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Effect of tibolone and raloxifene on the tail temperature of oestrogen-deficient rats

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Abstract

Oestradiol, clonidine, tibolone and raloxifene were tested for their effects on the tail temperature of oestrogen deficient rats, a potential new model that can be used to test compounds that may be of use in the treatment of hot flushes in humans. Rats underwent ovariectomies or sham operations and their tail temperature and physical activity were measured telemetrically. Oestrogen depletion affected tail temperature in the rats' active, but not their resting phase. During the transition from the resting to the active phase, tail temperature in normal rats dropped by about 6°C, but only by approximately 1°C after ovariectomy. Treatment of the ovariectomised rats with oestrogen, clonidine or tibolone dose-dependently restored the drop in tail temperature. However, raloxifene did not change the tail temperature of ovariectomised rats. Thus, tibolone and raloxifene have different effects on the temperature regulation in the tail. This method of measuring tail temperature free of stress in ovariectomised rats may serve as a useful procedure for selecting compounds that are of potential use in the treatment of hot flushes.

Keywords: Menopause; Hot flush; Tail temperature; Telemetry; Tibolone; Raloxifene

1. Introduction

Probably the most disturbing and annoying symptom associated with the menopause is the hot flush, which occurs in over 80% of all women (Nagamani et al., 1987; Dennerstein, 1996). A hot flush is characterised by a sudden sensation of heat or burning starting in the head or neck and passing over the entire body. Although the precise mechanism underlying hot flushes is not yet known, oestrogen deficiency seems to play a prominent role since the hot flushes are experienced in the periods when blood levels of oestrogens are low and since oestrogen replacement prevents them. Hot flushes have been clearly linked to a transiently disturbed thermoregulatory mechanism that activates a heat-loss response consisting of sweating and increased peripheral blood flow (Kronenberg et al., 1984; Lomax, 1991; Berendsen, 2000).

Animal models with which to study hot flushes are scarce. In fact, only two models have been described in the literature: a nonhuman primate and a rat model. Two different research groups tested the primate model using only a limited number of animals. One group used two ovariectomised monkeys and found that the undulating temperature pattern of the skin of the scalp was suppressed after treatment with ethinyl oestradiol and clonidine, but not with domperidone or naloxone (Jelinek et al., 1984). The other group used three rhesus monkeys and found a higher frequency of increased skin temperature of the ear pinna following ovariectomy (Dierschke, 1985).

In rodents, Simpkins et al. (1983) developed a model for hot flushes in rats. They saw a strong rise in tail-skin temperature, an increase in heart rate and a surge in luteinising hormone (LH) after morphine withdrawal with naloxone in ovariectomised rats. In menopausal women, a rise in peripheral skin temperature, palpitations and a LH surge are seen. On the basis of these similarities, it was concluded that the morphine withdrawal model could be a suitable animal model to study the possible neuronal mechanisms underlying the menopausal syndrome and to predict the effects of unknown compounds on hot flushes. Subsequently, several authors have used this model (e.g. Kasson and George, 1983; Katovich and O'Meara, 1987; Katovich et al., 1986, 1990; Merchenthaler et al., 1998).

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However, findings relating to the interaction of morphine with oestrogens stimulated us to find and develop a new animal model in which it is possible to measure oestrogen withdrawal effects. Using the Simpkins model, it was shown that chronic oestrogen treatment prevented a naloxone-induced rise in tail-skin temperature in morphine-dependent rats. In later experiments, however, it was shown that morphine dependence was prevented by chronic oestrogen or neurosteroid treatment (Nomikos et al., 1987; Reddy and Kulkarni, 1997) and the morphine withdrawal syndrome was shown to be stronger in male than in female rats (Craft et al., 1999). Thus, the presence of oestrogen or the effect of chronic treatment with oestrogen does not protect the animal from the consequences of morphine withdrawal, but, rather, prevents the development of morphine dependence. Hence, the lack of a naloxone-induced increase in tail-skin temperature following pretreatment with oestrogens is not due to the protective effect of oestrogens against the temperature rise, but due to a lack of morphine dependence caused by oestrogen treatment. In addition, a similar prevention or weakening of morphine dependence was seen after treatment with the benzodiazepine diazepam (Tejwani et al., 1998), a compound that, to our knowledge, has never been shown to protect against hot flushes.

