ACCELERATED COMMUNICATION

Neuroprotection against Oxidative Stress by Estrogens: Structure-Activity Relationship

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SUMMARY

Oxidative stress-induced neuronal cell death has been implicated in different neurological disorders and neurodegenerative diseases; one such ailment is Alzheimer's disease. Using the Alzheimer's disease-associated amyloid β protein, glutamate, hydrogen peroxide, and buthionine sulfoximine, we investigated the neuroprotective potential of estrogen against oxidative stress-induced cell death. We show that 17-β-estradiol, its nonestrogenic stereoisomer, 17-α-estradiol, and some estradiol derivatives can prevent intracellular peroxide accumulation and, ultimately, the degeneration of primary neurons, clonal hippocampal cells, and cells in organotypic hippocampal slices. The neuroprotective antioxidant activity of estrogens is dependent on the presence of the hydroxyl group in the C3 position on the A ring of the steroid molecule but is independent of an activation of estrogen receptors.

Ovarian steroids are of prime importance in the normal maintenance of brain function; the loss of these steroids at menopause may account, at least in part, for the cognitive decline and neurodegeneration that are associated with AD (1). Consistent with this are the results of population-based studies, which suggest that the increased incidence of AD in older women may be caused by the deficit of the female sex hormone estrogen after menopause and that the use of estrogen during the postmenopausal period can delay the onset and lower the risk of AD (2). Estrogen acts on a number of different target organs, including the brain, that express specific estrogen receptors, and it is a key modulator of processes involved in differentiation, homeostasis, and development of the female reproductive function (3, 4). In addition to a genomic mode of action via transcriptional activation, estrogen and other steroids, such as progesterone derivatives, have been found to produce short term nongenomic actions. These include the modulation of the electrical properties of neurons and of transmitter release processes (5). The non-

genomic antioxidant activity of estrogens has received increasing attention recently (6). Oxidative stress and free radical-mediated cell death have been linked to diseases such as atherosclerosis (7) and to a number of neurodegenerative disorders such as Parkinson's disease and AD (8–11).

Because it has been suggested that estrogens, in contrast to all other natural steroids, are antioxidants of membrane phospholipid peroxidation in cell free systems because of their phenolic structure (6), and because initial data indicate a neuroprotective effect of 17-β-estradiol *in vitro* (12, 13), we investigated the neuroprotective potential of estrogen and some of its derivatives against oxidative stress-induced neurodegeneration. Oxidative damage and lipid peroxidation can be caused by the neurotoxic amyloid β protein (11) that accumulates in plaques in the brains of AD patients (14) or by excitatory amino acids such as glutamate, which has also been implicated in various neurodegenerative diseases (15), via a glutamate receptor-dependent (16) or a glutamate receptor-independent pathway (17, 18). The latter can be mediated through the induction of an imbalance in antioxidant enzyme systems followed by a reduction in the levels of intracellular antioxidant glutathione in neurons (17, 18). The

ABBREVIATIONS: AD, Alzheimer's disease; BSO, buthionine sulfoximine; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide; PC, phase contrast; DCF, dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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main goals of this study were 1) to investigate any possible protective effects of estrogen for neurons and 2) to identify a possible structure-activity relationship. Therefore, we tested 17-α-estradiol, 17-β-estradiol, estriol, estrone, ethinyl estradiol, mestranol, quinestrol, and the catechol estrogens 2-OH-estradiol and 4-OH-estradiol (Fig. 1) for their neuroprotective potential against the oxidative stressors Aβ and H_2O_2. The latter is a mediator of Aβ toxicity (11) and a precursor of highly oxidizing, tissue-damaging radicals such as the lipid peroxidizing hydroxyl radical (19). In addition, glutamate and BSO, which blocks the *de novo* biosynthesis of glutathione (19), were used.

