0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(11):5080–5086

doi: 10.1210/en.2004-0973

Glia Mediates the Neuroprotective Action of Estradiol on β-Amyloid-Induced Neuronal Death

M. A. SORTINO, M. CHISARI, S. MERLO, C. VANCHERI, M. CARUSO, F. NICOLETTI, P. L. CANONICO, AND A. COPANI

Departments of Experimental and Clinical Pharmacology (M.A.S., M.Ch., S.M.), Internal Medicine (C.V., M.Ca.), and Pharmaceutical Sciences (A.C.), University of Catania, 95125 Catania, Italy; DISCAFF, University of Piemonte Orientale (P.L.C.), 28100 Novara, Italy; Department of Human Physiology and Pharmacology, University of Rome La Sapienza (F.N.), 00185 Rome, Italy; and INM Neuromed (F.N.), 86077 Pozzilli, Italy

17β-Estradiol (17β-E_2) is known to exert neuroprotective activity against β-amyloid, but its exact target and mechanism of action in this effect have not been elucidated. The involvement of astroglia in neuroprotection of 17β-E_2 against the β-amyloid fragment [$\beta AP_{(25–35)}$] has been evaluated using an experimental paradigm in which medium conditioned from rat astroglia pretreated with 17β-E2 was transferred to pure rat cortical neurons challenged with 25 μM $\beta AP_{(25–35)}$ for 24 h. The toxicity of $\beta AP_{(25–35)}$ was assessed by flow cytometry, evaluating the ability of the peptide to induce an aberrant mitotic cell cycle in neurons. The results obtained indicate that conditioned medium from astrocytes preexposed to 17β-E_2 for 4 h increased the viability of cortical neurons treated with $\beta AP_{(25–35)}$. This effect was not modified by treatment with the estrogen receptor antagonist ICI 182,780, added directly to neurons, nor was it mimicked by direct addition of 17β-E_2 to neuronal cultures during exposure to $\beta AP_{(25–35)}$. A soluble factor stimulated by 17β-E_2 seemed to be involved, and accordingly, the intracellular and released levels of TGF-β1 were increased by 17β-E_2 treatment, as established by Western blot analysis. In addition, the intracellular content of TGF-β1 in immunopositive cells, as detected by flow cytometry, was reduced, suggesting that 17β-E_2 stimulated mainly the release of the cytokine. In support of a role for TGF-β1 in astrocyte-mediated 17β-E_2 neuroprotective activity, incubation with a neutralizing anti-TGF-β1 antibody significantly modified the reduction of neuronal death induced by 17β-E_2-treated astrocyte-conditioned medium. (*Endocrinology* 145: 5080–5086, 2004)

AFTER THE FIRST clinical report by Paganini Hill and Henderson (1), considerable evidence has accumulated over the years supporting the inverse relationship between exposure to estrogen and Alzheimer's disease (AD) (reviewed in Refs. 2 and 3). Moving from that and on the bases of the well known neuroprotective effects exerted by estrogen under different *in vitro* conditions that mimic *in vivo* pathological states (2, 3), attention has been focused on the effects of estrogen on neuronal damage induced by β-amyloid peptide (βAP) that is known to accumulate in the brain and to be involved in the pathogenesis of AD. Although results obtained in clinical trials evaluating the efficacy of hormone replacement therapy in AD have failed to provide definite positive results, the enormous body of evidence demonstrating the neuroprotective activity of estrogen are strongly supportive of its potential beneficial use in neurodegenerative conditions. Certainly a deeper knowledge of the still undisclosed mechanisms underlying the neuroprotective activity of estrogen will help in the attempt to translate the positive effects observed *in vitro* in clinical efficacy.

Estrogen acts by multiple mechanisms. It is known that micromolar concentrations of estrogen are endowed with antioxidative properties (4–7), but classical genomic (8, 9) or alternative, rapid, nongenomic responses (10–12) also seem to be involved in the neuroprotective effects of estrogen against βAP toxicity.