The starting point for the development of a new animal model was the stress-free measurement of the temperature of extremities and of oestrogen withdrawal. Thus, using a telemetric system, the tail temperature of normal and ovariectomised rats was monitored over 24 h. In this paper, we present the effects of oestrogen deficiency caused by ovariectomy and by treatment with the gonadotrophine-releasing hormone (GnRH) receptor antagonist, Org 30850, on tail temperature. For further validation, the effects of oestrogen replacement and of clonidine treatment are presented. Clonidine is an α-adrenoceptor agonist and is often prescribed as a nonhormonal treatment for hot flushes. The effects of tibolone and raloxifene are also investigated. Tibolone is a tissue-specific steroid used for the treatment of climacteric complaints, such as hot flushes, and osteoporosis. Raloxifene is a selective oestrogen receptor modulating (SERM) compound used for the prevention or treatment of osteoporosis, but which may induce hot flushes.

2. Materials and methods

Animal handling was in accordance with the "Dutch Law on Animal Experimentation (WOD)" and the "European Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European Union Directive #86/606/CEE)". "The Committee for Experiments on Animals" (DEC) of NV Organon approved the experiments.

2.1. Animals

Female Wistar rats (HSD/Cpb:WU, Harlan Sprague–Dawley, Horst, the Netherlands) weighing 225–250 g were used. The rats were individually housed in macrolon cages (dimensions: $38 \times 22 \times 15$ cm) on sawdust bedding, under a controlled 14-h light, 10-h dark cycle. As rats are most active in the dark, the light–dark cycle was reversed so that the lights came on at 1930 h. The room temperature was maintained at 20–21°C and the rats had free access to standard food pellets and tap water. Each experimental group consisted of five to eight animals.

2.2. Surgery

The rats were ovariectomised or underwent a sham operation, which left their ovaries intact, and were implanted with a temperature and physical activity transmitter (TA10TA-F40, Data Sciences International) under isoflurane (Forene®, Abbott) anaesthesia. The body of the transmitter was placed in the peritoneal cavity through a small ventral laparotomy. The ovaries were also taken out through this laparotomy. The transmitters were fixed with two stitches to the abdominal muscles. The tip of the temperature probe was adapted for placement in a rat's tail by stripping the silastic layer from the tip. The probe tips were tunnelled subcutaneously into the tail, placed 2 cm beyond the tail base and fixed in place with 0.05 ml Vet Seal® (Braun Melsungen). Immediately following wound closure with wound clips, the rats were treated with the analgesic, buprenorphine (Temgesic®, Schering-Plough, 0.6 μg/rat) and put back in their home cage.

2.3. Procedure

After implantation and ovariectomy, the rats were left undisturbed to recover for at least 2 weeks. Then they were placed in their own cage on receivers for measuring tail temperature and locomotor activity (RLA 1020, Data Sciences International). The receivers were attached to a consolidation matrix (BCM 100) and to a data acquisition system (Dataquest IV). In the first experiment, the tail temperature and locomotor activity of intact and ovariectomised rats was measured over 24 h. In subsequent experiments, the rats were treated with a test compound for 5 days at 1100 h, 90 min after the light in the animal room was turned off. The tail temperature and locomotor activity of the rats was measured continuously, but for calculation of the treatment effects, the measurements between 1200 and 1400 h were used. During this time period, the rats remained completely undisturbed. Thereafter, the animals were cared for routinely. The last 3 days before a compound treatment was started, the tail temperature of the rats was monitored in order to be sure that the differences in tail temperature between the intact and ovariectomised rats were significantly different. After completion of a