Experimental Procedures

Materials, cell lines, and cell culture. Cells were cultured in DMEM supplemented with 10% fetal calf serum under standard culture conditions. Rat primary cultures from E19 embryonic hippocampi and mouse primary cortical neurons from E19 embryonic cortices were prepared as previously described (11, 21). Primary cells were cultured on poly-L-lysine–coated dishes in a 50% DMEM/50% Ham's F12 medium that contained N2 supplements. Under these minimal culture conditions, more than 90% of the cells were stained positive for neuron-specific enolase. Primary neurons were used after 7–10 days *in vitro*. Mouse clonal hippocampal HT22 cells were cultivated in DMEM supplemented with 10% fetal calf serum. All media, sera, and medium supplements were from GIBCO (Eggenstein, Germany). The amyloid β protein used (fragment 25–35) was from Bachem/Saxon (Hannover, Germany). Glutamate, H_2O_2, and BSO were from Sigma (Deisenhofen, Germany), as were all other chemicals. Stock solutions for the toxins were prepared and diluted in H_2O.

Cytotoxicity and viability assays. Cell viability was assessed using a modified MTT assay as previously described (9, 11, 12). Cell lysis induced by different toxins was assessed with the trypan blue exclusion test followed by cell counting (11, 12). In addition, the fluorescing DNA label PI was used to differentiate between dead cells and living cells (12). All toxicity assays were repeated five times in triplicate determinations. For trypan blue and PI stainings, cells were plated in 60-mm dishes, and the different reagents were added. After 24 hr, trypan blue (at a concentration of 0.12%) or PI (at a concentration of 5 μg/ml) was added, and the number of viable cells (trypan blue-excluding or PI-negative) per low magnification field were determined. For statistical comparisons, analysis of variance followed by a Scheffe's *post hoc* test was used.

Preparation and culture of organotypic hippocampal slices. Organotypic hippocampal slices were prepared and maintained as previously described (24). Briefly, after decapitation, the brains of 5- to 6-day-old male Sprague-Dawley rats were removed and transferred to cold DMEM. Using a tissue chopper, the hippocampus and the entorhinal cortex were cut into 250-μm slices and were placed on a sterile, porous (0.4 μm) membrane (Millicell; Millipore, Eschborn, Germany). The membranes were transferred into a tissue culture plate and covered with culture medium (final volume 1 ml). The culture medium consisted of 50% DMEM, 25% horse serum, 25% Hanks' balanced salts, and 100 units/ml penicillin/100 μg/ml streptomycin. The culture medium was changed routinely three times a week. Slice cultures were used for experiments after 14 days *in vitro*.
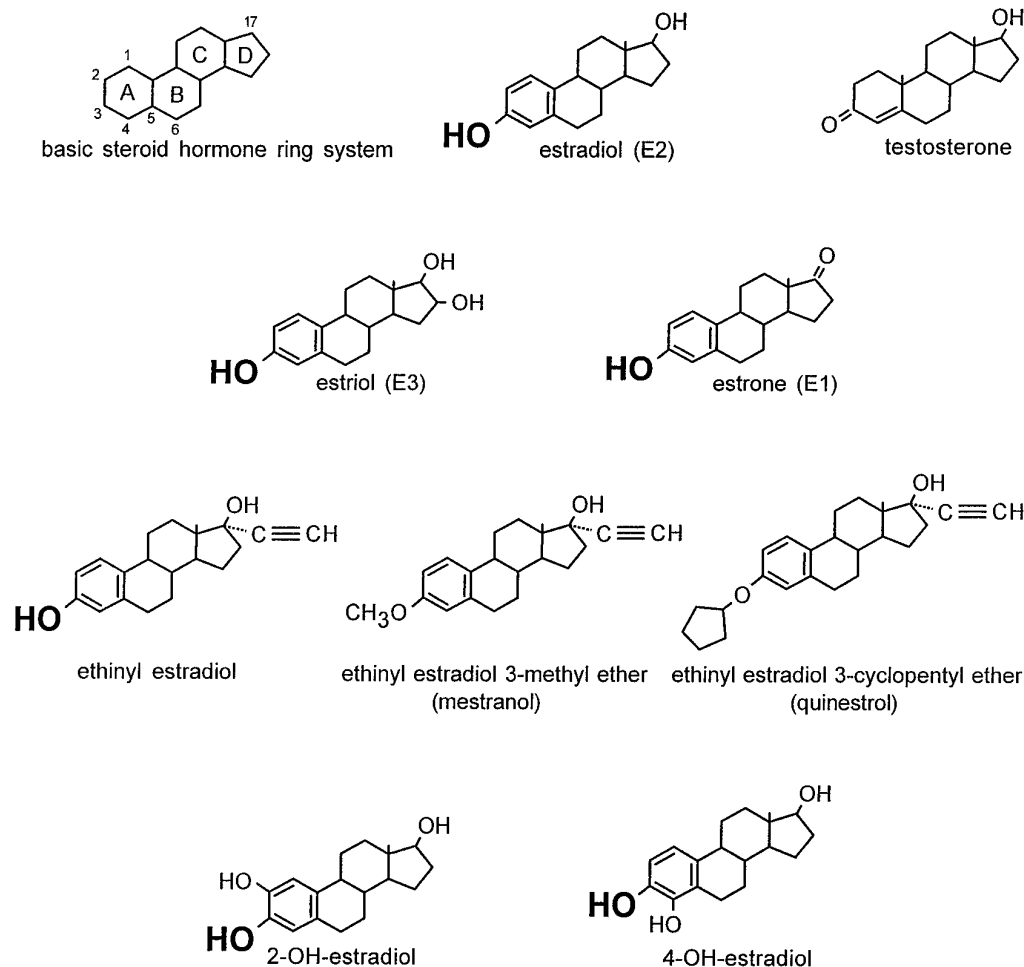