Astroglia represents a main target for estrogen in the brain; it expresses estrogen receptors α (ERα) and ERβ (13) and plays a key role in estrogen-induced developmental processes, such as synapse formation, plasticity (14), and neuronal morphology (15). A role for astrocytes in estrogen-induced neuroprotection has also been invoked (16), based mainly on the ability of estrogen to modulate astrocyte activation that occurs after brain lesion (14) and on the property of astrocytes to produce trophic factors (17, 18). In this regard, it appears particularly intriguing that an up-regulation of ERα and/or ERβ occurs in astrocytes under conditions of neuronal damage, including AD (19, 20), suggesting a prominent role for astrocytes in the estrogen effects that occur after injury.

Multiple mechanisms have been involved in the neurotoxic effect of βAP, and increasing evidence supports a tempting mitotic activity of the peptide that cannot be achieved in neurons, because of their nature of postmitotic cells, leading to their death. Thus, in pure cultures of rat cortical neurons, βAP induces an abortive mitotic cycle, assessed by the entrance of neurons into the S phase of the cycle and the expression of specific cell cycle proteins (21). Based on this, compounds with differentiating and/or cell cycle-inhibiting activity have been suggested as potential neuroprotective drugs (21, 22).

Abbreviations: AD, Alzheimer's disease; βAP, β-amyloid peptide; 17β-E_2, 17β-estradiol; ER, estrogen receptor; MFI, mean fluorescence intensity; MTT, 3-[4,5-dimethlthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

By exploiting the ability of βAP to induce a mitotic cell cycle, in the present study we have evaluated whether estrogen protects cortical neurons from the neurotoxic effect of this peptide and whether astrocytes can take part in the estrogen neuroprotective activity.

Materials and Methods

Primary astrocyte culture

Cortical glial cells were prepared from 1- to 3-d-old Sprague-Dawley rats (Morini, Reggio Emilia, Italy). All animal experimental procedures were carried out in accordance with the directives of the Italian and European Union regulations for the care and use of experimental animals (DL116/92) and were approved by the Italian Ministry of Health. In brief, after removal of meninges and isolation of cortexes, cells were dispersed by mechanical and enzymatic dissociation using a 0.25% solution of trypsin (Invitrogen Life Technologies, Inc., Milan, Italy) in PBS. Cells were plated onto 75-mm^2 flasks and maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml)/streptomycin (100 μg/ml), and glutamine (2 mM). All medium constituents were obtained from Invitrogen Life Technologies, Inc., and all plastic materials were purchased from Corning Life Sciences (Acton, MA). Confluent cultures at 8–10 d *in vitro* were shaken overnight at 37 C to remove microglia and oligodendrocytes. Astrocytes were collected by trypsin digestion, seeded onto 35- or 100-mm dishes, and used for experiments 6–8 d after replating.

Pure culture of cortical neurons

Cultures of pure cortical neurons were obtained from rats on embryonic d 15 (Morini) and prepared according to a procedure previously described (21). Briefly, cortexes were dissected in Ca^{2+}/Mg^{2+}-free buffer and mechanically dissociated. Cortical cells were plated at a density of 2×10^6 cells/dish on 35-mm dishes (Nunc, Rochester, NY) precoated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich Corp., Milan, Italy) in DMEM/Ham's F-12 (1:1) medium supplemented with the following components: 10 mg/ml BSA, 10 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 2 mM glutamine, 6 mg/ml glucose (all from Sigma-Aldrich Corp.), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cytosine-D-arabinofuranoside (10 μM) was added to the cultures 18 h after plating to avoid the proliferation of nonneuronal elements and was kept for 3 d before medium replacement. This method yields greater than 99% pure neuronal cultures, as judged by immunocytochemistry for glial fibrillary acidic protein and flow cytometry for neuron-specific microtubule-associated protein 2 (21).

