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The effect of estrogens and antiestrogens in a rat model for hot flush

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Abstract

The present studies evaluated the effect of estrogens and the selective estrogen receptor modulator (SERM) tamoxifen and raloxifene in a rat model for hot flush. In this model, ovariectomized rats were treated for 8 or 9 days either sc or po. Rats were dependent to morphine by implanting a morphine pellet (75 mg each) sc on days 3 and 5 of treatment. On the last day of treatment, a thermistor, connected to a data acquisition system, was placed on the tail of each animal and morphine addiction was withdrawn by naloxone injection (1.0 mg/kg, sc). Temperature measurements were taken for 1 h under ketamine (80 mg/kg, im) anesthesia. In general, vehicle treated rats showed a 5–6°C elevation of their tail skin temperature with the peak occurring about 15 min after naloxone injection. 17α-Ethinyl estradiol (EE) was evaluated both sc and po using a broad range of doses. The IC_{50} for inhibition of tail skin temperature rise was approximately 0.1 mg/kg, sc and 0.2 mg/kg, po. 17β-Estradiol and 17α-estradiol were also active in this model whereas non-estrogenic steroids were inactive. Raloxifene and tamoxifen were tested for estrogen agonist and antagonist activity administered sc and po. Raloxifene did not demonstrate reproducible estrogen agonist activity at doses up to 10 mg/kg, whereas it demonstrated significant antagonistic activity at the 10 mg/kg dose regardless of the route of administration. Tamoxifen exhibited significant estrogen agonist activity at all doses tested (0.1–10.0 mg/kg) and was a significant antagonist of EE at the 1.0 mg/kg dose. Our results demonstrate the potential utility of this model to evaluate and discriminate among classes of compounds with varying degrees of estrogen agonist and antagonist activity.

Keywords: Estrogens; Antiestrogens; Rat model; Hot flush

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1. Introduction

The most common and characteristic symptom associated with the female climacteric is the hot flush which occurs in up to 75% of women after the physiological menopause or following oophorectomy [1]. Although the precise mechanism underlying hot flushes is not known, the vasomotor instability is associated with the decline in secretion of ovarian steroids [2], particularly in the free fraction of circulating estradiol [3]. In support of the role of estrogen in body temperature regulation, estrogen replacement therapy has been regarded as the most effective treatment of hot flushes since the beginning of the century [4,5]. Although estrogen replacement therapy is effective in most cases of hot flushes, the association between low serum levels of estrogen and hot flushes is not perfect. Hot flushes occur in about 25% of women before the onset of menopause when circulating levels of estradiol are higher than in postmenopausal women [6,7,1,2]. In addition, some women with low serum estradiol levels do not exhibit hot flushes postmenopausally [7,1]. Hot flushes may be considered as events of adaptation of the central nervous system to ever reducing levels of estradiol. This idea is supported by the observations that the incidence of hot flushes decreases with increasing time after menopause despite persistence of low ovarian steroid secretion [6,7].

The rat hot flush model first introduced by Simpkins et al. [8] and Katovich et al. [9] utilizes morphine-dependent, ovariectomized rats. During opiate withdrawal with the morphine antagonist naloxone, tail skin temperature rises and this rise is accompanied by a small drop in core temperature [8,9]. Interestingly, the rise in tail skin temperature is accompanied by a surge in luteinizing hormone (LH) secretion and a transient tachycardia [10]. Chronic estrogen treatment suppresses the rise in tail skin temperature and the associated hormonal changes [11]. These physiological events are similar in magnitude, duration and temporal patterns to those observed in women undergoing the menopausal hot flush [12,4,5]. Therefore, the morphine-dependent rat has been used as a model to study the mechanisms underlying the hot flush syndrome [10,9,11,13].

In the present study, the rat hot flush model introduced by Simpkins et al. [8] Katovich et al. [9] and modified by our group has been further characterized and used to evaluate the estrogenic and antiestrogenic activities of the partial estrogen receptor agonists/antagonists, also called selective estrogen receptor modulator (SERM), tamoxifen and raloxifene.