Fig. 1. Molecular structure of estrogens and estrogen derivatives used in the experiments. The 3-OH group on the A ring of the steroid molecule necessary for the neuroprotective activity is highlighted.

Detection of intracellular H_2O_2 and related peroxides. The formation of intracellular peroxides was detected by using DCF-DA as previously described (11, 17). DCF-DA is a nonfluorescent compound that, upon entering cells, is de-esterified and then becomes a substrate to oxidation by intracellular H_2O_2 and related peroxides. Primary cortical neurons or clonal hippocampal HT22 cells were plated, and toxins were added. After 6 hr, 10 μM DCF-DA was added for 1 hr at 37°. Then, the cells were washed with phenol red-free HEPES-buffered DMEM supplemented with 2% fetal calf serum, and the cultures were viewed with a fluorescence microscope using fluorescein optics. Cultures were compared with treatment condition by an observer blinded to the study, and fluorescence was determined qualitatively by counting the cells first under PC and then under fluorescence. For quantification, >200 cells per low magnification field were counted in five separate experiments, fluorescing cells were determined, and results were expressed as the percentage of fluorescent cells.

Results and Discussion

Rat primary hippocampal neurons and mouse clonal hippocampal HT22 cells pretreated for 20 hr with 10 μM 17-β-estradiol, estriol, or estrone and with the nonestrogenic stereoisomer 17-α-estradiol, which does not bind to estrogen receptors and is therefore biologically inactive (20), were protected against a 24-hr challenge by Aβ_{25-35} (2 μM), the toxic fragment of Aβ (21) (Fig. 2, A and B). Cells of both of these hippocampal cell culture systems were also protected against H_2O_2 (30 μM or 60 μM) and BSO (500 μM) by these estrogens (Table 1 and data not shown). A significant increase in cell survival ($p < 0.01$) could only be observed at the 10-μM concentration of these estrogens. Lower concentra-

tions were not effective, as shown in detail for the survival of HT22 cells that were pretreated with ethinyl estradiol after a challenge with either BSO, Aβ_{25-35}, or glutamate (Fig. 2C)

