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AGE-RELATED ALTERATIONS IN ESTROGEN RECEPTOR DYNAMICS ARE INDEPENDENT OF CYCLING STATUS IN MIDDLE-AGED C57BL/6J MICE

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Summary—The objective of this study was to determine whether changes in estrogen receptor (ER) levels and dynamics that were previously observed in old acyclic mice were present in middle-aged mice and whether the cycling status of the mice influenced those changes. Young (3–6 months) regularly cycling and middle-aged (12–14 months) C57BL/6J mice that were either acyclic or still cycling regularly were injected with a dose of E2 (0.05 μg/10 g body wt) sufficient to achieve maximal levels of nuclear ER (ERn) in all tissues examined: hypothalamus (HYPO), pituitary (PIT), and uterus (UT). The rise and fall of ERn and the replenishment of cytosolic ER (ERc) were measured 0, 1, 2, 4, 8, 12, and 24 h later.

Cycling status did not affect ER binding profiles in middle-aged tissues. Therefore, data from cycling and acyclic subgroups were pooled for comparison with young mice. The increase in ERn following E2 injection, measured as the integrated area under the ERn profile, was reduced 33, 23, and 17%, respectively, in HYPO, PIT, and UT of middle-aged mice. In addition, the duration of elevated ERn was selectively reduced in middle-aged HYPO. ERc levels were reduced in middle-aged HYPO and UT, but replenishment rates were not altered. Reductions in total ER (ERn + ERc) were sufficient to account for the decline in ERn in middle-aged HYPO and UT, but factors in addition to ER loss appear to contribute to reduced ERn in middle-aged PIT. These results indicate that alterations in ER levels and dynamics occur prior to the transition to acyclicity, that these alterations are not secondary to hormonal or other changes associated with acyclicity, and that receptor loss appears to account for most of the age-related reduction in nuclear ER binding.

INTRODUCTION

As female rodents age, alterations appear in neuroendocrine, ovarian and uterine functions [1–3]. These changes lead to declining reproductive capacity and, ultimately, acyclicity and infertility. Because estradiol (E2) becomes progressively less able to elicit responses from aged tissues [2, 4], reduced sensitivity of tissues to E2 may play an important role in these reproductive aging processes. Since many of the effects of E2 are mediated by the estrogen receptor (ER), studies directed towards determining the molecular mechanisms that underlie reduced sensitivity to E2 have focused on this protein.

It is well established that the levels of nuclear ER (ERn) in hypothalami (HYPO), pituitaries (PIT) and uteri (UT) of aged rodents are reduced following an E2 challenge [5–12]. However, why ERn is reduced is poorly understood. It is not even known whether reduced ERn is the consequence of age-related changes intrinsic to ER containing cells, or is the result of systemic changes (i.e. hormonal or metabolic). Because E2 and progesterone profiles change as female rodents age [4], and because both hormones influence ER levels [13–15], reduced ERn in aged tissues could be secondary to these changes.

We previously reported that ER levels as well as intracellular ER dynamics are altered in old mice (22–24 months) [12]. However, because all of the old mice were acyclic, we were unable to determine whether the alterations in ER were independent of their acyclic state. Furthermore, it was not known if the alterations found in old mice were present earlier, when mice are undergoing reproductive decline, and might thus play a role in reproductive failure.

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The objective of this study was to address these questions by determining whether ER levels and intracellular dynamics are altered in middle-aged mice, and if so, whether the changes are similar in cycling and acyclic mice. We report that ER levels are reduced and dynamics are altered in middle-aged mice, and that these changes are independent of cycling status.

EXPERIMENTAL

Animals and experimental design

Female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Me), housed 3 per cage, and allowed to age in our colony as described previously [16]. Mice were studied at 3–6 months (young) and 12–14 months (middle-aged). Middle-aged mice were separated into regularly cycling and acyclic subgroups as determined by vaginal cytology (see below).

Vaginal smears were obtained daily and classified by day of cycle (Day (D)1 = proestrus) [17]. Tissues from regularly cycling mice were collected late in the cycle (D5 or D6), when total and nuclear ER binding are greatest [15]. A preliminary study showed no difference in the binding levels on those two days of the cycle (data not shown). Cycle lengths were 4.7 ± 0.1 days (mean $\pm$ SE) in young mice and 5.5 ± 0.2 days in middle-aged cycling mice. Tissues from middle-aged acyclic mice were obtained following a period of more than 14 consecutive days of cornified vaginal smears (persistent vaginal cornification). Middle-aged mice in persistent diestrus were not studied because there are insufficient numbers at this age [18]. Mice showed no signs of disease.