2. Materials and methods

2.1. Reagents

17α-Ethinyl estradiol (EE), 17β-estradiol (17β-E2), 17α-estradiol (17α-E2), tamoxifen citrate, and aldosterone were obtained from Sigma (St. Louis, MO). Raloxifene, trimegestone, and 5α-dihydrotestosterone (DHT) were synthesized by Wyeth-Ayerst Research. Morphine sulfate was obtained from Mallinckrodt (Paris, KY). Morphine pellets were prepared from the morphine free base in the laboratory of Dr M.J. Katovich (University of Florida) as previously described [9]. Naloxone was obtained from Research Biochemicals International (Natick, MA) and ketamine was obtained from Phoenix Pharmaceuticals (St. Joseph, MO).

2.2. Animals

The research animals were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The study was approved by the Institutional Animal Care and Use Committee of Wyeth-Ayerst Research. Ovariectomized female (60-day-old) Sprague–Dawley rats were obtained following surgery from Taconic Farms (Germantown, NY). The surgeries were performed a minimum of 7 days prior to the first treatment. Upon arrival the rats were randomized and given a minimum of 72 h to acclimate to the surroundings. The animals were housed individually or in pairs under 12 h light/dark cycle and given Purina 5001 rodent chow (North Penn Feeds, North Wales, PA) and water ad libitum.

2.3. Dosing

Vehicle and EE controls (0.1 mg/kg, sc or 0.3 mg/kg, po) were included in every study. Doses were prepared based on mg/kg mean group body weight in either 10% ethanol or 10% DMSO in sesame oil (Sigma) vehicle prior to sc treatment or in 1% Tween 80 (Sigma) in saline for oral administration. When tamoxifen citrate and raloxifene were tested for antagonist activity, EE was coadministered at 0.1 or 0.3 mg/kg for sc or po studies, respectively.

2.4. Experimental protocol

The experimental protocol is shown in Fig. 1. Following the 72 h acclimation period, the animals were treated once daily with the compound(s) of interest. There were ten animals per treatment group. Administration of the compound was either by subcutaneous (sc) injection of 0.1 ml in the nape of the neck or orally by gavage (po) in a volume of 0.5 ml. On the third day of treatment, a morphine pellet (75 mg morphine sulfate) was implanted subcutaneously. On the fifth day of treatment, two additional morphine pellets were implanted similarly. On the eighth day, approximately half of the animals were injected with ketamine (80 mg/kg, im) and a thermocouple, connected to a MacLab Data Acquisition System (API Instruments, Milford, MA), was taped on the tail approximately 1 in. from the root of the tail. This system allowed the continuous measurement of tail skin temperature. Baseline temperature was measured for 15 min, then naloxone (1.0 mg/kg) was given sc (0.2 ml) to block the effect of morphine and tail skin temperature was measured for 1 h thereafter. On the ninth day, the remaining animals were set up and analyzed similarly. Our studies have shown that neither ketamine administration nor the duration of treatment significantly affect either the temperature rise caused by the morphine withdrawal or the suppression of this response by estrogen.

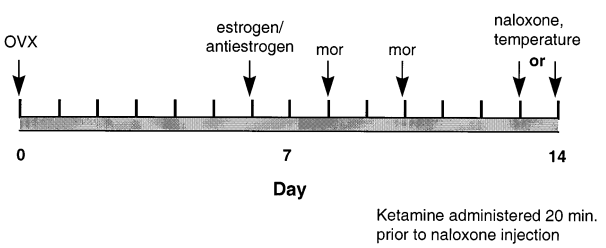

Fig. 1. Protocol for the rat hot flush model. Approximately 60-day-old ovariectomized Sprague–Dawley rats were treated for 8 or 9 days either sc or po with the compound(s) of interest. Rats were dependent to morphine by implanting a morphine pellet sc on days 3 and 5 of treatment. On the last day of treatment, a thermistor, connected to a data acquisition system, was placed on the tail of the animals and morphine addiction was withdrawn by naloxone injection (1.0 mg/kg, sc). Temperature measurements were taken for 1 h under ketamine (80 mg/kg, im) anesthesia.