17-α-Estradiol, ethinyl estradiol, quinestrol, mestranol, and the catechol estrogens 2-OH-estradiol and 4-OH-estradiol were tested for their neuroprotective activity at 1 μM and 10 μM against BSO and H_2O_2 in mouse primary cortical neurons and against glutamate and H_2O_2 in HT22 cells. Estradiol is rapidly converted to 2-OH- and 4-OH-estradiol by an NADPH-dependent cytochrome P450-linked monooxygenase system *in vivo* (22). All tested estrogens that carry an OH group at the C3 position on the A ring of the steroid molecule afforded neuroprotection in these experimental paradigms at a concentration of 10 μM (Fig. 3; Table 1). Again, steroid concentrations lower than 10 μM did not afford protection, as shown in detail for ethinyl estradiol (Fig. 2C). Steroid molecules with an ether-modified OH group at the C3 position (Fig. 1), such as the 17-α-ethinyl estradiols quinestrol (3-cyclopentyl ether) or mestranol (3-methyl ether) and testosterone with a keto group at the C3 position, did not prevent oxidative stress-induced cell death (Fig. 3; Table 1). After the glutamate challenge, PC microscopy revealed dramatic changes in cellular morphology, and PI stainings demonstrated cell death of the clonal hippocampal HT22 cells. Some experimental data argue for a receptor-independent pathway of neuroprotection by estrogens because 1) the addition of the estrogens 2 or 20 hr before the toxic challenge did not influence the protection afforded (data not shown), 2) high concentrations must be used to achieve a protective
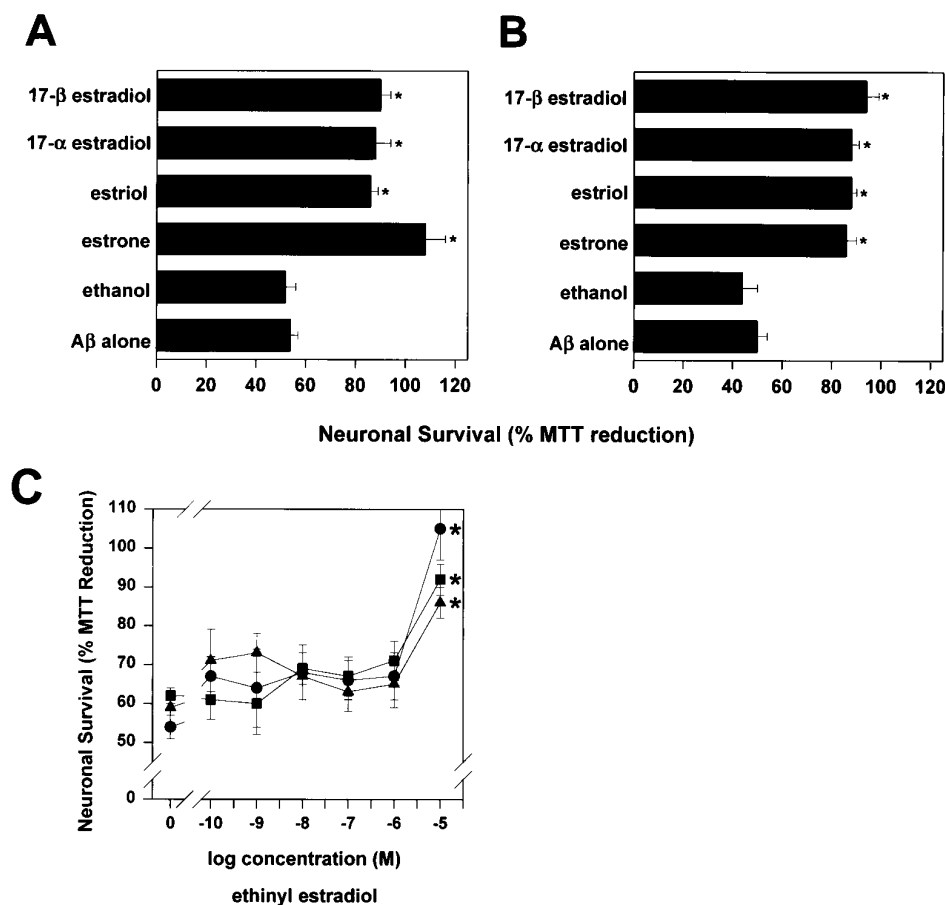