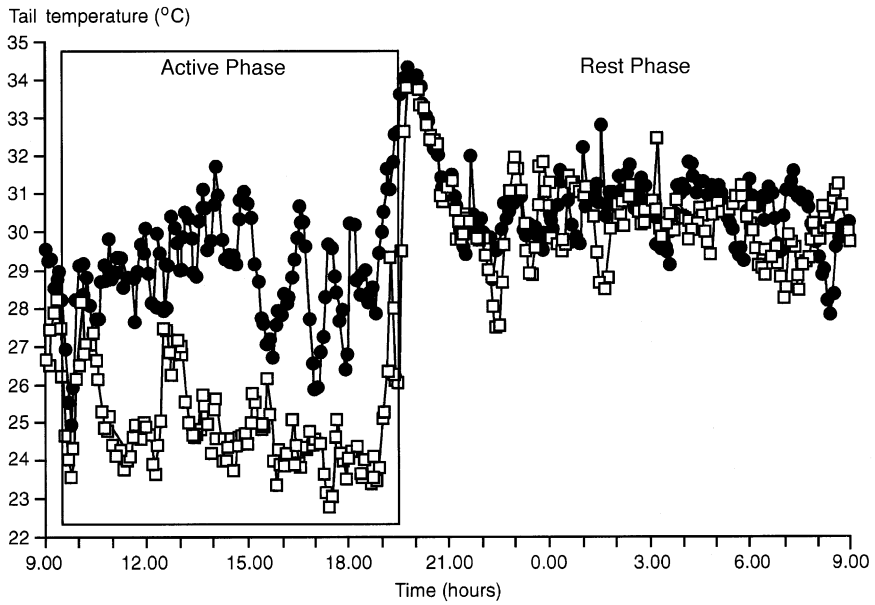

Fig. 1. Tail temperature of sham-operated (□) and ovariectomised (●) female rats in their active (dark) and rest (lights on) phase. Tail temperature was measured on day 15 after surgery ($N = 7$).

series of experiments, the rats were killed and inspected for any remnants of the ovaries.

2.4. Drugs and solutions

The compounds used in this study were 17β-oestradiol, [Ac-D-pClPhe^{1,2}, D-Bal^{3}, D-Lys^{6}, D-Ala^{10}]-GnRH (Org 30850), tibolone (Org OD 14, Livial®), raloxifene and clonidine HCl. All compounds were synthesised in the Medicinal Chemistry Department of NV Organon. Org 30850 and clonidine were dissolved in a sterile saline solution (0.9% NaCl in water); tibolone and raloxifene were suspended in an aqueous solution of gelatin (5 mg/ml) and mannitol (50 mg/ml); and 17β-oestradiol was suspended in a solution of 5% mulgofen (EL 719®, GAF) in saline. All drugs were injected subcutaneously into the loose skin at the back of the rat's neck. A dose volume of 1 ml/kg body weight was used. Control animals were injected with an equivalent volume of the vehicle.

2.5. Statistics

The tail temperature for each rat was measured for 7 s every 5 min. The mean tail temperature over the 2-h period for which the measured tail temperature was used for calculation (see Section 2.3) was calculated for each rat. For each day, the individual temperature was compared with the temperature on the first day of the experiment. The mean temperature change relative to the first day was then calculated for each experimental group together with the standard error of the mean (S.E.M.). The results were statistically evaluated using the analysis of variance (ANOVA). The locomotor activity of the rats was detected by the receivers and measured by detecting the changes in signal strength that occurred if the animals moved about their cages. A digital pulse was generated for each quantum of implant movement, with the number of pulses being roughly proportional to the distance the animal moves. These pulses were counted by the Dataquest system as an index of the relative degree of movement. The results were also evaluated by ANOVA.

