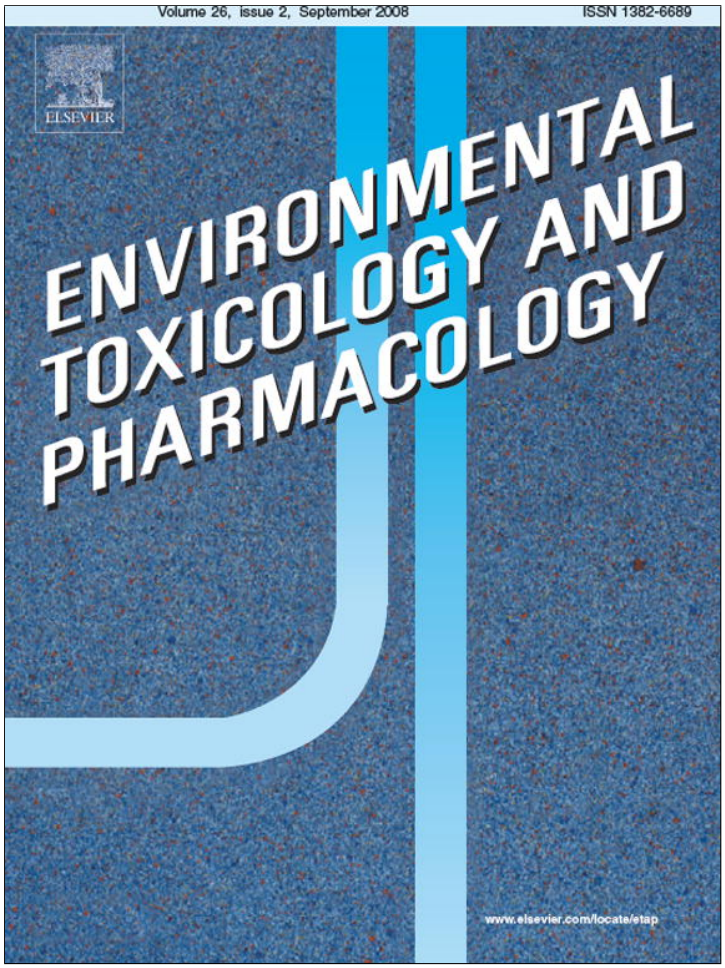

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Low dose 4-MBC effect on neuroendocrine regulation of reproductive axis in adult male rats

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ABSTRACT

4-Methylbenzylidene camphor (4-MBC) is an ultraviolet absorbent. The objective of this paper was to evaluate the effect of 4-MBC low-dose exposure on the neuroendocrine reproductive regulation in male rats. Wistar male adult rats were injected sc. with 4-MBC during 5 days with a dose of 2 and 10 mg/kg or during 2 days with a dose of 2 and 20 mg/kg. In all rats serum prolactin, LH and FSH concentration were assayed. The hypothalamus of rats injected during 2 days were also dissected to study GnRH release. Rats that received 2 and 10 mg/kg of 4-MBC during 5 days showed a decrease in the LH and FSH serum concentration. In rats injected during 2 days, serum LH decreased with 2 and 20 mg/kg and FSH decreased with 2 mg/kg of 4-MBC. *In vitro* hypothalamic GnRH release also decreased in these animals. These results show that low doses of 4-MBC inhibit the reproductive axis in adult male rats.

1. Introduction

It has been determined that certain chemical substances called endocrine disrupters (ED) are either voluntarily or involuntarily incorporated into the organism and are capable of altering the different endocrine axis regulation, particularly the reproductive one. Some of them have been involved as potential activators of sexual steroid signalling. These ED with estrogenic or antiandrogenic effects are responsible for alterations in males such as sperm anomalies, cryptorchidism, hypospadia and testicular cancer, and in females are responsible for increasing the incidence of mammary cancer (Damgaard et al., 2002; Sharpe, 2003).