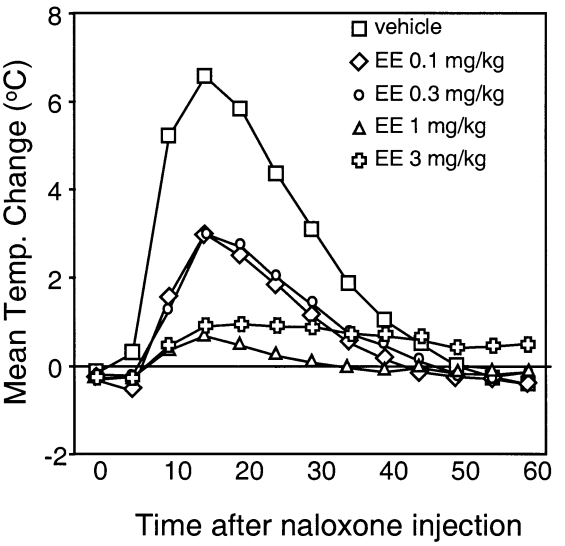

Fig. 2. Dose-response effect of 17α-ethinyl estradiol administered orally on the mean change in tail skin temperature vs time after naloxone administration. In general, a 5–6°C increase in the vehicle treated group is observed 15 min after naloxone which is suppressed by EE.

2.5. Statistical evaluation of results

A two factor repeated measures, with one factor repeated, analysis of variance model was used to analyze the change of tail skin temperature (induced by naloxone) in morphine-dependent rats. The factors were 'treatment' and 'time' (repeated). The data were analyzed at 5 min intervals from the time of naloxone administration (time

zero) to 60 min after the treatment. With the exception of Fig. 2, the data presented in this report is from the 15 min time point. The change in tail skin temperature was greatest at this time and provided the best data for statistical analysis. The data were transformed by square root to stabilize the variability. Following the transformation, the Huber M-estimation weighting was used to down weight the outlying transformed observations. The JMP software (SAS Institute, Cary, NC) was used for the repeated measure analysis of the transformed and weighted observations. Multiple comparisons (LSD P-values) were made among the treatment groups at each time point.

3. Results

3.1. The effect of 17α-ethinyl estradiol on naloxone-induced tail skin temperature rise

EE administered once daily, either sc or orally, to ovariectomized rats resulted in a dose-dependent suppression in the rise in tail skin temperature evoked by morphine withdrawal (Figs. 2 and 3). Fig. 2 shows a study which demonstrates that tail skin temperature began to rise 5 min after naloxone injection, reached its highest level (approximately 4–6°C) 15 min after morphine withdrawal, then declined and returned to baseline within 45–60 min. Since the rise in tail skin temperature was greatest 15 min after naloxone injection, data collected at this time point were analyzed for statistical differences and potency estimation. The estimated IC_{50} values for EE obtained from the pooled data of several experiments were 0.1 and 0.2 mg/kg following sc and po administration, respectively (Fig. 3).

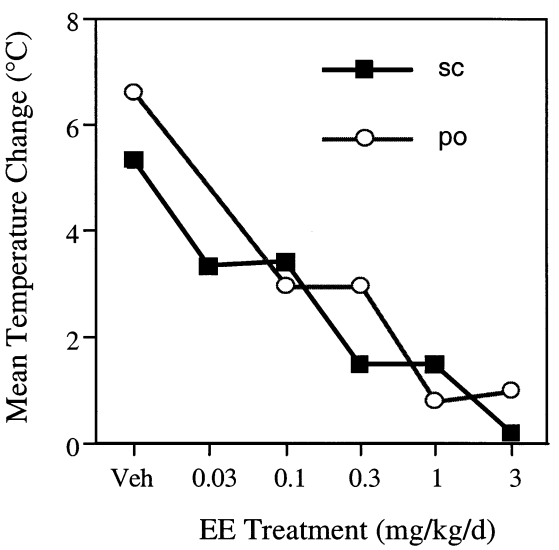

Fig. 3. Representative dose-response studies comparing the effect of EE administered either orally or subcutaneously on the mean change in tail skin temperature observed 15 min after naloxone administration.