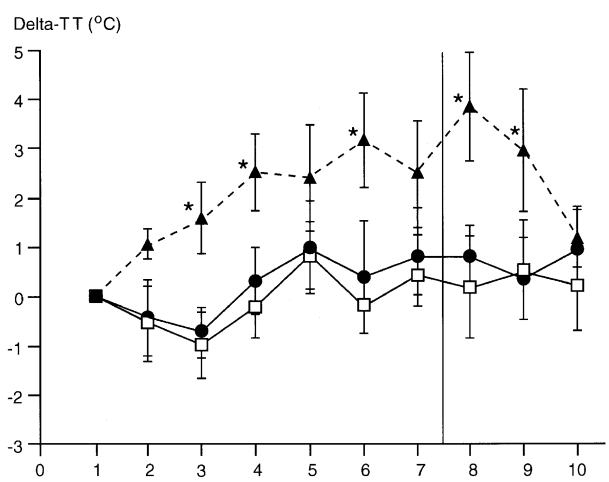

Fig. 2. Effect of treatment with the GnRH receptor antagonist Org 30850 (50 μg/kg/day) on the tail temperature of intact female rats. Shown are the differences in tail temperature on the treatment days compared with those on day 0 (Δt). The tail temperature was measured during the active phase of the rats, from 90 to 210 min after treatment. The vertical line between days 7 and 8 indicates that the last compound treatment was given on day 7. □, Sham-operated rats treated with placebo; ●, ovariectomised rats treated with placebo; ▲, sham-operated rats treated with 50 μg/kg/day Org 30850. $^{*}P < 0.05$ if compared with sham-operated group ($N = 6$–7).

3. Results

After the recovery period of 2 weeks, the rats appeared healthy and the ovariectomised rats behaved in a similar manner to the sham-operated rats. Their food and water intakes were normal.

3.1. Effects of oestrogen deficiency on tail temperature

Fig. 1 shows that the temperature of the tail varies during the active and rest phase (night and day successively). During the rest phase, the tail temperature fluctuated around 31°C, whereas in the active phase the temperature fluctuated around 25°C. After ovariectomy, the tail temperature of the rats during the rest phase was not different to that of intact rats. However, after ovariectomy, the drop in tail temperature during the transition from rest to active phase was strongly inhibited and fluctuated around 29°C in the active phase. The effect of ovariectomy on tail temperature reached statistical significance from the third day after removal of the ovaries. In further experiments studying the effects of compounds, only the tail temperature during the active phase was used. To clarify treatment effects, mean temperature changes are shown relative to temperature on day 0 (Δt), the day prior to treatment, in Figs. 2–4.

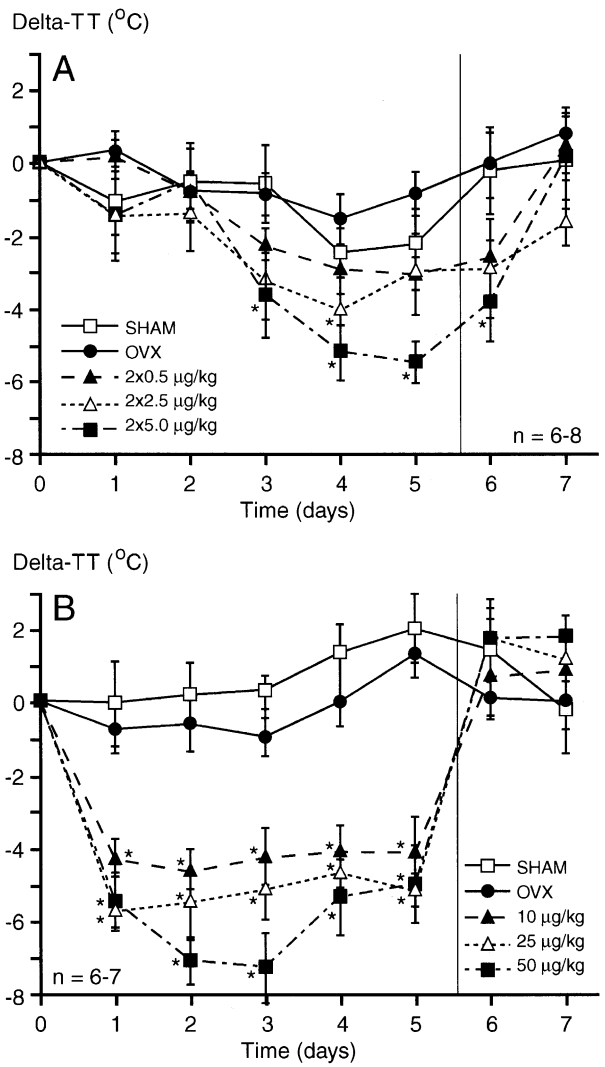

Fig. 3. Effect of treatment with estradiol (A) and clonidine (B) on the tail temperature of ovariectomised rats. Shown are the mean changes in tail temperature with the standard error of the mean (S.E.M.) compared to the temperature on day 0 (Δt). Tail temperatures are measured in the active phase of the animals from 90 to 210 min after the daily treatment. The vertical lines between days 5 and 6 indicate that the last compound treatment was given on day 5. $^{*}P < 0.05$ if compared to ovariectomised group.

