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Brief Communication

Estrogen Induction of Glial Heat Shock Proteins: Implications for Hypothalamic Aging

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MYDLARSKI, M. B., A. LIBERMAN AND H. M. SCHIPPER. *Estrogen induction of glial heat shock proteins: Implications for hypothalamic aging.* NEUROBIOL AGING **16**(6) 977–981, 1995.—In the aging mammalian hypothalamus, a unique subpopulation of glial cells accumulates peroxidase-positive cytoplasmic inclusions distinct from lipofuscin. In adult rodents, this senescence-dependent glial granulation is accelerated by administration of estradiol valerate. In the present study, brain sections derived from male rats given 3 monthly intramuscular injections of estradiol valerate (0.2 mg or 2.0 mg) were immunostained for heat shock proteins and glial fibrillary acidic protein to determine whether a glial stress response is implicated in estrogen-induced granulation. Our findings indicate that estrogen elicits a heat shock response and subsequent granulation in astrocytes residing in estradiol receptor-rich brain regions including the arcuate nucleus and the wall surrounding the third ventricle but not in estradiol receptor-deficient regions such as the striatum and corpus callosum. The heat shock proteins induced by estrogen, namely, the 27, 72, and 90 kDa stress proteins, are upregulated in astrocytes in response to oxidative challenge supporting our hypothesis that estrogen mediates senescent changes in the rodent hypothalamus through oxidative mechanisms.

Aging Astrocyte Estrogen Heat shock protein Inclusion

IN THE course of normal aging, chronic exposure to ovarian estrogen promotes the development of pathologic changes in the hypothalamic arcuate nucleus (ARC), a neuroendocrine locus rich in estradiol receptors and implicated in the regulation of gonadotropin secretion (1,3,23). The appearance of pathologic changes in the ARC can be markedly accelerated in male rats following multiple intramuscular injections of estradiol valerate (EV), and in female rats, by EV injection or continuous exposure to high-physiologic levels of estradiol resulting from continuous illumination or steroid-releasing Silastic implants (3,4,18). The estrogen-related ARC lesion is characterized by the accumulation of peroxidase (Gomori-)-positive astrocyte granules that are histochemically and morphologically distinct from the aging pigment, lipofuscin (22,23,26). Loss of β-endorphin (8), dendritic damage, synaptic remodeling and microgliosis have also been reported in the ARC of EV-treated rats (1,3,5).

The mechanism by which estrogen promotes the accumulation of Gomori-positive astroglial inclusions in the hypothalamus remains unclear. In primary astroglial cultures and in rodent astrocytes in situ (28,29), the aminothiol compound, cysteamine (CSH), greatly accelerates the time-dependent accumulation of identical peroxidase-positive astrocytic inclusions. We recently demonstrated that damaged, iron-laden mitochondria are the subcellular precursors of mature, Gomori-positive astrocyte granules both in CSH-treated glial cultures (2) and in the aging periventricular brain (6). Moreover, CSH-induced astrocyte granulation appears to be dependent upon the antecedent induction of a robust cellular stress (heat shock) response characterized by the upregulation of heat shock proteins (HSP) 27, 72, and 90 in these cells (15,16,28). Studies with CSH, H_2O_2 and ionizing radiation, all of which elicit astrocyte granulation (15,28,29,31), strongly suggest that intracellular oxidative stress may be a "final common pathway" promoting both a heat shock response and the biogenesis of cytoplasmic inclusions in these cells. In the present study, we set out to determine whether activation of a cellular heat shock response similarly plays an incipient role in the biogenesis of redox-active astrocyte granules following chronic estrogenization.

METHOD

Four-week-old male Sprague–Dawley rats were obtained from the Canadian Breeding Farms, maintained under a 12L:12D cycle and allowed free access to standard rat chow and water.

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Long-Term EV study

Beginning at seven weeks of age, the animals received three consecutive monthly intramuscular injections of (a) 2.0 mg EV (Delestrogen, Squibb Canada Inc.), (b) 0.2 mg EV, or (c) sesame oil vehicle (control). Each treatment group contained 4 animals. At $17\frac{1}{2}$ weeks of age (2 weeks following the last injection) the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 100 ml saline followed by 250 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were immediately removed and immersed in cold fixative for 24 h. Forty-micron-thick coronal sections were cut on a vibratome (Lancer series 1000) in preparation for immunohistochemistry.

48-h (Short-Term) EV Study

Additional groups of 7-week-old animals were perfused 48 h following a single injection of 0.2 mg EV or sesame oil vehicle.