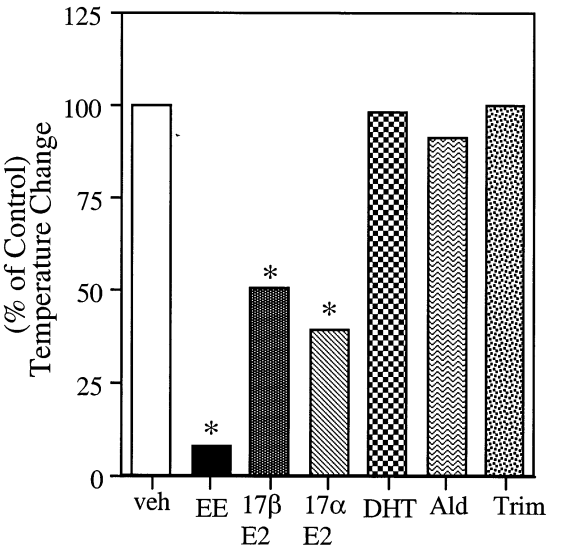

Fig. 4. The effect of 17α-ethinyl estradiol (EE, 0.1 mg/kg), 17β-estradiol (17β-E2, 0.125 mg/kg), 17α-estradiol (17α-E2, 10 mg/kg), 5α-dihydrotestosterone (DHT, 1 mg/kg), aldosterone (Ald, 0.1 mg/kg) and trimegestone (Trim, 1 mg/kg) administered subcutaneously on the mean change in tail skin temperature observed 15 min after naloxone administration. Doses shown are in mg/kg body weight. * $P < 0.05$ compared to the vehicle group.

3.2. The effect of estrogenic and non-estrogenic steroids on naloxone-induced tail skin temperature rise

We also examined the ability of other steroids to suppress the rise in tail skin temperature following naloxone treatment. Only the estrogenic compounds 17β- and 17α-estradiol, in addition to

EE, were able to suppress the rise in tail skin temperature (Fig. 4). The other steroids tested, including the androgen 5α-dihydrotestosterone (1.0 mg/kg)), the mineralocorticoid aldosterone (0.1 mg/kg) and the progestin trimegestone (1.0 mg/kg) had no effect on the naloxone-induced tail skin temperature rise (Fig. 4.).

3.3. The agonist and antagonist effects of raloxifene and tamoxifen on naloxone-induced tail skin temperature change

The partial estrogen receptor agonists/antagonists, raloxifene and tamoxifen, were also analyzed in the model in both agonistic and antagonistic mode. Raloxifene, when tested in the agonist mode at 0.1, 1.0, and 10.0 mg/kg, sc did not exhibit reproducible estrogenic activity, i.e. it did not blunt the naloxone-induced tail skin temperature rise in most of the experiments (Figs. 5 and 6). When tested as an antagonist following sc administration, raloxifene exhibited anti-estrogenic activity at 10.0 mg/kg (Fig. 6). In a representative experiment as shown in Fig. 7, raloxifene administered orally at 0.1, 1.0 and 10.0 mg/kg doses, was effective in reducing the rise in tail skin temperature at a dose of 0.1 mg/kg, but not at higher doses. However, in six independent studies, no reproducible agonist activity was seen, although EE always blunted significantly the naloxone-induced tail skin temperature rise. In contrast, raloxifene in the antagonist mode, was reproducibly active at 10.0 mg/kg (Fig. 7).

