in male rats, but not in mice (Murakami et al. 1995).

The present study also revealed that long-term dietary administration of the non-steroidal phytoestrogen genistein to rats affected their pituitary-gonadal axis in vivo and ex vivo steroidogenesis by their Leydig cells in a fashion different from procymidone. Despite the lack of any changes in their serum levels of LH or testosterone, Leydig cells isolated from genistein-treated rats lost their responsiveness to hCG and (Bu)2cAMP almost completely, suggesting that genistein and/or its metabolites have direct effects on Leydig cell function. This reduced steroidogenesis is presumably due to a decrease in the expression and activity of P450 scc, although there were no apparent decreases in serum testosterone levels. Similarly, a decrease in the activity of the steroidogenic enzymes without apparent reduction in serum testosterone levels after treatment by phthalate has been reported earlier (Akingbemi et al. 2001). It might be suggested that the inhibition of P450 scc was not profound enough to affect testosterone production by Leydig cells at the time of serum testosterone measurement or that compensatory mechanisms were activated in vivo to counteract the effect of decreased enzyme activity. Another possible explanation for this finding is that phytoestrogens can inhibit the activities of 5α-reductase and aromatase in Leydig cells, as has been shown in peripheral tissues (Kells & Vickery 1984, Evans et al. 1995), thereby decreasing the rate of testosterone metabolism and supporting the level of circulating androgen despite suppression of upstream steroidogenic enzyme.

To date, relatively little is known concerning the effects of genistein on steroidogenesis by Leydig cells. It has been reported that long-term dietary administration of this substance reduces serum levels of testosterone and androstenedione without affecting the expression of StAR in rats (Weber et al. 2001) and, furthermore, suppresses both basal and LH-stimulated androgen production by rooster Leydig cells in vitro (Opalka et al. 2004). Moreover, genistein has been shown to inhibit follicle-stimulating hormone- and forskolin-stimulated production of progesterone by primary cultures of rat ovarian cells (Whitehead & Lacey 2000). In contrast to the study by Weber et al. (2001), we and others (Makela et al. 1995, Ohno et al. 2003) could not find significant changes in plasma androgen levels in longer-term studies of phytoestrogen effects on reproductive function. This discrepancy may be due to a difference in the composition of the phytoestrogens used to treat the animals. In our study, rats received a chow containing genistein only, whereas in the report by Weber and co-workers (2001) animals were fed with a phytoestrogen-rich diet containing seven different derivatives of genistein, with genistein being a minor component. It can be speculated that these phytoestrogens, due to their synergistic or additive effects on Leydig cells, could have a more potent suppressive effect on steroidogenesis than genistein alone.

In the present study we have demonstrated that dietary genistein down-regulates the expression of mitochondrial P450 scc, which catalyses the conversion of cholesterol to pregnenolone. The mechanism remains unknown, but may involve activation of the estrogen receptor by genistein. This suggestion is supported by the recent report that the xenoestrogen methoxychlor (a derivative of DDT) alters steroidogenesis by immature rat Leydig cells by suppressing the expression of P450 scc and that the antiestrogen ICI 182 780 prevents this effect (Akingbemi et al. 2000). In addition, genistein and its derivatives were found to be potent inhibitors of P450c21 and human adrenocortical 3β-hydroxysteroid dehydrogenase in vitro (Ohno et al. 2002). It should be also noted that the mechanisms underlying the biological actions of genistein are complex, since in addition to its estrogen-like effects (Makkes 1998), this compound is also a tyrosine kinase inhibitor (Akiyama et al. 1987). Therefore, the observed biological responses to genistein will be determined by both activation of the expression of specific estrogen-dependent genes and alterations in signalling pathways in the target cells.

The present investigation also reveals that chronic dietary exposure of rats to genistein significantly reduces the rate of meiotic DNA synthesis in preleptotene spermatocytes during stages VIII and IX of spermatogenesis, without affecting the other stages examined. This observation is consistent with the previous finding that genistein inhibits both proliferation and differentiation of spermatogonia and primary spermatocytes in primary testis cell cultures, isolated from the medaka (Song & Gutzeit 2003). Similarly, pre- and postnatal dietary high-dose exposure of rats to this phytoestrogen has been shown to cause aberrant and delayed spermatogenesis, including degeneration of

spermatocytes and depletion of spermatids, resulting in a deficit of sperm in the epididymis (Delcos et al. 2001).

In summary, our findings demonstrate that long-term dietary administration of procymidone or genistein to rats exerts different effects on the pituitary-gonadal axis in vivo and on Leydig cell steroidogenesis ex vivo. Possibly as a result of disruption of hormonal feedback control due to its anti-androgenic action, procymidone activates this endocrine axis, thereby causing hypergonadotropic activation of testicular steroidogenesis. In contrast, genistein influences spermatogenesis and significantly inhibits Leydig cell steroidogenesis ex vivo without altering the serum level of either LH or testosterone.

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