Peroxidase Histochemistry-GFAP Immunostaining

Free-floating sections were washed in Tris-buffered saline (TBS; pH 7.6, 4°C) and incubated in modified Karnovsky medium for the demonstration of (brown) endogenous peroxidase activity (10) followed by overnight incubation at 4°C with a rabbit-derived polyclonal antibody directed against glial fibrillary acidic protein (GFAP; 1:40 dilution; kindly provided by E. Wang of our institution) exactly as previously described (27,28). The tissue was subsequently processed by the Vectastain avidin-biotin-peroxidase complex (ABC; Vector, Burlingame, CA) technique using goat anti-rabbit IgG (11). GFAP was visualized as a pink precipitate with α-naphthol as chromogen and pyronin B intensification as previously described (27,28). Peroxidase-positive astrocytes in these brain regions were examined and photographed using a Leitz Diaplan photomicroscope. Control sections were processed as above with omission of either (a) DAB (with preincubation in 3% H_2O_2 in TBS for 30 min to eliminate endogenous peroxidase activity), (b) the anti-GFAP antibody, or (c) H2O2 in the α-naphthol reaction.

Quantitation of DAB-Positive Astrocyte Granules

In the long-term study, numbers of peroxidase-positive astrocyte granules were quantitated in ×1000 fields of corpus callosum (CC) and caudate-putamen (CP) with the aid of an ocular grid by a single investigator unaware of the tissue source. Similar counts were performed in the ARC in the short-term study.

Stress Protein-GFAP Double Labelling

Free-floating rat brain sections were preincubated in 3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity and washed in PBS. Sections derived from the long-term study were subsequently incubated with mouse monoclonal antibodies directed against HSP27, HSP72 (inducible), HSP90, or GRP 94 (StressGen, Victoria, BC) as described in full detail elsewhere (28). Antibody dilutions were 1:200 for anti-HSP27 and 1:100 for the other stress proteins. Immunohistochemistry was performed using peroxidase-conjugated goat anti-mouse IgG (secondary antibody) and the ABC kit. Following incubation in DAB-H_2O_2, the stress proteins were visualized as brown precipitates. GFAP immunolabeling using α-naphthol-pyronin was subsequently performed on these sections as described above. To control for possible background HSP staining, some sections were processed with nonspecific ascites fluid (1:100–1:200 dilutions) in place of the stress protein monoclonal antibody. Rat brain sections from the short-term study were processed for HSP90-GFAP double labeling. Stained sections were examined and photographed using a Leitz-Diaplan photomicroscope. Analysis was restricted to the ARC, the area surrounding the third ventricle (peri-III), the CC and the CP. For each animal, a minimum area of 0.14 mm^2 per region was evaluated and the total number of GFAP-positive astrocytes as well as the percentage of GFAP-positive astrocytes expressing HSP were determined. Differences between groups were compared using Student's two-tailed t test with $p < 0.05$ indicating statistical significance.

RESULTS

Long-Term EV Study

Peroxidase-positive astrocyte granules. Peroxidase-positive cytoplasmic granules were visualized as brown (oxidized DAB) deposits surrounded by pink (oxidized α-naphthol-pyronin) GFAP-positive cytoplasm (see reference 27,28 for colour illustrations depicting peroxidase-positive astrocytes). EV treatment has previously been shown to increase numbers of peroxidase-positive astrocyte granules in the ARC and third periventricular region of adult male and female rats (1,3,4,26). In the present study, chronic estrogenization did not increase numbers of peroxidase-positive glial inclusions in the CC or CP, or total numbers of GFAP-positive astrocytes in any of the brain regions surveyed, relative to controls (data not shown). In the absence of anti-GFAP antiserum or H_2O_2 in the α-naphthol medium, brown DAB-positive granules were readily visualized but astrocytes remained unlabeled. With the elimination of DAB and following preincubation in H_2O_2, all GFAP-positive astrocytes appeared devoid of endogenous peroxidase activity.

Stress protein immunohistochemistry. Colour illustrations depicting astroglia immunoreactive for GFAP and various HSPs are provided in ref. 28. The percentages of GFAP-positive astrocytes exhibiting immunoreactivity for each stress protein surveyed following long-term estrogen treatment are shown in Table 1.

HSP27. In animals receiving multiple injections of 0.2 mg EV, there were significant increases in the percentages of astrocytes expressing HSP27 in the ARC and peri-III ($p < 0.05$ relative to controls) but not in the other regions examined ($p > 0.05$). Increases in the proportion of astrocytes expressing HSP 27 in ARC and peri-III following 2.0 mg EV treatment did not achieve statistical significance ($p > 0.05$ for each comparison relative to controls) due to large standard deviations.