Tamoxifen when administered sc or po, exhibited mixed agonist and antagonist activity. When administered sc, it significantly suppressed the rise in tail skin temperature at both 1.0 and 10.0 mg/kg and blocked the action of EE at 1.0 mg/kg, but not at the 10.0 mg/kg dose (Fig. 8). When administered orally, tamoxifen demonstrated significant agonist activity at 0.1, 1.0, and 10.0 mg/kg, and like the sc treatment, blocked the action of EE at 1.0 mg/kg but not at 10.0 mg/kg (Fig. 9).

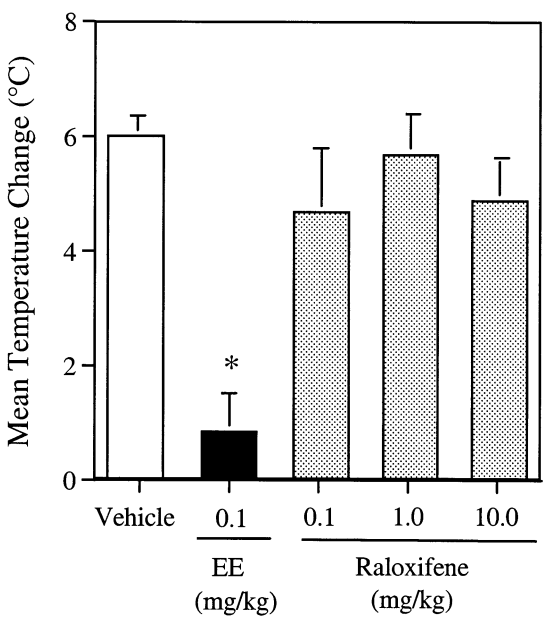

Fig. 5. The effect of raloxifene administered subcutaneously in the agonist mode on the mean change in tail skin temperature observed 15 min after naloxone administration. In this study, raloxifene had no significant estrogen agonist effect at any dose tested. * $P < 0.05$ compared to the vehicle group.

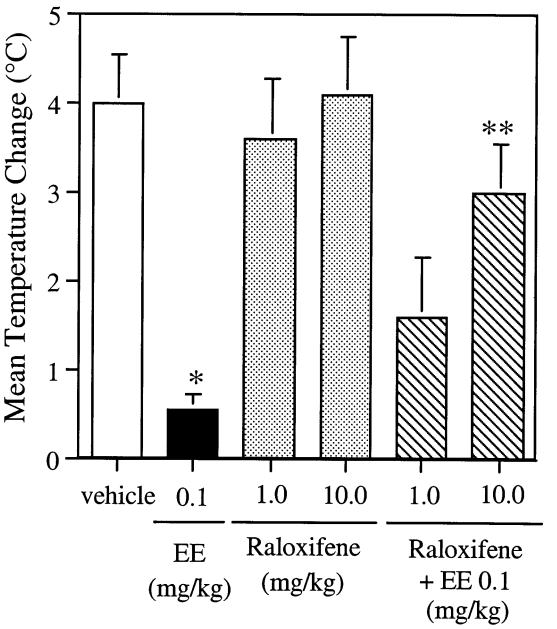

Fig. 6. The effect of raloxifene administered subcutaneously in both the agonist and antagonist modes (with EE) on the mean change in tail skin temperature observed 15 min after naloxone administration. Raloxifene demonstrated significant estrogen antagonist activity at 10 mg/kg, sc. * $P < 0.05$ compared to the vehicle group, ** $P < 0.05$ compared to the EE group.

4. Discussion

There is ample evidence that estrogen, morphine and endogenous opioids (EOPs) modify body temperature. Opioids are thought to participate by altering the hypothalamic set point for thermoregulation [14,15], although the precise mechanism is not known. Furthermore, morphine-responsive temperature-sensitive neurons have been identified in the preoptic area of the hypothalamus, a major site of temperature regulation [15].