A

B

C

Neuronal Survival (% MTT reduction)

Neuronal Survival (% MTT Reduction)

log concentration (M)

ethinyl estradiol

Fig. 2. Different estrogens protect rat primary hippocampal neurons (A) and mouse clonal hippocampal cells (HT22) (B) against Aβ-induced cell death. Primary neurons and HT22 cells were plated and pretreated with the indicated estrogens (10 μM) for 20 hr. Then, cultures were challenged with 2 μM Aβ_{25-35} for an additional 20 hr, and neuronal survival was determined by performing MTT assays as previously described (9, 11, 12). C, HT22 cells were preincubated for 20 hr with increasing concentrations of ethinyl estradiol and then challenged by either 500 μM BSO ([triaf]), 2 μM Aβ_{25-35} (●), or 1 mM glutamate (■) for 20 hr, and MTT assays were performed. Results are expressed as the percentage of reduction of MTT relative to the corresponding control. The experimental data are presented as triplicate determinations across five independent experiments (mean ± standard error). Comparisons between cultures incubated with estrogens (or ethanol) alone and cultures treated with estrogens (or ethanol) plus toxin were made by analysis of variance followed by a Scheffe's *post hoc* test. ∗, $p < 0.01$ (considered significant). A final concentration of 0.1% of the steroid solvent ethanol was not toxic to the cells and did not interfere either with the toxins or with the MTT reduction. An increase in cell survival caused by treatment with the estrogens alone was below 10% in these short time assays.

Fig. 3. 17-α-Estradiol (*17-α E*), 17-β-estradiol (*17-β E*), and ethinyl estradiol (*EE*) prevent glutamate-induced intracellular accumulation of H_2O_2 and related peroxides and, ultimately, cell death in clonal hippocampal HT22 cells, whereas the ethinyl estradiol mestranol (*M*) is not protective. HT22 cells were plated in 60-mm dishes and either pretreated with the indicated estrogens (10 μM) for 20 hr or left untreated. Cells were challenged with 1 mM glutamate, and intracellular peroxide formation was determined after 6 hr using DCF stainings, as described in Experimental Procedures. After an additional 14 hr, parallel cultures were stained for dead cells, using PI at 5 μg/ml, as previously reported (12). Magnification of PC, PI, and DCF images is 100$\times$.

TABLE 1

Protection of hippocampal neurons against oxidative stress-induced cell death by different estrogens

HT22 cells and primary cortical neurons were incubated with the various estrogens, and testosterone for 20 hr at 10 μM before the indicated toxins were added. After 6 hr, the intracellular formation of H_2O_2 and related peroxides was determined using DCF fluorescence. Parallel cultures were treated for an additional 14 hours. At that time, either the reduction of MTT as a measure of cellular viability (9, 11, 12) or trypan blue exclusion and cell countings as a measure of cell lysis (11, 12) was performed. The data in parentheses refer to the treatment of the cells with H_2O_2. Steroids did not interfere with the colorimetric MTT assay. All results were normalized to control values (no addition of reagent) as 100. The data of the survival assays are presented as means $\pm$ standard error of five independent experiments. Peroxide formation as detected with DCF fluorescence is expressed as percent fluorescent cells. Presented data are the means $\pm$ standard error of five independent experiments. Comparisons were made by ANOVA followed by a Scheffe's *post hoc* test.