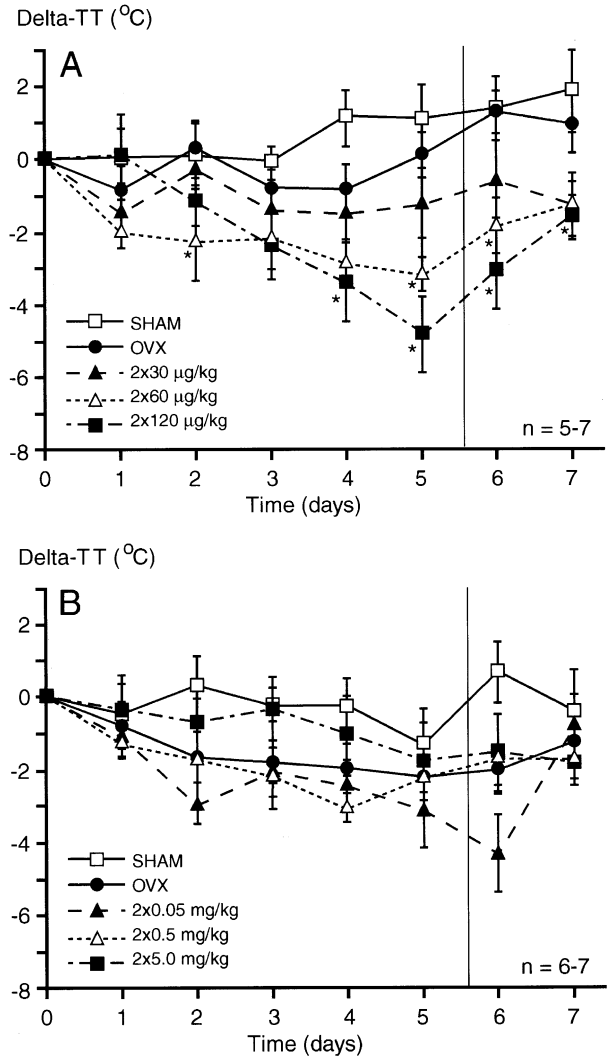

Fig. 4. Effect of treatment with tibolone (A) and raloxifene (B) on tail temperature of ovariectomised rats. Shown are the mean changes in tail temperature with the standard error of the mean (S.E.M.) compared to the tail temperature on day 0 (Δt). Tail temperatures are measured in the active phase of the animals from 90 to 210 min after the daily treatment. The vertical lines between days 5 and 6 indicate that the last compound treatment was given on day 5. $^{*}P < 0.05$ if compared to the ovariectomised group.

Treatment with the GnRH receptor antagonist, Org 30850, at a dose of 50 μg/kg/day also caused an attenuated drop in tail temperature during the active phase. During the treatment period, small fluctuations were seen in the tail temperatures of sham-operated and ovariectomised rats, but the tail temperature of sham-operated rats treated with Org 30850 increased by about 3°C and approached the temperature rise seen following ovariectomy (Fig. 2). On the third day after treatment with Org 30850 was stopped, the tail temperature returned to pretreatment levels. Both after ovariectomy and after treatment with Org 30850, the increase in body weight was significantly greater than that following a sham operation. The body weight increase was 5.0 ± 2.3 g after a sham operation, and 12.3 ± 2.7 and 17.3 ± 4.2 g after ovariectomy and Org 30850 treatment, respectively ($P < 0.05$).