HSP72. Chronic treatment with 2.0 mg EV resulted in a significant increase in the percentage of astrocytes expressing HSP72 in the peri-III region ($p < 0.05$) but not in the other brain areas surveyed ($p > 0.05$). A trend toward increased HSP72 expression in the ARC failed to reach statistical significance ($p > 0.05$).

HSP90. In the ARC and peri-III regions, low- and high-dose EV treatment induced a robust increase in glial HSP90 staining ($p < 0.05$ relative to controls). High-dose EV treatment induced a smaller, but statistically significant ($p < 0.05$), increase in glial HSP90 expression in the CP, whereas no effect ($p > 0.05$) was observed in the CC.

GRP94. A trend toward increased GRP94 immunoreactivity in astrocytes surrounding the third ventricle in EV-treated rats relative to controls was not statistically significant ($p > 0.05$). Similarly, administration of EV had no appreciable effects on glial GRP94 expression in any of the other areas examined ($p > 0.05$ for each comparison).

48-h EV study. As in the case of long-term EV treatment, GFAP expression was unaffected by acute administration of 0.2

TABLE 1

PERCENT OF GFAP-POSITIVE ASTROCYTES (MEAN ± SD) PER REGION EXPRESSING HSP 27, 72, or 90 or GRP 94 AFTER LONG TERM EV TREATMENT

	ARC			Peri-III			CC			CP		
	Control	EV 0.2 mg	EV 2.0 mg	Control	EV 0.2 mg	EV 2.0 mg	Control	EV 0.2 mg	EV 2.0 mg	Control	EV 0.2 mg	EV 2.0 mg
HSP 27	16.7 ± 6.9	68.2 ± 7.1*	56.2 ± 27.2	28.5 ± 4.4	58.6 ± 14.2*	55.9 ± 29.3	26.3 ± 26.0	38.2 ± 24.9	46.8 ± 22.3	33.0 ± 4.5	36.6 ± 12.7	43.3 ± 8.4
HSP 72	7.6 ± 7.8	17.2 ± 18.1	23.8 ± 11.7	4.4 ± 3.4	16.8 ± 10.9	30.6 ± 5.9*	13.2 ± 16.6	9.3 ± 9.4	15.2 ± 4.8	7.4 ± 10.2	4.6 ± 4.8	2.7 ± 4.0
HSP 90	2.7 ± 1.9	37.8 ± 15.5*	37.1 ± 6.4*	6.1 ± 7.9	35.5 ± 9.0*	32.8 ± 6.0*	19.5 ± 15.4	12.2 ± 8.1	14.9 ± 9.1	3.7 ± 3.1	4.8 ± 6.0	12.9 ± 5.3*
GRP 94	16.0 ± 14.4	23.5 ± 24.4	15.6 ± 18.6	12.0 ± 13.9	24.5 ± 17.0	25.7 ± 25.2	16.1 ± 22.4	13.1 ± 11.0	5.8 ± 2.9	8.3 ± 4.1	9.8 ± 5.6	7.6 ± 4.0

Abbreviations: ARC = arcuate nucleus; EV = estradiol valerate; CC = corpus callosum; CP = caudate-putamen; HSP = heat shock protein; Peri-III = third periventricular region.
* = Significantly increased from control values ($p = 0.05$).

mg EV (data not shown). Short-term EV treatment did not significantly alter numbers of DAB-positive astrocyte granules in the ARC (Fig. 1), a locus exhibiting intense granulation after long-term exposure to estrogen (3,4,5). Additional rat brain sections were processed for HSP90-GFAP double immunolabeling to determine whether an astrocytic stress response precedes EV-induced granulation. In the ARC, peri-III region and CC, there were significant increases in the percent of glia expressing HSP90 after short-term EV treatment (Fig. 1). In the CP, an increase in the proportion of astrocytes expressing HSP90 after short-term estrogen treatment failed to achieve statistical significance ($p < 0.05$).

DISCUSSION

We previously demonstrated that estrogen administration or withdrawal accelerates or attenuates, respectively, the aging-related accumulation of peroxidase-positive astrocyte granules in the rodent periventricular brain (3,4,22,23). The pharmacological doses of EV utilized in this study induce many pathologic changes in the ARC of male rats akin to those which arise spontaneously in senescent female rats and in castrated younger females exposed to high-physiological concentrations of exogenous estradiol (1,3,4,23). In the present study, we observed that estrogen-mediated granulation is restricted to regions rich in estrogen-receptors such as the ARC and the third ventricular subependymal zone and does not occur in estrogen-receptor-deficient areas such as the CP and CC. We recently reported that in primary glial cultures and in the intact periventricular brain, the sulfhydryl agent, CSH, induces the appearance of identical glial inclusions in the context of an antecedent cellular stress response (15,16,28). Moreover, certain stress proteins upregulated by CSH, such as HSP27 and ubiquitin, become incorporated within the nascent astrocytic inclusions (17).