βAP, drugs, and antibodies

The βAP fragment 25–35 [$\beta AP_{(25–35)}$] was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Different lots of $\beta AP_{(25–35)}$ were tested, and the same batch was used throughout the study to rely on a consistent profile of toxicity. Peptides were solubilized in sterile, double-distilled water at an initial concentration of 2.5 mM and were stored frozen at −20 C. $\beta AP_{(25–35)}$ was used at a final concentration of 25 μM in the presence of the glutamate receptor antagonists MK-801 (10 μM) and 6,7-dinitroquinoxaline-2,3-dione (30 μM) to avoid the potentiation of endogenous glutamate toxicity.

17β-Estradiol (17β-E_2; Sigma-Aldrich Corp.) and ICI 182,780 (Tocris Cookson Ltd., North Point, UK) were dissolved in ethanol and dimethylsulfoxide, respectively, at an initial concentration of 10 mM. Subsequent dilutions were made in aqueous solutions.

The antibodies used were rabbit anti-ERα and anti-ERβ (Zymed Laboratories, Inc., South San Francisco, CA), mouse anti-TGF-β1 (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), mouse anti-TGF-β1 neutralizing antibody (Chemicon International, Temecula, CA), and mouse anti-β-actin (Sigma-Aldrich Corp.). All secondary antibodies were obtained from Santa Cruz Biotechnologies, Inc.

Apoptosis and cell cycle analysis

Mature cortical neurons at 7–8 d *in vitro* were harvested with 0.25% trypsin, fixed in 70% ethanol, and stored at −20 C until use. After repeated washing, cells were incubated for 1 h with 100 μg/ml ribonuclease and stained with 50 μg/ml propidium iodide (both from Sigma-Aldrich Corp.). DNA content and ploidy were assessed by a Coulter Elite Flow Cytometer. The prediploid peak, indicative of fragmentation and reduced content of DNA, was evaluated as an index of apoptosis. The Multicycle AV software program was used to analyze cell cycle distribution profiles on diploid and tetraploid populations.

Flow cytometry

Astrocytes were detached, fixed for 30 min in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. After blocking with BSA for 30 min, cells were incubated with a rabbit anti-TGF-β1 antibody (1:300) at room temperature for 60–90 min. After washing, a fluorescein isothiocyanate-conjugated antirabbit secondary antibody (1:200) was added for 1 h at room temperature, and samples were analyzed by flow cytometry.

Western blot analysis

Cultures were harvested at 4 C in 10 mM Tris lysis buffer containing 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 25 μg/ml leupeptin, and 0.5% aprotinin (all from Sigma-Aldrich Corp.). After sonication, an aliquot of the samples was processed for protein concentrations according to the method of Bradford. Samples were diluted in sample buffer and boiled for 5 min. Electrophoresis was performed in 8–12% SDS-PAGE (40 mA/h) using 60–80 μg cell proteins/lane. After separation, proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences Europe GmbH, Milan, Italy) for 2 h at room temperature using a Transblot semidry transfer cell. After blocking, the membranes were incubated with rabbit anti-ERα (1:100), anti-ERβ (1:500), and mouse anti-TGF-β1 (1:300) overnight at 4 C. Membranes were then thoroughly washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Specific bands were visualized using the SuperSignal chemiluminescent detection system (Pierce Chemical Co., Rockford, IL).

3-[4,5-Dimethlthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

MTT (Sigma-Aldrich Corp. 0.9 mg/ml final concentration) was added to the cell culture for 2 h at 37 C. The solubilization solution, containing acidified isopropanol and 20% sodium dodecyl sulfate, was added and left for 20 min to extract the produced formazan, which was then evaluated in a plate reader (absorbance, 560 nm).

Statistical analysis

All data were analyzed by one-way ANOVA, followed by Bonferroni *t* test for significance. $P < 0.05$ was taken as the criteria for statistical significance.