In addition to opioids, ovarian steroid hormones have also been reported to modify body temperature in rats [16–18] and humans [19]. Interestingly, female rats appear to be more sensitive than males to the thermotropic effects of morphine and ovariectomy, but not orchidectomy, alters the response to morphine in rats [10,20].

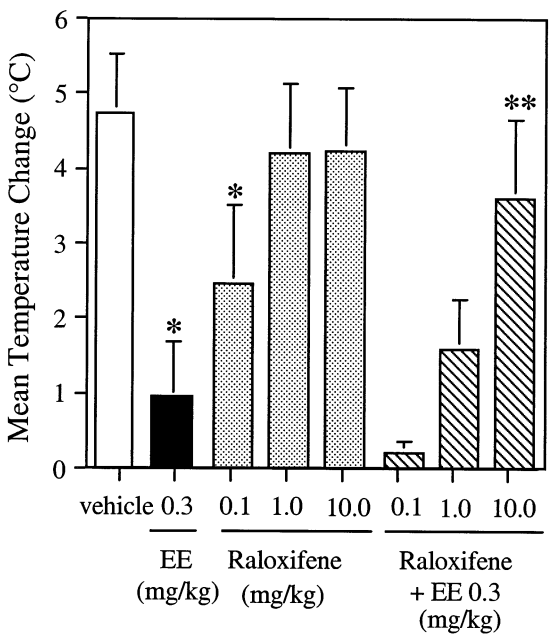

Fig. 7. The effect of raloxifene administered orally in both the agonist and antagonist mode (with EE) on the mean change in tail skin temperature observed 15 min after naloxone administration. Raloxifene demonstrated significant estrogen antagonist activity at 10 mg/kg, po and significant agonist activity at 0.1 mg/kg, po. * $P < 0.05$ compared to the vehicle group, ** $P < 0.05$ compared to the EE group. However, the low dose agonist activity was not consistently observed.

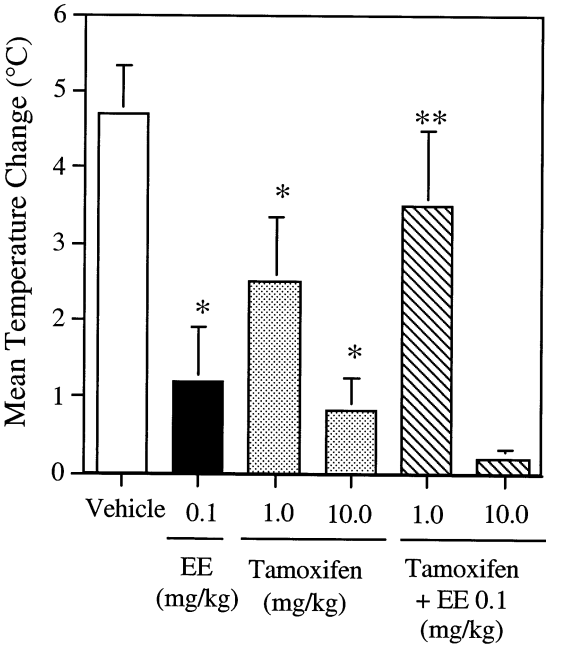

Fig. 8. The effect of tamoxifen administered subcutaneously in both the agonist and antagonist mode (with EE) on the mean change in tail skin temperature observed 15 min after naloxone administration. Tamoxifen demonstrated both significant estrogen agonist activity at 1 and 10 mg/kg, sc and significant antagonist activity at 1 mg/kg, sc. * $P < 0.05$ compared to the vehicle group, ** $P < 0.05$ compared to the EE group.

Several studies indicate an interaction between sex steroids and EOP activity. For example, the levels of β-endorphin in the rat brain are dramatically reduced following ovariectomy and can be restored by estrogen replacement [21–23]. In women, EOP activity fluctuates and follows the changes of estrogen levels during the menstrual cycle [24]. In menopausal women [25] and aged female rats [26–28], β-endorphin levels are reduced. Moreover, naloxone is unable to elicit LH secretion in postmenopausal women; however, it increases LH secretion is estrogen-treated patients [29,30]. Similar observations were reported in chronically ovariectomized rats [31]. Naloxone infusion to postmenopausal women results in a decrease in body temperature. This change is greater when postmenopausal women are under estrogen replacement therapy [29]. Taken together, these observations suggest that EOP activity is absent or diminished when estrogen levels

are low and the reduced opioid activity is responsible, at least in part, for the genesis of hot flushes.