Reagents	Concentration	MTT	Cell count	Peroxide
	μM			
HT22 cells				
Control		100	100	100
Glutamate (or H_2O_2)	1000 (60)	9 ± 7 (15 ± 4)	16 ± 5 (19 ± 4)	168 ± 4
Estrone	10	91 ± 6 (74 ± 2)[a]	96 ± 3 (91 ± 6)[a]	107 ± 4[b]
17-α Estradiol	10	86 ± 3 (63 ± 6)[a]	92 ± 5 (89 ± 5)[a]	105 ± 3[b]
17-β Estradiol	10	91 ± 6 (66 ± 5)[a]	90 ± 6 (93 ± 5)[a]	108 ± 4[b]
Estriol	10	80 ± 6 (80 ± 2)[a]	95 ± 9 (96 ± 2)[a]	103 ± 2[b]
Ethinyl estradiol	10	85 ± 2 (65 ± 4)[a]	83 ± 5 (90 ± 5)[a]	110 ± 4[b]
Mestranol	10	5 ± 3 (21 ± 5)	6 ± 7 (14 ± 4)	164 ± 2
Quinestrol	10	3 ± 5 (10 ± 3)	5 ± 4 (17 ± 5)	165 ± 2
2-OH Estradiol	10	78 ± 6 (85 ± 5)[a]	74 ± 6 (69 ± 4)[a]	105 ± 4[b]
4-OH Estradiol	10	83 ± 5 (46 ± 4)[a]	82 ± 6 (73 ± 4)[a]	107 ± 4[b]
Testosterone	10	6 ± 4 (11 ± 7)	5 ± 4 (13 ± 5)	160 ± 5
Primary cortical neurons				
Control	100	100	100	100
BSO (or H_2O_2) alone	500 (30)	39 ± 4 (43 ± 3)	50 ± 5 (55 ± 2)	145 ± 4
Estrone	10	85 ± 4 (69 ± 5)[a]	88 ± 2 (72 ± 2)[a]	105 ± 5[b]
17-α Estradiol	10	82 ± 3 (90 ± 3)[a]	86 ± 4 (90 ± 5)[a]	104 ± 6[b]
17-β Estradiol	10	89 ± 5 (95 ± 4)[a]	89 ± 4 (88 ± 4)[a]	106 ± 4[b]
Estriol	10	103 ± 6 (76 ± 4)[a]	93 ± 5 (75 ± 2)[a]	104 ± 5[b]
Ethinyl estradiol	10	96 ± 4 (70 ± 4)[a]	92 ± 4 (70 ± 4)[a]	108 ± 4[b]
Mestranol	10	34 ± 5 (33 ± 7)	42 ± 8 (42 ± 6)	155 ± 6
Quinestrol	10	41 ± 6 (35 ± 6)	49 ± 6 (40 ± 9)	144 ± 4
2-OH Estradiol	10	78 ± 4 (74 ± 2)[a]	84 ± 5 (82 ± 2)[a]	105 ± 7[b]
4-OH Estradiol	10	90 ± 6 (88 ± 4)[a]	83 ± 3 (84 ± 1)[a]	106 ± 4[b]
Testosterone	10	31 ± 6 (53 ± 5)	39 ± 5 (54 ± 7)	154 ± 5

[a] $p < 0.01$.
[b] $p < 0.05$.

effect, 3) the addition of actinomycin D as the inhibitor of RNA synthesis did not block the protective effect (data not shown), 4) HT22 cells that lack endogenous estrogen receptors can be protected (12), and 5) 17-α-estradiol, which does not bind to estrogen receptors, is also neuroprotective.