Mice were sacrificed 1–24 h after a s.c. injection of E2 (0.05 μg/10 g body wt) in corn oil. This was the lowest dose that produced maximal nuclear binding in all 3 tissues of both age groups (data not shown). For baseline control values (time 0), animals were injected with vehicle 1 h before sacrifice.

Receptor nomenclature

In this report ERc is defined as estrogen receptors that are present in the cytosolic fraction, presumably because they are only loosely bound to chromatin and are solubilized during tissue disruption. ERn refers to receptors retained in the partially purified nuclear pellet, presumably because they are more tightly associated with the nuclear material. ERt refers to total receptor (ERn + ERc). Depletion is defined as the loss of ERc following an E2 injection, and replenishment refers to the subsequent return of ERc to baseline levels (time 0) or higher.

Tissue preparation and receptor assay

A detailed description of the procedures, chemicals, and nomenclature used in this study, as well as the validation of the exchange assay for mouse tissues, has been published [15]. Mice were exsanguinated under anesthesia between 0900 and 1100 h, tissues were removed, and plasma was prepared. All subsequent procedures were carried out in a cold room at 0–4°C. Each determination of receptor content utilized tissues pooled from 2 animals. Tissues were homogenized in ice-cold TED buffer (10 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol and 0.02 g/dl sodium azide, pH 7.4 at 23°C). The nuclear fraction was obtained by centrifuging tissue homogenates through a 1.2 M sucrose pad at 6900 *g* for 30 min and resuspending the pellet in TED. The supernatant above the sucrose pad was centrifuged at 105,000 *g* for 1 h at 5°C to prepare the cytosolic fraction. Nuclear and cytosolic fractions were incubated with [^{3}H]E2 ([2,4,6,7-^{3}H]E2 (95.0 Ci/mmol), New England Nuclear Canada Ltd, Lachine, Quebec) in the presence or absence of a 100-fold excess of non-radioactive E2. A single saturating concentration of [^{3}H]E2 (6.0 nM in the UT, 1.4 nM in the HYPO and PIT) was used in the incubations. These concentrations were derived from Scatchard studies [19] of tissues from young and old animals [12]. In both nuclear and cytosolic fractions, NaSCN (final concentration: 0.5 M) was added to the incubations to extract ER from nuclei and to potentiate exchange between free and receptor-bound E2 at 0°C. ERn samples were incubated for 16–20 h to allow complete exchange. Cytosolic ER (ERc) samples were incubated an extra 24 h to enable a larger number of tissues to be assayed per experiment. There was no loss of binding over these incubation periods (data not shown). Bound [^{3}H]E2 was separated from free [^{3}H]E2 by LH-20 chromatography and measured by liquid spectrophotometry. The difference in the amount of bound [^{3}H]E2 with and without excess unlabelled ligand was considered specific high-affinity E2 binding. Portions of the initial

homogenate were saved for determination of DNA content [20], and binding was normalized to DNA (fmol of E2 bound per μg DNA).

E2 RIA

Plasma E2 was assayed using an E2 antibody (ovine) provided by Dr J. Challis, (University of Western Ontario, London, Ontario) as described previously [21].

Statistical analyses

Plasma E2 levels were analyzed by 2-way analysis of variance (ANOVA), with age (or cycle status, for comparison of middle-aged subgroups) and time after injection as main effects. ER levels were analyzed by 2-way analysis of covariance (ANCOVA), using age (or cycle status) and time after injection as main effects, and plasma E2 concentration as the covariate to ensure that differences were independent of any differences in plasma E2 levels due to age or cycle status. The Student–Newman–Keuls (SNK) multiple comparisons *a posteriori test* [22] was used to determine the significance of differences between individual means. P values less than 0.05 were considered significant.

RESULTS

Plasma E2

Plasma E2 levels were measured to ensure that differences among groups in ER profiles were not secondary to differences in circulating E2 concentrations. Plasma E2 levels did not differ in middle-aged acyclic and cyclic subpopulations ($P > 0.05$, ANOVA; data not shown) and were therefore pooled. As shown in Fig. 1, E2 profiles did not differ between young and middle-aged mice ($P > 0.05$, ANOVA). In both groups plasma E2 peaked at 1 h and returned to baseline by 4 h.