Results

All studies were carried out using an experimental model in which cortical neurons were exposed to $\beta AP_{(25–35)}$ in the presence of medium conditioned by astrocytes that had been pretreated with 17β-E_2. By applying this paradigm, neuronal cultures were never exposed to 17β-E_2, but only to conditioned medium from glia previously treated with the hormone. Confluent astrocyte cultures grown in DMEM and 10% fetal calf serum were shifted into serum-free DMEM/F-12 supplemented as described in *Materials and Methods* and exposed to 10 nM 17β-E_2 for different lengths of time (4–24 h). An equal amount of ethanol, used as a vehicle for 17β-E_2, was added to control cultures. The shift into this medium was necessary to carry out both preincubation and final incubation of astrocytes with medium suitable for neuronal cultures to be exposed to βAP (chemically defined, serum-free medium). After this time, cultures were thoroughly

washed (twice, 7.5-min wash in serum-free DMEM/F-12), medium was replaced with fresh DMEM/F-12 and after 24 h was transferred to the neuronal culture, to which 25 μM $\beta AP_{(25–35)}$ was added, 30 min later, for the following 24 h. The concomitant presence of glutamate receptor antagonists when neurons were challenged with $\beta AP_{(25–35)}$ excluded potentiation of toxicity by endogenously released glutamate (21).

Exposure of cortical neurons to conditioned medium from glia previously treated with 10 nM 17β-E_2 reduced by about 50% the occurrence of apoptosis in response to 25 μM $\beta AP_{(25–35)}$ (Fig. 1A). Apoptosis was measured as the percentage of a prediploid population at cell cycle analysis by flow cytometry. This effect was consistently observed when glia was treated for 4 h with 17β-E_2 and then shifted to 17β-E_2-free medium for additional 24 h (Fig. 1A). Similar results were observed when astrocytes were exposed to the hormone for longer periods of time (8–12 h; not shown), but in all subsequent experiments the 4- plus 24-h protocol was chosen. The protective effect of 17β-E_2-treated astrocytes finds support in MTT reduction assay that demonstrated a similar 40–50% inhibition of the toxic effect induced by $\beta AP_{(25–35)}$ (Table 1). The reduction of the apoptotic population at flow cytometry was accompanied by changes in the percentage of neurons in the G_0/G_1 phase of the cycle and a parallel reduction of the S phase population induced by $\beta AP_{(25–35)}$ (Fig. 1B).

To ascertain whether the protective effect of 17β-E_2-conditioned glial medium was due to transferring of estradiol, either exogenously added or produced by astrocytes, the same experiment was carried out in the presence of 1 μM of the ER antagonist ICI 182,780, added directly to neuronal cultures during the 24-h exposure to $\beta AP_{(25–35)}$. However, under these conditions, the protective activity of glia-conditioned medium was fully maintained, and ICI 182,780 *per se* appeared devoid of any effect (Fig. 1C). To strengthen further the lack of a direct effect of estrogen on the neuronal population, when 17β-E_2 was added directly to cortical neurons that express ERα, but not ERβ (Fig 2A), no significant protective effect against $\beta AP_{(25–35)}$ toxicity was observed by either MTT reduction (not shown) or appearance of a prediploid population (Fig. 2B), and there was no change in the cell cycle distribution profiles (Fig. 2C). Furthermore, addition of 17β-E_2 to cortical neurons exposed to medium from astrocytes pretreated with 17β-E_2 did not increase the protective effect produced by glia-conditioned medium (Fig 2D).

Searching for a soluble factor released by 17β-E_2-stimulated astrocytes, attention has been focused on TGF-β1. When astrocyte cultures were exposed to 17β-E_2 for 4 h and then shifted to fresh medium for an additional 12 h, an increased expression of TGF-β1 was evident by Western blot (Fig. 3A). The antibodies used usually detected a single band of about 30 kDa corresponding to the dimer of TGF-β1 protein (Fig. 3A), but occasionally a smaller band of 14 kDa was present (not shown). Both isoforms of TGF-β1 were increased after treatment with 17β-E_2. A pronounced increase after 17β-E_2 treatment was detected also in the levels of TGF-β1 protein collected in the incubation medium (Fig. 3B). Interestingly, analysis of TGF-β1 levels by flow cytometry revealed a peculiar temporal profile, with a reduction of the intracellular content, measured as mean fluorescence intensity (MFI), of the growth factor (18 and 24 h after the 4-h incubation in the presence of 17β-E_2; Fig. 3C). To ascertain the