3.2. Effects of oestrogen and clonidine on the tail temperature of ovariectomised rats

The inhibition of the drop in tail temperature of ovariectomised rats at the transition from their resting to their active phase was dose-dependently restored by treatment with oestradiol. An oestradiol dose of 2×0.5 μg/kg/day caused a small nonsignificant effect, while a dose of 2×5 μg/kg/day completely restored the fall in tail temperature (Fig. 3A). The effect of this higher dose was statistically significant on day 3. On day 5, the drop was 3.0 ± 1.1°C and 5.4 ± 0.6°C after doses of 2×2.5 and 2×5.0 μg/kg/day, respectively ($P < 0.05$). After stopping treatment on day 5, the effects of oestradiol disappeared within 2 days (day 7). During their rest phase, the tail temperature of the rats was not affected by oestradiol treatment (not shown). The gain in body weight of the ovariectomised rats was attenuated by the lowest dose of oestradiol and body weight decreased after treatment with the two higher doses. The decrease was 1.2 ± 2.4 and 4.8 ± 2.1 g, respectively, after 2×2.5 and 2×5.0 μg/kg/day oestradiol, whereas the increase in body weight in sham-operated and ovariectomised rats was 5.5 ± 1.6 and 10.9 ± 2.0 g, respectively.

Clonidine completely restored the inhibited drop in tail temperature in a dose-dependent manner. After a dose of 10 μg/kg, a clear, and statistically significant, drop of 4.2 ± 1.3°C was already seen by the first day of treatment (Fig. 3B) ($P < 0.05$). Twenty four hours after stopping treatment, the effect disappeared. The body weight gain of the rats was attenuated by clonidine treatment. The weight gain of sham-operated and ovariectomised rats was 5.6 ± 1.1 and 5.1 ± 1.1 g, respectively, whereas after clonidine 10 and 50 μg/kg, the respective weight gain was 1.6 ± 1.4 and 0.0 ± 1.7 g. After treatment with clonidine 25 μg/kg, the rats lost 1.7 ± 1.2 g of body weight.

3.3. Effects of tibolone and raloxifene on the tail temperature of ovariectomised rats

The fall in tail temperature, inhibited after ovariectomy, was dose-dependently restored after treatment with tibolone (Fig. 4A). A tibolone dose of 2×30 μg/kg/day was ineffective, but doses of 2×60 and 2×120 μg/kg/day were effective. On day 5 of treatment, the temperature

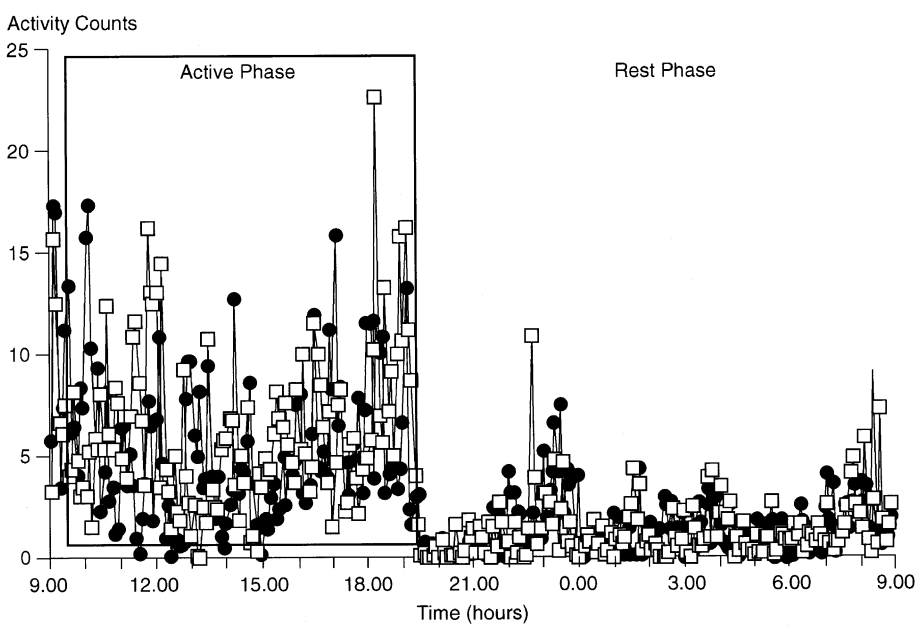

Fig. 5. Locomotor activity of sham-operated (□) and ovariectomised (●) female rats in their active and rest phase. Locomotor activity was measured on day 15 after surgery in the same animals in which tail temperature was measured (Fig. 1).

drop was 3.2 ± 0.5°C and 4.8 ± 1.0°C after 2×60 and 2×120 μg/kg/day, respectively ($P < 0.05$). The body weight gain of the rats was dose-dependently inhibited by tibolone. The doses that caused a restoration of tail temperature also caused a reduction in body weight. Compared to body weight on day 0, these reductions were 1.4 ± 2.6 and 9.4 ± 1.6 g on day 5 after 2×60 and 2×120 μg/kg/day, respectively. The body weight of the sham-operated and ovariectomised rats was increased by 6.2 ± 1.8 and 6.0 ± 2.2 g, respectively.