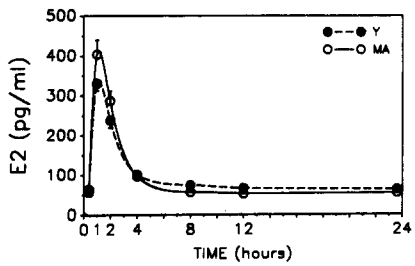

Fig. 1. Plasma E2 profiles following an injection of E2. Young (Y) (3–6 months) and middle-aged (MA) (12–14 months) mice were injected s.c. with E2 in corn oil (0.05 μg/10 g body wt) and exsanguinated under anesthesia from the right ventricle 1, 2, 4, 8, 12, and 24 h later (time 0 = vehicle). Each point is the mean ± SE of 5–13 determinations with 2 animals per determination. At some time points SEs are too small to be seen.

ER binding in cyclic and acyclic middle-aged mice

Figure 2 shows the effect of cycling status on ERn binding profiles in middle-aged mice following an injection of E2. ERn profiles did not differ between cycling and acyclic subgroups in any tissue ($P > 0.25$, ANCOVA). ERc and ERt binding profiles also did not differ between these two groups ($P > 0.35$, ANCOVA; data not shown). The data from these two subgroups were therefore pooled for subsequent comparisons with young mice.

Nuclear ER dynamics

The increase of ERn following injection of E2 was reduced in all tissues of middle-aged mice, as indicated by a significant main effect of age ($P < 0.001$, ANCOVA, Fig. 3). In addition, the

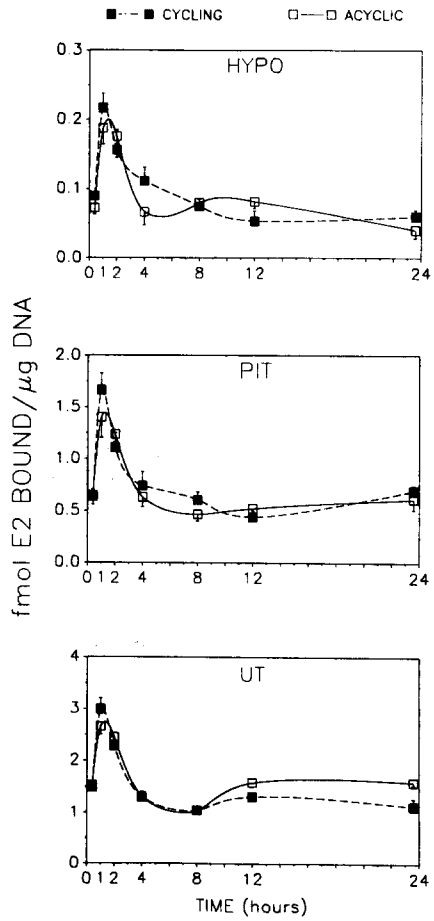

Fig. 2. The effect of cycle status on nuclear ER binding (ERn) following E2 injection in middle-aged cycling and acyclic mice. Each point is the mean ± SE of 4–7 determinations. For further details, see Fig. 1.

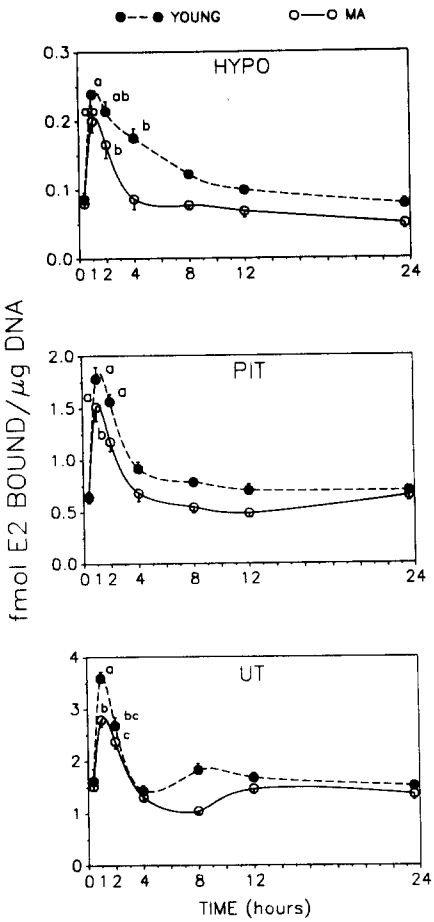

Fig. 3. The effect of age on nuclear ER binding (ERn) following E2 injection in young and middle-aged (MA) mice. In this and all other figures, data from middle-aged cycling and acyclic mice has been pooled, because they were indistinguishable. Points with alphabetical letters are significantly elevated above baseline (time 0), and those not sharing a common letter are significantly different from each other ($P < 0.05$, SNK). For further details, see Fig. 1.