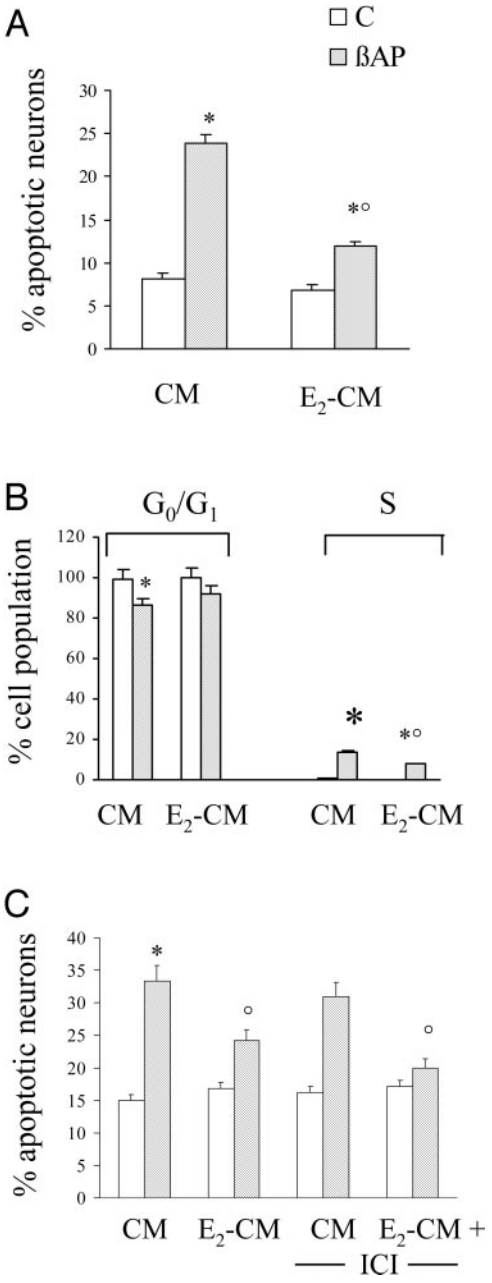

FIG. 1. Neuroprotective effect of conditioned medium from 17β-E_2-treated astrocytes on neuronal death (A and C) and induction of cell cycle (B) by $\beta AP_{(25–35)}$ in pure cortical neurons. Apoptosis induced by 25 μM $\beta AP_{(25–35)}$, evaluated as the percentage of a prediploid population at cell cycle analysis (A), was significantly reduced in cultures exposed to astrocyte 17β-E_2-conditioned medium (E2-CM) compared with untreated glia-conditioned medium (CM). B, The distribution profiles of neurons exposed to $\beta AP_{(25–35)}$ significantly differed in the presence (E2-CM) or absence (CM) of 17β-E_2-conditioned medium. The protective effect was not modified by the ER antagonist ICI 182,780 (1 μM), added directly to neurons during the 24-h exposure to $\beta AP_{(25–35)}$ (C). Data are the mean ± SE of three to five independent studies, each run in triplicate. *, $P < 0.05$ *vs.* respective control; °, $P < 0.05$ *vs.* CM.

role of TGF-β1 in glia-mediated neuroprotection against $\beta AP_{(25–35)}$ toxicity, neuronal viability was evaluated in the presence of a neutralizing anti-TGF-β1 antibody. Under these conditions, both MTT viability assay (Fig. 4A) and analysis of a prediploid population by flow cytometry (Fig. 4B) revealed a significant reduction of the 17β-E_2-mediated protective effect.