A number of ultraviolet (UV) light absorbents, particularly those of the camphor and cinnamate line, are suspected to be endocrine disrupters (Holbech et al., 2002; Inui et al., 2003; Mueller et al., 2003). The 3-4-methylbenzylidene camphor (4-MBC: Eusolex 6300), and the octylmethoxy-cinnamate (OMC) are the major UV absorbents used in sunscreens and belong to this group of endocrine disrupters (Schlumpf et al., 2001). Both substances have the individual feature of being absorbed through the skin (Janjua et al., 2004).

It has been shown that 4-MBC produces an increase of the estrogen-like effects in female immature rats (Schlumpf et al., 2001) and in the MCF-7 cell line, derived from an estrogen sensitive mammary tumor (Tinwell et al., 2002). Nevertheless, in binding studies with estrogen receptors, 4-MBC has shown weak activity (Mueller et al., 2003; Klann et al., 2005). In spite of this fact, micromolar concentrations of this disrupter are capable of producing – as potently as estradiol does – the induction of the estrogen receptor gene expression (Klann et al., 2005), which indicates that 4-MBC has the potential to alter physiologic and developmental processes, mediated by signaling mechanisms of the estrogen receptor. On the other hand, *in vitro* studies have shown that 4-MBC has the capacity to increase the activation of the alpha and beta estrogen receptor, though with greater potential for the last one (Mueller et al., 2003; Schlumpf et al., 2004a,b).

The 4-MBC has shown effects at the reproductive system level. Such high doses as 500–1000 mg/kg of 4-MBC produce uterine weight increase when administered by subcutaneous and oral route to immature animals (Tinwell et al., 2002). Similar results have been observed in immature rats that received the drug in their food,

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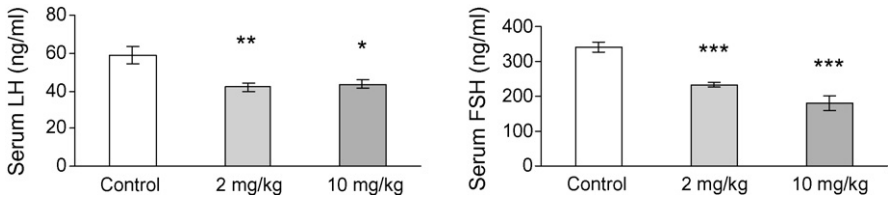

Fig. 1. Serum gonadotrophins concentration in adult male rats ($n = 10–12$ per group) exposed to 4-MBC during 5 days ($^{*}p < 0.02$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control).

whereby the uterus trophy effect is greater in the group receiving 4-MBC than in the OMC group (Schlumpf et al., 2001).

The administration of 4-MBC through skin prevents hepatic first-pass and the following 4-MBC metabolization (Volkel et al., 2006), and thus achieves greater plasma concentrations than the ones obtained with oral administration, in addition to being the dermal pathway the main drug entrance in humans.

Taking into account results found *in vivo* with high doses of 4-MBC, the objective put forward in this paper was to evaluate the effect of 4-MBC low doses administered *in vivo* during a short period over the neuroendocrine parameters that regulate the reproductive axis in male adult rats.

2. Material and methods

2.1. Animals

Male adult Wistar rats from the Department of Physiology, School of Medicine, University of Buenos Aires were used for this experiment. The animals were kept in a light, temperature and humidity controlled environment (lights on from 07.00 to 19.00 h; *T*, 22 °C). Animals were fed ad libitum and had free access to chow and water during the experiment, being 70 days old at the moment of the experiment. They were treated with a single 4-MBC subcutaneous dose, every day for 5 or 2 days. Each group consisted of about 10–12 animals. In all cases, adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in the European Communities Council Directives (86/609/EEC) and FRAME guidelines (FRAME Reduction Committee November 1999).

2.2. Experimental protocols

The 4-methylbenzylidene camphor (4-MBC, purity 99.9%), was purchased from BASF (Ludwigshafen, Germany). The drug was dissolved in olive oil and administered subcutaneously once a day in all cases, for which the two following treatment schemes were used: one group of animals was injected with 4-MBC at a dose of 2 and 10 mg/kg (MBC 2 and MBC 10), during 5 consecutive days. A second group of rats was injected during a shorter period of time with 4-MBC, receiving 2 and 20 mg/kg (MBC 2 and MBC 20), during 2 consecutive days. Control animals only received the vehicle. All rats were sacrificed by decapitation between 14.00 and 16.00 h, and trunk blood was collected to determine serum LH, FSH and prolactin levels. In the rats injected during 2 days hypothalamus samples were dissected to tissue incubation for study the GnRH secretion.