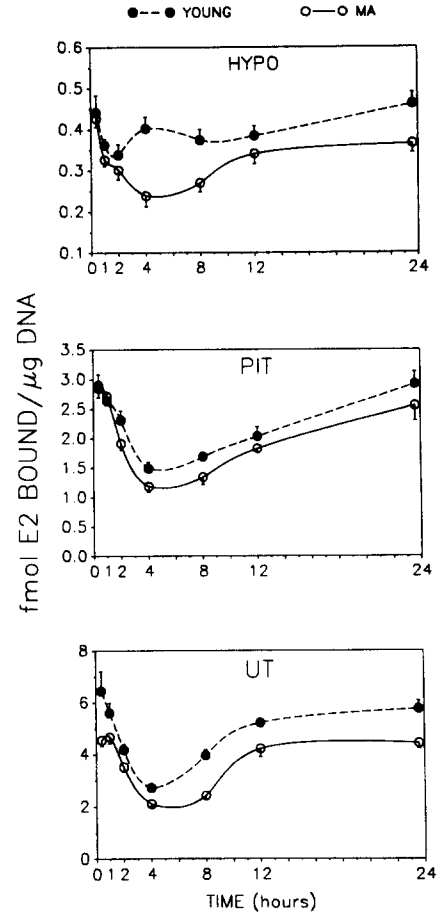

Fig. 4. The effect of age on total ER content (ERn + ERc) following E2 injection in young and middle-aged (MA) mice. For further details, see Fig. 1.

fall of ERn following attainment of peak binding was accelerated in HYPO of middle-aged mice. Whereas ERn levels remained elevated for 8 h in young HYPO, ERn returned to baseline by 4 h in middle-aged HYPO ($P < 0.05$, SNK). Together, these changes resulted in integrated areas under the nuclear ER profiles of middle-aged HYPO, PIT, and UT that were 33, 23, and 17% lower, respectively, than young values.

The reduced Ern in tissues of middle-aged mice could be the consequence of either a loss of ERt, and hence a reduction in ER available for binding, or an inability of ER to bind nuclei. If the reduced ERn was secondary to a loss of ERt, the reduction in ERt would be proportional to the reduction of ERn. As shown in Fig. 4, integrated areas of the ERt profiles of middle-aged HYPO, PIT, and UT were 27, 12, and 23% lower, respectively, than in young tissues. The proportionality of the reductions of ERt and ERn in HYPO and UT indicate that the loss of ERt can largely account for the decline in nuclear binding in these tissues. Reduced nuclear binding in middle-aged PIT, however, was nearly twice the loss of total ER, suggesting that factors in addition to ER loss (e.g. alterations in the ER protein or its nuclear binding sites) contribute to reduced ERn binding in this tissue.

Cytosolic ER dynamics

Figure 5 shows the depletion-replenishment profiles of ERc following E2 injection in young and middle-aged mice. ERc profiles were reduced in middle-aged HYPO and UT ($P < 0.005$, ANCOVA), but not in PIT ($P > 0.5$, ANCOVA). The shape of the replenishment profiles in UT and PIT were similar in young and middle-aged mice, indicating that the rates of replenishment in these two tissues did not differ with age. In HYPO, however, the shape of the two profiles differed. Although

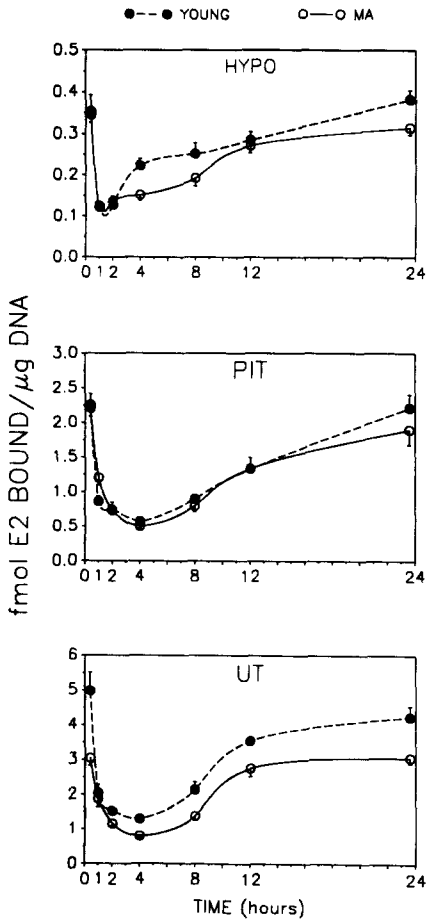

Fig. 5. The effect of age on depletion and replenishment of cytosolic ER (ERc) following E2 injection in young and middle-aged (MA) mice. For further details, see Fig. 1.

replenishment was completed by 12 h in both age groups, the initial recovery of ERc from maximal depletion at 1–2 h was more rapid in young than in middle-aged HYPO (young vs middle-aged at 4 h, $P < 0.05$, SNK).