Discussion

A protective effect of 17β-E_2 against β-amyloid-induced cell death has been demonstrated in different neuronal cell lines as well as in primary neuronal cultures (4, 6–8, 11, 12, 23–28). Besides those conditions in which the antioxidative activity of estrogen was proven (4, 5, 7), often neuronal cell lines were used (6, 9, 10, 26–28), and some times they were made responsive to estrogen by transfection of ERs (8, 11); in addition, primary neuronal cultures were often mixed cell cultures, also including a glial cell population (11, 12). We demonstrate in this study that astroglia mediates the neuroprotective effects of 17β-E_2 against $\beta AP_{(25–35)}$-induced death in cortical neurons. Exposure of the neuronal culture to conditioned medium from 17β-E_2-pretreated astrocyte in fact reduced $\beta AP_{(25–35)}$-induced neuronal death and significantly modified the distribution profiles of neuronal cells within the cell cycle. The extent of protection observed and the modifications of cell cycle distribution were consistent with our previous data (21) obtained with cell cycle inhibitors and differentiating agents that induce cell cycle arrest in proliferating cells (29).

TABLE 1. Neuroprotective effect of conditioned medium from astrocytes previously exposed to 10 nM 17β-E_2 on the viability of pure cortical neurons exposed to 25 μM $\beta AP_{(25–35)}$ for 24 h

Treatment	MTT reduction (% of control)	
	C	$\beta AP_{(25–35)}$
Vehicle	100 ± 2.3	37 ± 4.9[a]
17β-E_2	109 ± 8.2	73 ± 5.4[a,b]

Data are the mean ± SE of four independent experiments, each performed in triplicate.
[a] $P < 0.05$ *vs.* respective control.
[b] $P < 0.05$ *vs.* vehicle plus $\beta AP_{(25–35)}$.

The effect of 17β-E_2 on astrocyte cultures seems to be related to interaction with classical ERα and ERβ that are expressed in cortical glial cells, as determined from our own results obtained by RT-PCR and immunocytochemistry (not shown) and from previous observations (13). The maintenance of the protective effect of conditioned medium from glia previously exposed to 17β-E_2 in the presence of the ER antagonist ICI 182,780 ruled out the possibility that hormone still present in the culture medium was acting through classical genomic mechanisms on cortical neurons. This experimental paradigm proved also that estrogen, produced by astrocytes through aromatase activity, as previously suggested (30), was not exerting its protective action by activating IC1–182,780-sensitive pathways on neurons. These results together with the lack of effect of 17β-E_2 added directly to neurons completely excluded the involvement of estrogen in the neuroprotection obtained with 17β-E_2-astrocyte-conditioned medium.

We found that direct treatment of cortical neurons with 17β-E_2 did not reduce or prevent neuronal death induced by $\beta AP_{(25–35)}$, nor did it modify induction of cell cycle by the peptide. Although estrogen has been shown to exert direct neuroprotective activity in neurons under several different experimental conditions of neurodegeneration (reviewed in Ref. 2), Harms *et al.* (31) have shown that the neuroprotective activity of 17β-E_2 against an apoptotic stimulus can reveal region specificity, occurring in hippocampal and septal, but