2.3. Hypothalamic incubation

Brain tissues were rapidly removed after decapitation, and the hypothalamus dissected out with a single razor blade and weighed. The hypothalamic samples containing the anterior preoptic and medial basal areas (APOA-MBH) were dissected with the help of a stereomicroscope. The hypothalamic samples were bordered laterally by the hypothalamic sulci, rostrally, 3 mm anterior to the optic chiasma; caudally, by the mammillary bodies; the depth was 3–4 mm. The thickness of each sample was less than 2 mm. This tissue included the median eminence.

After dissection, the anterior preoptic area (APOA) and the medial basal hypothalamic area (MBH) were kept in plastic chambers (similar hypothalamic samples were used in each incubation) containing 300 μl of Earle's medium with glucose (1 mg/ml) and bacitracin (20 mM). The pH was adjusted to 7.4. Each chamber was incubated in a Dubnoff shaker at 37 °C with constant shaking (60 cycles/min) in an atmosphere of 95% O_2, 5% CO_2. After 30 min of pre-incubation, time necessary to stabilize all tissue GnRH release, the medium was discarded and fresh medium was added. The samples were incubated for 60 min, the medium collected (basal samples) and immediately frozen at −80 °C for GnRH assay. The viability of tissue at the end of incubation was confirmed by adding high levels of KCl (56 mM) to the medium, showing an increase of GnRH secretion in response to depolarization produced by ClK.

2.4. Determination of LH, FSH, prolactin and GnRH

Gonadotropins and serum prolactin were determined in duplicate by using a double antibody radioimmunoanalysis (RIA) and precipitated with polyethylene glycol. The material was kindly provided by the NIAMD rat pituitary program. Results were expressed in nanogram per milliliter of serum (ng/ml), in terms of the reference preparation (rat LH RP-1, rat FSH RP-1 and prolactin RP-1). Intra and interassay coefficients of variation were 6 and 11%, respectively. All samples were analyzed in the same assay.

GnRH release determination in the samples obtained from the incubation medium was performed by single antibody RIA and precipitated with ethyl alcohol. Antiserum was kindly handed over by Dr. Wuttke (Goettingen, Germany). Results were expressed in picograms/milliliter/10 mg of fresh tissue. The limit for GnRH detection was 0.2 pg/ml. Intra and interassay coefficients of variation were 7 and 14%, respectively.

2.5. Statistical analysis

Results are expressed as the mean ± S.E.M. Significance was assessed by analysis of variance (ANOVA) and the Bonferroni post test. Student *t*-test was also used and a $p < 0.05$ was considered significant.

3. Results

Rats receiving 4-MBC during 5 days showed significant decrease in LH (Co, 58.71 ± 4.47; MBC 2, 42 ± 2.13 $p < 0.01$; MBC 10, 43.67 ± 2.28 ng/ml $p < 0.02$) and FSH (Co, 341.25 ± 14.45; MBC 2, 232.86 ± 6.8 $p < 0.001$; MBC 10, 180 ± 20.82 ng/ml $p < 0.001$) serum concentration (Fig. 1). Prolactin serum concentration changes were not observed in these animals.