DISCUSSION

The present investigation confirms and extends previous studies showing reduced nuclear ER binding in aging female rodents [5–12]. The results indicate that alterations in ER levels and dynamics occur prior to the transition to acyclicity in C57BL/6J mice, and that the magnitude of the changes is similar in cycling and acyclic mice.

Most of the changes that were observed in middle-aged mice were found previously in older mice [12]. For example, the elevation of ERn following E2 injection was reduced similarly in middle-aged and old tissues relative to their young controls [the present study, 12]. The most pronounced loss occurred in HYPO (middle-aged and old; 33 and 34%, respectively) and the smallest loss occurred in UT (17 and 19%); PIT was intermediate (23 and 28%). The accelerated loss of peak ERn in middle-aged HYPO had also been observed previously in old HYPO [12]. The presence of these changes in both cycling and acyclic middle-aged mice as well as in old acyclic mice indicate that they are not attributable to the altered hormonal milieu of persistent vaginal cornification, to the hormonal withdrawal associated with persistent diestrus, or to any other characteristic of acyclicity. ERn levels are also reduced in HYPO and PIT of middle-aged rats before they stop cycling [7, 8]. Thus, changes in the concentration of ER and its dynamics are relatively early phenomena of reproductive aging in both mice and rats, and are caused by events occurring during or prior to middle-age. Although it is possible that these alterations are a consequence of hormonal changes associated with the lengthening cycles of middle-age [23], this is unlikely, since those changes are relatively small [23], and it is doubtful that they could cause alterations that the more marked hormonal changes of acyclicity did not amplify.

The causes of the age-related changes in ER levels and dynamics are not well understood. A major question is the relative importance of receptor loss vs qualitative changes in the receptor to the reduced cell nuclear binding of ER. Our results indicate that receptor loss is sufficient to account for the reduced nuclear binding of ER in HYPO and UT. Similar results led to the same conclusion in older mice [12]. In contrast, ER loss only accounted for half of the reduction of ERn in middle-aged PIT, suggesting that an impaired capacity of ER to form ERn also contributes to reduced ERn in this tissue. Alternatively, nuclear acceptor sites may be altered in middle-aged PIT. This apparent reduction in ability of ER to bind nuclei was not observed in PIT of older mice [12].

As in older mice [12], the loss of elevated ERn in middle-aged HYPO was accelerated and may be a consequence of slower replenishment of ERc. In young HYPO, the rapid recovery of ERc at 4 h sustained total ER content at pre-injection levels, providing an undiminished pool of ER sufficient to maintain ERn at elevated levels, assuming that all ERc is available for nuclear binding. By contrast, ERc levels were significantly reduced at 4 h in middle-aged HYPO, and the pool of ER available for nuclear binding was thereby diminished.

The magnitude of the ER reductions observed in this and most other studies are modest. However, as noted before [12], altered ER comprises only one of numerous cellular and molecular changes which occur in the hypothalamic–pituitary–gonadal axis during aging, many of which are relatively small [1, 2]. It is important to recognize, however, that these changes are numerous, and that in combination, they may produce the more substantial deficits often observed at physiological endpoints. Moreover, many studies [24–29], though not all [30], show strong correlations between steroid responsiveness and the magnitude as well as duration [24, 25] of nuclear binding. Thus, relatively small changes in ER levels and dynamics may contribute to the alterations of estrogen-dependent reproductive functions in middle-aged mice (e.g. the positive feedback response of luteinizing hormone to E2 [31], estrous cycle lengthening [32]). It is unlikely, however, that ER alterations in HYPO and PIT contribute to the loss of cyclicity, since the aging ovary is the major determinant of the cessation of cyclicity in this mouse strain [32, 33]. Moreover, changes in ER were present in middle-aged mice before they stopped cycling and did not differ in middle-aged acyclic mice.

In conclusion, this study shows that changes in ER levels and dynamics occur as early as midlife and that those changes are similar in cycling and acyclic mice. Thus, alterations of ER in middle-aged tissues are independent of the altered hormonal milieu associated with acyclicity. As in older mice, most of the changes were attributable to reduced ER content and altered processing of ER. The molecular basis for these changes and their role in altered responsiveness to E2 remains to be determined.

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