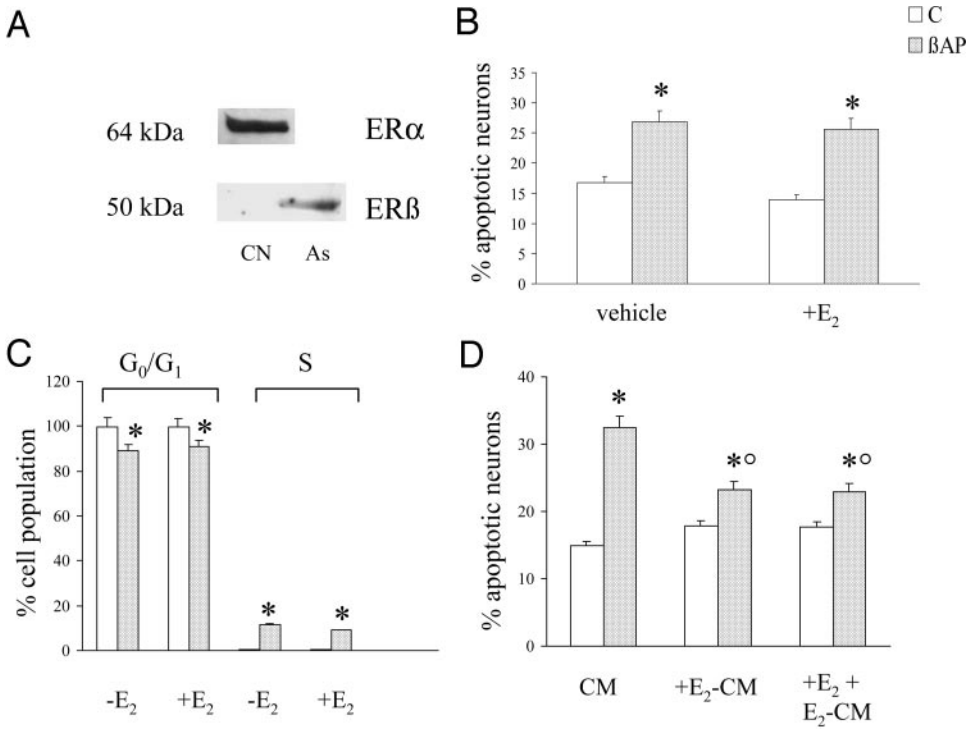

FIG. 2. Lack of effect of 17β-E_2 added directly to cortical neurons on cell death (B) and induction of cell cycle (C) by $\beta AP_{(25–35)}$. ERα, but not ERβ (*left lane*), are expressed in cortical neurons in culture (A). Proteins from astrocytes (*right lane*) were used as a positive control for detection of a specific ERβ band (A). B and C, 17β-E_2 (10 nM; +E_2) added to neuronal cultures 30 min before treatment with 25 μM $\beta AP_{(25–35)}$ failed to protect cortical neurons against $\beta AP_{(25–35)}$-induced apoptosis (B) and changes of cell cycle distribution (C). The protective effect induced by transferring of astrocyte 17β-E_2-conditioned medium (E2-CM) was not modified by addition of 17β-E_2 directly to the neuronal culture 30 min before 25 μM $\beta AP_{(25–35)}$. (D). Data are the mean ± SE of three or four independent studies. *, $P < 0.05$ *vs.* control; °, $P < 0.05$ *vs.* CM.