The rat hot flush model first introduced by Simpkins et al. [8] and Katovich et al. [9] utilizes morphine-dependent, ovariectomized rats. When the morphine effect is withdrawn by a single injection of naloxone, tail skin temperature rises and this rise can be blunted with the chronic estrogen treatment [13].The rats are immobilized in a restrainer when morphine addiction is withdrawn with naloxone. Immobilization, however, is stressful for the animal and the animal-to-animal variation in responses to naloxone and estrogen are variable, rendering the model difficult to use. In our experiments, the animals are slightly anesthetized with an intramuscular injection of ketamine prior to naloxone injection. As a result, the animals do not have to be restrained and, therefore, the stress, associated with restraining, has been avoided. Ketamine treatment does not jeopardize the morphine withdrawal-induced rise in tail skin temperature and the suppression of this temperature rise by estrogen. Reducing the stress-state of the experimental animals with ketamine produces data that are more homogenous and reproducible than those obtained from restrained animals (present paper vs [13]).

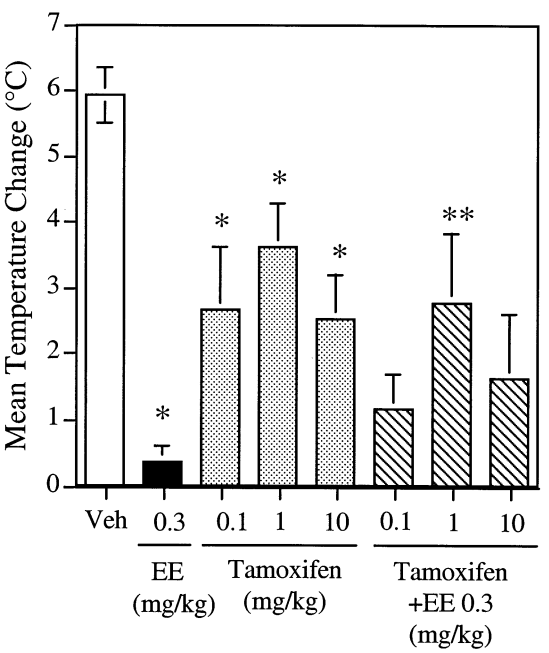

Fig. 9. The effect of tamoxifen administered orally in both the agonist and antagonist mode (with EE) on the mean change in tail skin temperature observed 15 min after naloxone administration. Tamoxifen demonstrated significant estrogen antagonist activity at 1 mg/kg, po and significant agonist activity at 0.1, 1, and 10 mg/kg, po. * $P < 0.05$ compared to the vehicle group, ** $P < 0.05$ compared to the EE group.

In addition to using ketamine anesthesia, we have also shortened the time of estrogen treatment and changed the reference estrogen administered. In the original model [9], high doses of 17β-estradiol, ranging from 0.4 to 200.0 mg/kg, were used in a pellet form, inserted sc, for 3 weeks. In our model, the length of treatment time has been reduced from 3 weeks to 8–9 days, thus providing a simpler and more economical model than introduced earlier. Moreover, EE rather than 17β-estradiol was used as the reference estrogen, because EE provided a more consistent response (blunting naloxone-induced tail skin temperature rise) than 17β-estradiol and is more potent when administered orally. Furthermore, the model was validated so that compounds could be given by either the oral or subcutaneous route. In our studies, the route of administration did not significantly effect the activity of EE, raloxifene or tamoxifen. The IC_{50} values of EE on hot flush were 0.1 and 0.2 mg/kg, administered sc or orally, respectively. The potency for EE administered orally on hot flush is similar to that reported for uterine wet weight increase and lowering of plasma cholesterol levels [32]. However, when EE is administered sc, the dose required to suppress the change in tail skin temperature is significantly greater than necessary to stimulate uterine growth, but similar to that required to lower plasma cholesterol [32]. Therefore, the doses of EE required to affect hot flush are similar to those necessary to affect other endpoints in vivo.