Incubation of primary cortical neurons with Aβ and BSO, and treatment of HT22 cells with glutamate induced an intracellular accumulation of H_2O_2 and related peroxides (11) that can be detected with DCF stainings after 6 hr. This increase could be blocked by the preincubation of cells with different estrogens, as shown qualitatively in Fig. 3 and quantitatively in Table 1. Peroxides are precursors of the highly reactive, lipid-peroxidizing hydroxyl radical (19). Estrogens with an intact 3-OH group on the A ring significantly reduced the percentage of fluorescent cells after the glutamate challenge ($p < 0.05$), whereas quinestrol, mestranol, and testosterone were inactive and could not block these intracellular oxidative events, which is consistent with a lack of protective activity (Fig. 3; Table 1).

The hippocampus is a major target of neuronal cell death in neurodegenerative disorders such as AD (23). Organotypic hippocampal slice cultures from postnatal rats preserve the intrinsic connections and regional differentiation specific to the hippocampus *in vivo* and are frequently used for the investigation of neurotoxins (24). These cultures were established and pretreated with the different estrogens for 20 hr, followed by a strong oxidative challenge with 250 μM H_2O_2 for 24 hr that induced massive neuronal death in the gyrus

dentatus region and the cornu ammonis region (Fig. 4A). The different 3-OH–containing estrogens protected the neuronal cells in the hippocampal slice, as exemplified by ethinyl estradiol, whereas its ether-modified derivatives quinestrol, mestranol, and testosterone were not protective (Fig. 4; data not shown).