The results of the current study further support our contention that induction of a cellular stress response is a prerequisite for astrocytic granulation in the aging rat brain. Using immunocytochemical double labeling, we observed marked enhancement of HSP 27 and 90 staining in GFAP-positive astrocytes of the ARC and peri-third ventricular regions of long-term EV-treated rats relative to controls. We also observed augmented

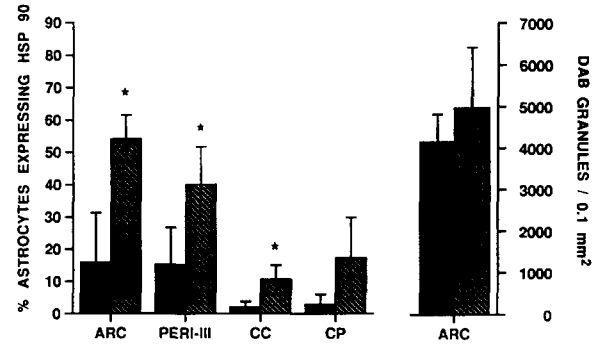

FIG. 1. The graph on the left shows the percent of GFAP-positive astrocytes (mean ± SD) expressing HSP90 per unit area 48 h following a single injection of 0.2 mg EV. The graph on the right indicates number of DAB-positive granules (mean ± SD) per unit area of ARC after identical, short-term EV exposure. Controls = solid bars. EV-treated = hatched bars.

expression of HSP 72 which was limited to the peri-third ventricular region following prolonged EV treatment. Olazábal and co-workers have reported the induction of neuronal but not glial HSP90 and HSP70 in rat hypothalamus following estrogen treatment (12,19,20). However, they did not specifically assess HSP expression in GFAP-positive astrocytes by dual label immunohistochemistry. To our knowledge, the present study is the first to demonstrate estrogen-related upregulation of HSPs in astrocytes. This estrogenic effect on HSP expression (and subsequent granulation) may be direct given the high concentration of estrogen-receptor-positive astrocytes residing in the hypothalamic-median eminence region of rodents (14).

Unlike HSP27, HSP90 expression following long-term EV treatment was also increased in the striatum, a region refractory to EV-induced granulation. The paucity of estrogen-receptors in the striatum (13,30,32) suggests that induction of glial HSP90 in this region may not represent a direct genomic effect of estrogen in this cell population. Estrogen induces HSP90 in various neuronal populations (12,19) where it may serve as a steroid receptor chaperone (21). Conceivably, estrophilic neurons residing outside the caudate-putamen may, upon innervating the latter, evoke a striatal glial HSP90 response. Similarly, estrophilic neurons projecting through the CC may mediate transient upregulation of glial HSP90 in this region after short-term EV exposure. Enhanced HSP90 expression in striatal (and callosal) astrocytes may, on the other hand, represent a direct, nonestrogen-receptor-mediated action of the steroid on these cells.

In all regions surveyed, GFAP expression appeared unchanged by chronic EV exposure. In contrast, long-term CSH administration induces a strong heat shock response and widespread granulation in astrocytes along with enhanced GFAP staining (28). Taken together, the results of the CSH and estrogen experiments indicate that the accumulation of Gomori-positive astrocyte granules may occur within the context of, or entirely independent of, classical astrocyte hypertrophy (gliosis).

Astrocytes exposed to EV (present study) or CSH (15) synthesize HSPs 27, 72, and 90, stress proteins which are highly responsive to oxidative challenge. In contrast, astrocytic expression of GRP94, a stress protein responsive to glucose deprivation and calcium ionophores but not to oxidative stress (9,33), remained unchanged following CSH or EV treatment (15; present study). These observations support the notion that intracellular oxidative stress is the mechanism responsible for estrogen-related HSP expression and granulation in aging periventricular astrocytes. The fact that heat shock protein expression rapidly occurs in astrocytes without evidence of concomitant granulation in the 48-h EV experiment (present study) indicates that stress protein expression is not a consequence of the accumulation of the iron-rich (redox-active) cytoplasmic inclusions. Nonetheless, the latter may oxidize catecholestrogens and catecholamines to free radical intermediates (25) and thereby promote further oxidative injury within the aging hypothalamus.

We recently observed that the administration of potent antioxidants such as vitamin E (7) or 21-aminosteroids (24) prevents estrogen-induced depletion of hypothalamic beta-endorphin, anovulatory sterility, and the development of polycystic ovaries in adult, female rats. These observations further support our hypothesis that estrogen-related degeneration within the aging rodent hypothalamus-pituitary-ovarian axis is mediated, at least in part, by oxidative stress.

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