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Activation of ERα is necessary for estradiol's anorexigenic effect in female rats

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ABSTRACT

While there is considerable evidence that the ovarian hormone estradiol reduces food intake in female rats, it is unclear which estrogen receptor (ER) subtype, ERα or ERβ, mediates this effect. While several studies have demonstrated that activation of ERα, but not ERβ, is sufficient to reduce food intake in ovariectomized (OVX) rats, there are limited data regarding which receptor subtype is necessary. Here we used the selective ERα and ERß antagonists, MPrP and PHTPP, respectively, to investigate this question. We found that antagonism of ERα, but not ERβ, prevented the decrease in food intake following acute administration of estradiol in OVX rats. In addition, antagonism of ERα prevented the estrous-related, phasic reduction in food intake that occurs in response to the rise in circulating levels of estradiol in cycling rats. We conclude that activation of ERα is necessary for the anorexigenic effects of exogenous and endogenous estradiol in female rats.

Introduction

The ovarian hormone estradiol appears to play a physiological role in the control of food intake in female rats. The best evidence in support of this statement comes from studies in which ovariectomy has been shown to cause hyperphagia and weight gain (Wade and Gray, 1979), both of which can be prevented by estradiol treatment alone (Asarian and Geary, 2002). Additionally, the pre-ovulatory rise in circulating estrogens in cycling rats promotes a reduction in food intake during the estrous stage of the ovarian reproductive cycle (Drewett, 1973; Blaustein and Wade, 1976; Eckel et al., 2000). This estrous-related decrease in food intake appears to be mediated by estradiol, with minimal involvement of estriol or estrone, since estradiol treatment alone can reinstate this cyclic reduction in food intake in OVX rats (Asarian and Geary, 2002).

Many of the behavioral effects of estradiol are not apparent until hours or days following the rise in circulating estradiol in female rats. For example, the decrease in food intake observed in estrous rats does not occur until about 60 h after the initial rise in circulating estradiol (Becker et al., 2005) and treatment with exogenous estradiol takes ~36 h before any behavioral change in food intake is detected in ovariectomized (OVX) rats (Asarian and Geary, 2002). Thus, the anorexigenic effect of estradiol likely involves a genomic mechanism that is initiated by activation of one or both of the nuclear estrogen receptors (ERs), ERα and ERβ.

Studies involving either activation or blockade of ERα/ERβ signaling have been used to investigate the relative contribution of each ER subtype to estradiol's anorexigenic effect. Multiple groups have shown that treatment with an ERα agonist decreases food intake in OVX rats and mice (Roesch, 2006; Santollo et al., 2007; Thammacharoen et al., 2009). In comparison, treatment with an ERβ agonist fails to alter either food intake or the ability of an ERα agonist to reduce food intake (Roesch, 2006; Santollo et al., 2007). While these studies suggest that activation of ERα alone is sufficient to reduce food intake in OVX rats, other studies involving disruptions in ERα/ERβ signaling have provided equivocal evidence regarding the necessity of each ER subtype in mediating estradiol's anorexigenic effect. For example, an examination of the feeding behavior of female mice with a null mutation of the ERα subtype (i.e., αERKO mice) revealed that they were insensitive to the effects of estradiol treatment on several feeding-related measures (Geary et al., 2001). This finding suggests that estradiol's anorexigenic effect requires ERα and extends previous demonstrations that ERβ alone is not sufficient. In another study, however, the anorexigenic effect of estradiol was blocked by intracerebroventricular administration of an ERβ-selective, but not an ERα-selective, antisense oligodeoxynucleotide in the OVX rat (Liang et al., 2002). This finding suggests that estradiol's anorexigenic effect in the rat requires a functional ERβ.

Progress in determining the relative necessity of ERα and/or ERβ signaling in mediating estradiol's anorexigenic effect has been further hampered by the lack of suitable ER-selective antagonists. For example, methyl-piperidino-pyrazole (MPP), a non-steroidal, pyrazole compound (Sun et al., 2002), was originally classified as an ERα antagonist based on *in vitro* tests of its ability to antagonize estrogen-regulated genes (Harrington et al., 2003). However, in subsequent tests of its *in*

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vivo actions, MPP increased uterine weight in mice and rats (Davis et al., 2006; Santollo and Eckel, 2009), reduced food intake in OVX rats (Santollo and Eckel, 2009), and failed to attenuate the anorexigenic effects of estradiol and an ERα agonist in OVX rats (Santollo and Eckel, 2009). Despite these multiple, estradiol-like effects, MPP did attenuate the estrous-related decrease in food intake in cycling rats (Santollo and Eckel, 2009). Taken together, these studies suggest that MPP acts as an ERα antagonist following *in vitro* applications, but exerts mixed ERα agonist/antagonist actions following *in vivo* applications. Thus, MPP, which was initially categorized as an ERα antagonist, better resembles a selective ER modulator (SERM; a compound that exerts mixed agonist/antagonist activities at ERs). Indeed, MPP's structure, which is comprised of a core, non-steroidal ER ligand with a basic side chain, is similar to the structure of most SERMs. Moreover, it appears that under certain conditions MPP's basic side chain may be metabolically cleaved resulting in a compound with agonist, rather than antagonist, qualities (Zhou et al., 2008). In light of these findings, Katzenellenbogen's group developed a novel MPP analog, called methyl-piperidinopropyl pyrazole (MPrP), which contains a basic side chain that cannot undergo metabolic cleavage. While this novel compound is highly selective for ERα in binding affinity assays and exerts potent ER antagonist activity in transcription activation assays (Zhou et al., 2008), its *in vivo* actions have yet to be evaluated.