The effects of raloxifene on the tail temperature of ovariectomised rats is shown in Fig. 4B. In doses of up to 10 mg/kg/day given for 5 days, this compound could not restore the attenuated drop in tail temperature. Body weight gain, however, was inhibited by raloxifene. After a dose of 2×0.05 mg/kg/day of raloxifene, the body weight increased by 2.7 ± 2.1 g. After doses of 2×0.5 and 2×5 mg/kg, the rats body weight decreased by 3.1 ± 3.1 and 2.1 ± 2.7 g, respectively. The body weight gain of the sham-operated and ovariectomised rats was 1.0 ± 0.9 and 9.1 ± 1.3 g, respectively.

3.4. Effects on locomotor activity

Changes in body temperature are often related to changes in locomotor activity. Therefore, in these experiments, the locomotor activity was also measured. In Fig. 5, it is shown that ovariectomy did not change the locomotor activity of the rats in the active phase nor in the resting phase. Treatment with Org 30850, oestradiol, clonidine, tibolone or raloxifene also did not affect the locomotor activity of ovariectomised rats (results not shown).

4. Discussion

In the experiments presented here, we show that ovariectomy-induced oestrogen withdrawal in rats affects the temperature of their tail. This effect is seen during the dark phase of the day–night cycle, which is the rats' active period. In the resting phase (light period), the tail temperature was not affected by ovariectomy. During the transition from rest to the active phase, the tail temperature of normal (sham-operated) rats drops by about 6°C. After ovariectomy, this temperature drop was only about 2°C. This effect on tail temperature became apparent on the third day after ovariectomy.

Treatment of intact rats with the GnRH receptor antagonist Org 30850 for at least 3 days at a dose of 50 μg/kg also significantly attenuated the drop in tail temperature. Org 30850 at a dose of 50 μg/kg has been shown to completely suppress the oestrous cycle in rats and to significantly decrease the serum levels of oestradiol and follicle stimulating hormone (FSH; Deckers et al., 1992). Thus, reduction of oestrogen production, both surgically and chemically, affects rat tail temperature during the active phase of the rat, with a delayed onset of action.

In accordance with findings from others (e.g. Zhang et al., 1999), reduction of oestrogen levels by ovariectomy or treatment with the GnRH receptor antagonist Org 30850 did not affect locomotor activity. Replacement therapy with oestrogen and tibolone also did not change the locomotor activity of the animals. This excludes the possibility that differences in tail temperature might be due to a change in locomotor activity after oestrogen withdrawal.

After restoration of the ovariectomy-induced reductions in oestrogen levels by injections with oestradiol, there were dose-dependent attenuations of the temperature change caused by ovariectomy. This suggests that difference in tail temperature between intact and ovariectomised rats depends on the oestrogen level. In the treatment of hot flushes in humans, oestrogens are often used as a replacement therapy. The effect of these compounds is seen after a few weeks of treatment (Haas et al., 1988). In our experiments with rats, the effect of oestradiol is apparent on the third day of treatment. This delay of efficacy of oestrogens both in the treatment of hot flushes and in our experiments may point to an indirect action of oestrogens. Indeed, a lowered oestrogen level may cause a lowered neurotransmitter action and thereby trigger a hot flush (Berendsen, 2000).