Rats injected with 4-MBC during a 2-day period showed a lower LH serum concentration in both treated groups (Co, 70 ± 4.83; MBC 2, 39 ± 1.53 $p < 0.001$; MBC 20, 41 ± 1.77 ng/ml $p < 0.001$) and an FSH decrease in the group injected with 2 mg/kg of 4-MBC (Co, 250 ± 51.48; MBC 2, 88.13 ± 40.83 $p < 0.05$; MBC 20, 238.57 ± 22.72 ng/ml) (Fig. 2). Also a significant decrease

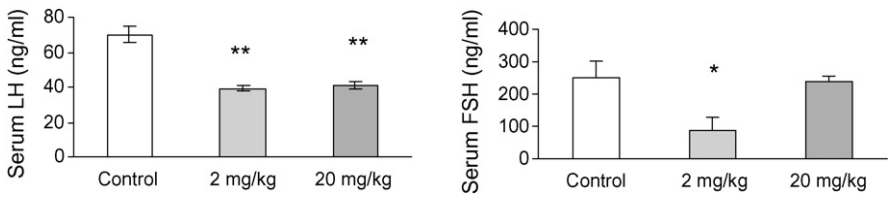

Fig. 2. Serum gonadotrophins concentration in adult male rats ($n = 10–12$ per group) exposed to 4-MBC during 2 days ($^{*}p < 0.05$ and $^{**}p < 0.001$ vs. control).

was observed in the GnRH release from the "hypothalamus" *in vitro* (Co, 6.37 ± 1.8; MBC 2, 1.74 ± 0.38 $p < 0.05$; MBC 20, 1.94 ± 0.84 pg/ml/10 mg of tissue $p < 0.05$).

4. Discussion

These results show that 4-MBC administered in low doses during short periods inhibits the reproductive axis in adult male rats, but does not modify the prolactin levels in blood. This endocrine disrupter generates changes in the reproductive axis through a weak estrogenic effect in rats (Schlumpf et al., 2001, 2004a,b). Many studies show that 4-MBC is a partial agonist of the estrogenic receptors alpha and beta (ERα and ERβ), and would show a preference for the ERβ (Mueller et al., 2003; Schlumpf et al., 2004a,b). Same as estradiol 17β would act on these receptors, on estrogen-sensitive organs and translated into a biological effect (Tinwell et al., 2002). Estrogens regulate the reproductive axis acting on the peripheral organs, but also change GnRH and gonadotropin secretion (Herbison, 1998). In males, estrogen participates in the mechanisms of negative feedback at the hypothalamic level (Mooradian et al., 1987; Rochira et al., 2006). Moreover, repression of GnRH promoter by estradiol has been proved in GT-1 cells producing GnRH (Roy et al., 1999). Therefore, a probable action of this disrupter would be to facilitate the negative feedback mechanism produced by estrogens in the hypothalamus with the following decrease of the gonadotropin concentration, as described in this paper.

In addition, other probable mechanisms that might be playing roles in these alterations would be changes in the expression of estrogen receptors in the hypothalamus induced by 4-MBC. It has been established that in male rats GnRH neuron in the preoptic area coexpresses the mRNA, as well as the protein, of beta type estrogen receptor (Hrabovszky et al., 2001). Micromolar concentrations of 4-MBC generate an increase in the amount of ER mRNA, that does not differ from the amount of mRNA induced by concentrations higher than 1 nmol/l of 17β-estradiol (Klann et al., 2005). On the other hand, male rats exposed to bisphenol A, another endocrine disrupter and also probably an estrogen agonist as 4-MBC, increases up to 4 times the expression of mRNA ERβ in the hypothalamic preoptic area (Ramos et al., 2003). All this would confirm the estrogenic agonist action of 4-MBC, since it has been established that other agonists of the estrogen receptor also modify the levels of this messenger receptor at the hypothalamic level (Zhou et al., 2002). It is expected that these changes in the estrogen receptor mRNA will be translated in hypothalamic changes of the protein as already described on some other tissues, e.g., uterus or prostate tissues (Durrer et al., 2005). The increase of estrogen receptor expression induced by the disrupter, would favor in this case the negative feedback over the gonadotrophic secretion of the axis. But Maerkel et al. (2007) have described an mRNA decrease of the alpha estrogen receptor in non-castrated male and female rats in the ventromedial areas as the hypothalamic preoptic medial area, when animals are exposed to 4-MBC from embryonic development up to adulthood, without change in the mRNA expression of the beta type receptor. It is necessary to make more studies to understand these differences and the mechanisms by which 4-MBC causes the neuroendocrine changes in the regulation of the male rat reproductive axis presented in this paper.

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