Currently, there is no evidence that ERβ-selective compounds, like the ERβ antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol (PHTPP), exert the mixed agonist/antagonist properties that are often seen in ERα-selective compounds. For example, PHTPP displays 36-fold selectivity for ERβ over ERα and displays complete antagonism for ERβ in reporter gene assays in co-transfected endometrial cells (Compton et al., 2004). The goal of the present study was to use these ER-selective antagonists (MPrP and PHTPP) to evaluate the relative necessity of ERα- and ERβ-activation in mediating estradiol's anorexigenic effect in OVX and cycling rats.

Materials and methods

Animals and housing

Female, Long–Evans rats (Charles River Breeding Laboratory, Raleigh, NC), weighing ~250 g at study onset, were individually housed in custom, shoebox cages. Each cage contained a spill-proof food cup, a water bottle, and a sleeping niche. Rats had free access to powdered chow (Purina 5001) and tap water. The colony room was maintained at 20 °C with a 12:12 h light/dark cycle (dark onset = 1300 h). Animal usage and all procedures were approved by the Florida State University Institutional Animal Care and Use Committee.

Surgery

Rats that underwent ovariectomy surgery (Experiments 1 and 2), were anesthetized by intraperitoneal (i.p.) injections of a mixture of ketamine (50 mg/kg; Ketaset, Fort Dodge Animal Health, IA) and xylazine (4.5 mg/ml; Rompun, Mobay, Shawnee, KS) and then bilaterally ovariectomized (OVX) using an intra-abdominal approach. Immediately following surgery, rats were given single, i.p. injections of butorphanol (0.5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and gentamicin (10 mg/ml; Pro Labs Ltd, St. Joseph, MO) to minimize post-surgical pain and the risk of infection, respectively. Rats were given 10 days to recover from surgery and then behavioral testing commenced once stable levels of food intake were observed.

Experiment 1. Does acute administration of either MPrP or PHTPP alter food intake in OVX rats?

It is becoming increasingly clear that pharmacological compounds designed to antagonize ERs can sometimes function as SERMs and exert mixed agonist/antagonist actions following *in vivo* administration (Wade and Heller, 1993; Bryant and Dere, 1998; Santollo and Eckel, 2009). As a first step in our efforts to determine the effects of selective ERα/β antagonism on food intake in the female rat, we investigated whether acute administration of either MPrP or PHTPP produces any estrogenic effects in OVX rats. Four hours prior to dark onset, rats ($n = 6$) received randomized, subcutaneous (s.c.) injections of either 0 or 25 μg of the ERα antagonist MPrP (synthesized by J.A. Katzenellenbogen, University of Illinois (Zhou et al., 2008)) dissolved in DMSO vehicle at 4-day intervals. A second group of rats ($n = 8$) received randomized, s.c. injections of 0, 25, or 50 μg of the ERβ antagonist PHTPP (Tocris) dissolved in DMSO vehicle at 4-day intervals. The dose of MPrP was chosen based on work involving MPP, the compound from which MPrP was derived. Previously, we demonstrated that a single injection of 25 μg MPP does not exert an estrogenic effect on food intake in OVX rats (Santollo and Eckel, 2009). In addition, this dose of MPP was sufficient to attenuate the estrous-related decrease in food intake that occurs in cycling rats (Santollo and Eckel, 2009). These findings, together with the fact that MPrP has greater ERα binding selectivity than MPP in a competitive, radiometric, binding assay (Zhou et al., 2008), suggested that a similar dose of MPrP (25 μg) was an appropriate starting point. We choose our doses of PHTPP (25 and 50 μg) based on previous *in vitro* studies (Chen et al., 2008; Ben-Jonathan et al., 2009). Although food intake was measured daily throughout the experiment, we were particularly interested in the 24-h period following drug treatment since this interval corresponds to the time in which SERMS such as MPP and tamoxifen decrease overnight food intake (Santollo and Eckel, 2009; Wade and Heller, 1993).

Experiment 2. Does either MPrP or PHTPP attenuate the anorexigenic effect of exogenous estradiol?

Rats ($n = 6$) received randomized, s.c. injections of vehicle, 2 μg estradiol benzoate (EB; Sigma), or 25 μg MPrP followed immediately by 2 μg EB. This yielded three treatment conditions: vehicle, EB and MPrP/EB. This series of injections was administered in random order 4 h prior to dark onset at 4-day intervals over a period of 12 days. A second group of rats ($n = 8$) received a similar series of drug injections but 50 μg PHTPP was administered in place of 25 μg MPrP. This yielded three treatment conditions: vehicle, EB, and PHTPP/EB. We choose to administer drug treatments at 4-day intervals based on a previous demonstration that injection of 2 μg EB every fourth day mimics the changes in endogenous estradiol secretion observed in cycling rats and, beginning approximately 24-h after injection, models the cyclic reduction in 24-h food intake that is observed during estrus in cycling rats (Asarian et al., 2002). In order to coincide with the period that models estrus, food intake was monitored for 24 h commencing at the start of the second dark phase following drug treatment.

Experiment 3. Does MPrP attenuate the anorexigenic effect of endogenous estradiol?

The estrous cycles of 8 female rats were monitored by examining the appearance and abundance of cells within vaginal cytology samples as previously described (Becker et al., 2005). Cycle stage labels were assigned to the previous 24-h period ending at the time of sampling. With this method, the light-phase peak in estradiol and luteinizing hormone secretion occurred during proestrus and estrus included the following dark phase when female rats display estrous-related decreases in food intake (Becker et al., 2005). Data collection did not begin until all rats displayed two consecutive 4-day, estrous cycles. Rats received s.c. injections of either 0 or 25 μg MPrP dissolved in DMSO vehicle just prior to the dark phase of proestrus. Twelve hours later, a second s.c. injection of either vehicle or 37.5 μg MPrP was administered. Drug treatment was administered in a counterbalanced manner across two consecutive