Treatment with clonidine also attenuated the change in tail temperature caused by ovariectomy. Clonidine is a centrally acting α-adrenoceptor agonist that is widely used in humans for the treatment of hot flushes in patients for whom hormone replacement therapy is contraindicated. The success of this treatment is variable. Some authors have shown that clonidine reduces the number and intensity of hot flushes (e.g. Tulandi et al., 1983; Bressler et al., 1993), whereas others failed to show a clear effect (e.g. Wren and Brown, 1986; Loprinzi et al., 1994). In the present study, clonidine dose-dependently attenuated the effect of ovariectomy on tail temperature with a short onset of action. It is not likely that clonidine's effect results from an effect on whole body temperature since it has been shown that a much higher clonidine dose (0.2 mg/kg) only marginally affects body temperature (Livingstone et al., 1984). In the present study, clonidine was found to be effective in reducing the tail temperature of ovariectomised rats in their active phase after just one injection. This may indicate that, in contrast to oestradiol, clonidine has a direct effect on tail temperature. A similar direct effect has been seen after treatment with the 5-hydroxytryptamine (HT) receptor antagonist, mirtazapine, which has been shown to be effective in the treatment of hot flushes with a short onset of action, in a number of clinical case studies (Tome and Isaac, 1998; Waldinger et al., 2000).

Treatment with tibolone attenuated the temperature change following ovariectomy. Tibolone displays tissue-specific actions (Kloosterboer and Sands, 2000) without

the oestrogen-like stimulation of breast and endometrial tissue (Speroff, 1996; Kloosterboer and Sands, 2000). In animal experiments, tibolone was also shown to possess tissue-specific oestrogenic, androgenic and progestagenic activities (De Visser et al., 1984). The effects of tibolone on temperature regulation were not unexpected since the compound was found to be active in a primate model of hot flushes (Jelinek et al., 1984) and central effects of the compound are indicated as it may have some "mood-elevating" properties (Tax et al, 1987). In postmenopausal women, tibolone was shown to prevent bone loss and to offer effective treatment for climacteric complaints, including hot flushes, while avoiding some of the problems associated with classical hormone replacement therapy, like recurrence of vaginal bleeding (Rymer et al., 1994; Ginsburg et al., 1995; Ross and Alder, 1995; Egarter et al., 1996). The similarity between the effects of oestrogen and tibolone in the present study suggests that the effect of tibolone on temperature regulation in the tail is mediated via its oestrogenic activity.

Even if given in doses as high as 10 mg/kg, raloxifene had no effect on the disturbed tail temperature in ovariectomised rats. Raloxifene is a nonsteroidal benzothiophene derivative that binds to the oestrogen receptor and is described as a selective oestrogen receptor modulator (SERM) (Blum and Cannon, 1998). It produces oestrogen-agonistic effects in some tissues (liver, bone) and oestrogen-antagonistic effects in others (breast, uterus) (Agnusdei et al., 1999; Khovidhunkit and Shoback, 1999; Grossman, 2000). Clinically, raloxifene is used for the prevention of osteoporosis. The most common adverse effects of the compound are the induction or worsening of hot flushes and leg cramps (Balfour and Goa, 1998; Scott et al., 1999). Since hot flushes are thought to be the result of disturbances of the central thermoregulation within the hypothalamus (Lomax and Schönbaum, 1993), this suggests that raloxifene acts as an antagonist on the hypothalamic oestrogen receptors. This agrees with our findings that raloxifene did not attenuate the drop in tail temperature after oestrogen withdrawal. It would be interesting to see whether treatment of intact rats with raloxifene causes similar effects on tail temperature to those seen after ovariectomy and after treatment with the GnRH receptor antagonist Org 30850.

In contrast to the effect on tail temperature and in-line with previous findings (Byrd and Francis, 1998), raloxifene displayed a similar effect on body weight gain to oestradiol and tibolone: i.e. a reduction in body weight. Raloxifene appears to have oestrogen-agonistic properties in this respect.

The present experiments were intended to aim the development of an alternative, better animal model for hot flushes as current models have a number of flaws. We think that the procedure described here has a number of advantages over other models: the rats are anaesthetised only once and treated with only one compound per experiment making the interpretation of the results easier. A telemetric system is used to measure the tail temperature, which does not expose the rats to undue stress.

Our results show that tibolone and raloxifene have different effects in this model. Since temperature is regulated within the hypothalamus, it is possible that tibolone and raloxifene have different central effects.

In conclusion, the procedure we present here may offer an effective model to test compounds that may have beneficial effects in the treatment of hot flushes in a stress-free manner. Tibolone modulates temperature regulation, whereas raloxifene has no detectable effect.

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