Our studies clearly show that EE given sc or orally is able to blunt the naloxone-induced rise in tail skin temperature in a dose-dependent manner. The naturally occurring and potent estrogen 17β-estradiol as well as a less potent estrogen 17α-estradiol were also active in this model, whereas, nonestrogenic steroids, such as trimegestone, dihydrotestosterone and aldosterone had no blunt-

ing effect in this model. These observations that 17α-estradiol was also able to blunt the naloxone-induced tail skin temperature rise when administered at a dose of 10 mg/kg is not surprising. It is well documented in the literature that 17α-estradiol, which is commonly believed to be inactive, does bind to the estrogen receptor [33] and increases uterine wet weight in the ovariectomized rat with a potency about 300-fold lower than 17β-estradiol [32]. Our experiments suggest that the blunting effect of estrogen on naloxone-induced tail skin temperature rise is specific to estrogen. It is likely that the effect is mediated via the estrogen receptor. However, experimental support for such a mechanism is not available and would require additional studies with a pure estrogen receptor antagonist such as ICI182780. However, ICI 182780 does not penetrate the blood–brain barrier [34] and therefore, cannot be used to support such an action.

Raloxifene, a selective estrogen receptor modulator (SERM), showed inconsistent estrogen agonist activity in the rat hot flush model, however, it exhibited antiestrogenic properties when coadministered with EE. Therefore, on this endpoint, raloxifene functions primarily as an estrogen antagonist. Tamoxifen, another estrogen receptor modulator, showed a mixed agonist/antagonist activity in the model. When administered alone, it exhibited weak estrogenic activity, but when administered together with EE, tamoxifen antagonized the action of the estrogen. Therefore, this model can discriminate between the relative levels of estrogenic and antiestrogenic activities of compounds such as tamoxifen and raloxifene.

Hot flushes are the major reason why postmenopausal women seek treatment from a physician. Estrogen replacement therapy, has been proven to alleviate hot flushes and exhibit other beneficial effects [35–37,4,5]. The estrogenic vs anti-estrogenic action of selective estrogen receptor modulators, among other factors, may depend on estrogen receptor subtype, the specific cell type, and promoter context. It is well known that tamoxifen exhibits considerable estrogenic activity in the uterus, bone and the cardiovascular system, but inhibits cellular proliferation in the mammary gland (for a review see [38–40]). Raloxifene also has estrogenic effects in bone and the cardiovascular system, inhibits proliferation in the mammary gland [41–44] and has no [38,42,43] or minor [44] proliferative action in the uterus. Based on our results with the rat hot flush model, raloxifene would not appear to be effective in treating hot flushes, whereas tamoxifen may have limited utility in treating hot flushes when circulating levels of estrogen are low. The only published report on the effect of raloxifene on hot flushes was from a randomized, double-blind, placebo-controlled, multicenter, 8-week study that indicated that raloxifene given at 600 mg per day increased the incidence of hot flushes [42]. These observations are consistent with our findings with raloxifene in the rat hot flush model, i.e. raloxifene behaves as an antiestrogen in the central nervous system.

The observations that estrogens blunt the naloxone-induced rise in tail skin temperature of morphine-dependent ovariectomized rats [11] indicate that the rat hot flush model provides a unique animal model to evaluate the effect of estrogen receptor ligands (both agonists and antagonists) on the regulation of surface temperature of the body. Although EE and 17β-estradiol reproducibly blunted naloxone-induced tail skin temperature rise, complete validation of the model would require a comparison of observations with several estrogen agonists and antagonists in women and this animal model.

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