In neuronal systems, estrogens can exert long term trophic actions and can stimulate the secretase metabolism of the amyloid β precursor protein via transcriptional activation after the binding of the estrogen receptor to estrogen-responsive elements on the DNA (25–27). These genomic actions of estrogen could potentially affect the AD risk. We report that estrogen and estrogen derivatives within the hydroxyl group in the C3 position on the A ring of the steroid molecule can also act as powerful neuroprotectants in an estrogen-receptor–independent short term manner because of to their antioxidative capacity. The concentrations required for a significant antioxidative neuroprotection in our *in vitro* system are higher than the estrogen levels that occur naturally *in vivo* but are consistent with those that have been previously shown to have antioxidant activity in different cellular and cell-free systems (6, 12). Plasma concentrations of 17-β-estradiol are in the nanomolar range, depending on sex and menopausal status (28). Interestingly, 17-β-estradiol has been shown to be a more potent antioxidant inhibitor (IC$_{50}$ = 21 μM) of iron-catalyzed lipid peroxidation in rat brain homogenates than vitamin E (IC$_{50}$ = 30 μM) (29); the latter is currently being tested in a clinical AD trial. Nevertheless, as
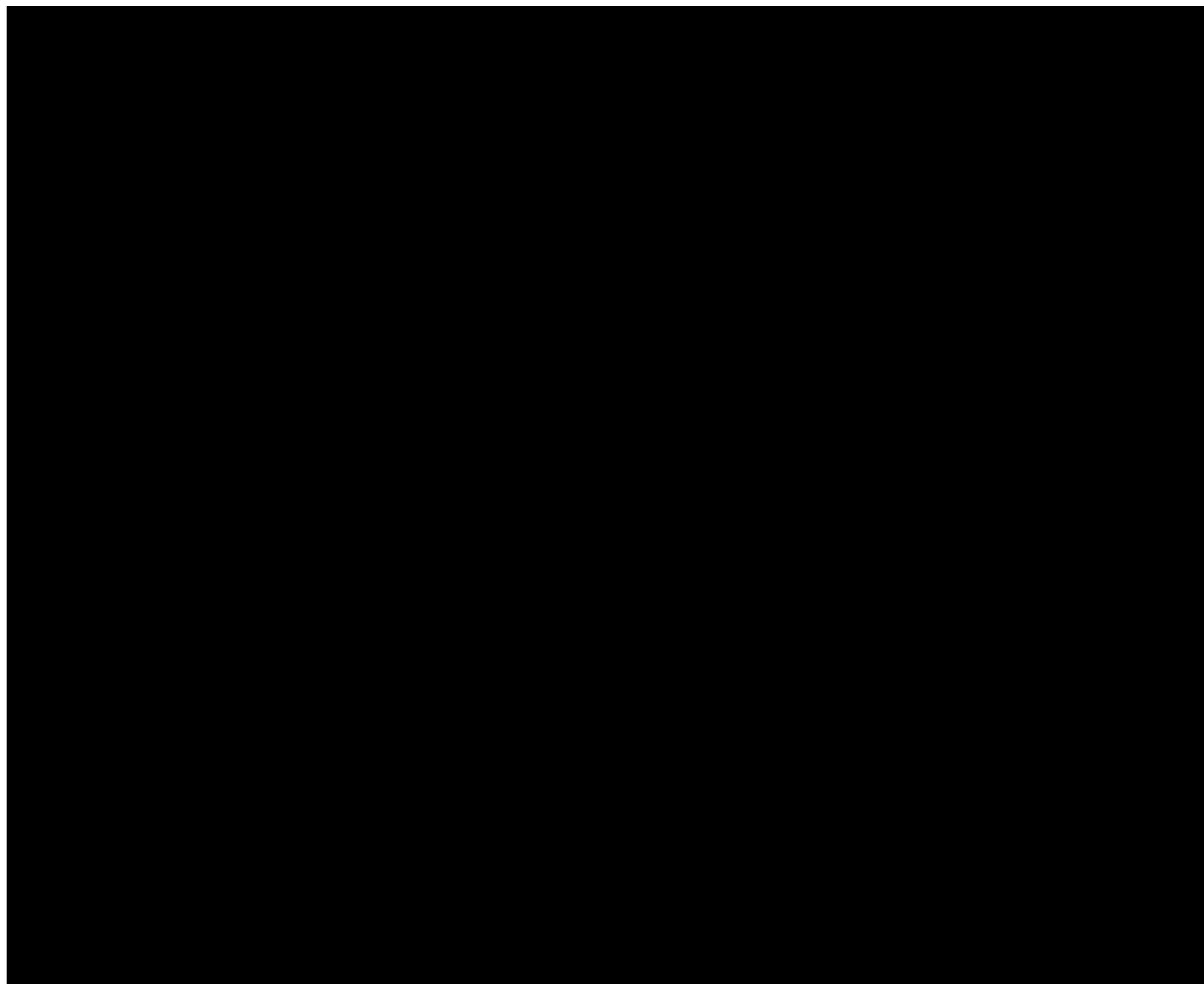
Fig. 4. Ethinyl estradiol prevents H_2O_2-induced oxidative cell death in organotypic hippocampal slice cultures. Organotypic hippocampal slices were pretreated for 24 hr with either 10 μM mestranol (B), quinestrol (C) (both of which had ether-modified 3-OH groups on the A ring), or ethinyl estradiol (D) (intact 3-OH group on the A ring). A, A slice challenged with H_2O_2 without further pretreatment. Then, 250 μM H_2O_2 was added to the slices; after 24 hr, cell death was determined by using PI (5 μg/ml). Images were obtained by using a fluorescence microscope (40×) and UV filters. *Red*, PI-positive (dead) cells. *Arrows*, gyrus dentatus region; *CA*, cornuammonis region.

shown for the steroid compound RU486 after oral administration, micromolar steroid concentrations can be attained *in vivo* (30).

It is of great significance that the neuroprotective effect against oxidative stressors in the different neuronal *in vitro* systems is also afforded by the nonestrogenic 17-α-estradiol, a compound that is biologically inactive with respect to binding to the estrogen receptor (20). In summary, our results pin down the neuroprotective activity of estrogens to the presence of the 3-OH group of the A ring in the steroid molecule and may therefore serve as a basis for the design and synthesis of other nonestrogenic antioxidants. One example could be the synthesis of estrogens with bulky alkyl substituents in both the 2- and 4-position on the A ring that do not bind to the estrogen receptor but that nevertheless exert an antioxidant potential (31).

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