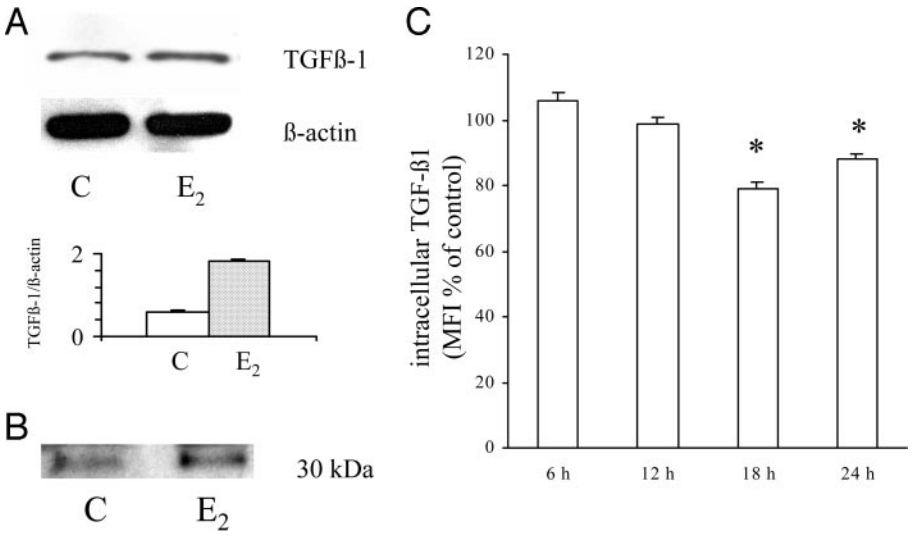

FIG. 3. Western blot analysis of TGF-β1 content in astrocytes (A) and in the incubation medium (B) of astrocytes exposed to 10 nM 17β-E_2 for 4 h and shifted to fresh medium for an additional 18 h. A dimer of about 30 kDa is visible. Normalization for protein loading was performed by blotting the same membrane with anti-β-actin (1:250). B, Protein loading was checked by staining the transferred proteins with Ponceau's solution. C, Time-course analysis by flow cytometry of the mean intracellular content (MFI) of TGF-β1 expression at different times after exposure of astrocytes to 17β-E_2 for 4 h. Data are the mean ± SE of three different experiments carried out in triplicate and are expressed as a percentage of the respective control value. *, $P < 0.05$ *vs.* respective control.

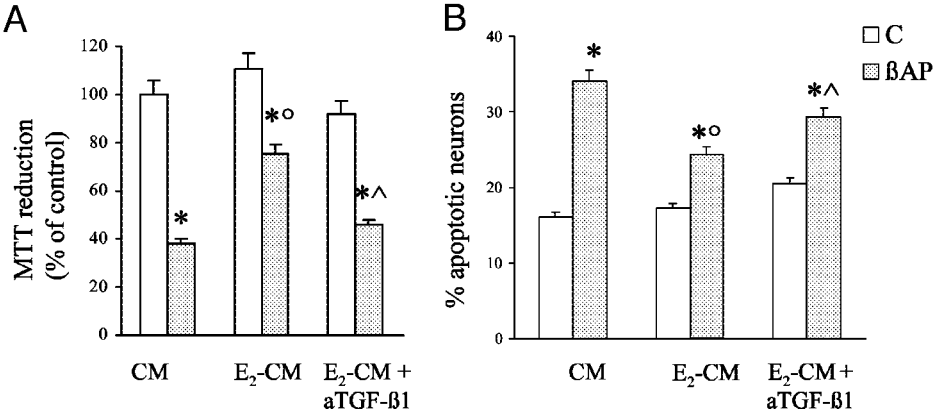

FIG. 4. Effect of a neutralizing anti-TGF-β1 antibody on the neuroprotective activity of 17β-E_2 astroglia-conditioned medium. Incubation medium from astrocytes previously exposed to 17β-E_2 (E_2-CM) was transferred to pure cortical neurons before treatment with $\beta AP_{(25–35)}$ (25 μM) and anti-TGF-β1 neutralizing antibody (5 μg/ml). After 24 h, neurons were processed for MTT reduction assay (A) or flow cytometric analysis for cell cycle distribution profiles (B). Data are the mean ± SE from three experiments, each performed in duplicate. *, $P < 0.05$ *vs.* respective control; °, $P < 0.05$ *vs.* CM; ^, $P < 0.05$ *vs.* E_2-CM.

not in cortical, neuronal cultures. Furthermore, it appears extremely important that in cortical neurons, as opposed to hippocampal and septal cultures, estrogen treatment failed to increase Bcl-2 levels (31) and that the comparative evaluation of ER expression in neuronal cultures from the three cerebral regions revealed a prevalence of neurons expressing low levels of ERα in the cortex (31).

With regard to the specific βAP toxicity in pure cultures of cortical neurons, very little information is currently available. A weak protective activity of estrogen against βAP toxicity, with the steroid added directly into the neuronal culture, has, in fact, been demonstrated (23). The discrepancy with our results can certainly be due to the different experimental conditions used, including methods applied for the assessment of cell death, maturation of neurons *in vitro*, slight differences in the cell cultures, *etc.* In both our and Cordey' studies (23), a short-term preincubation with 17β-E_2 was carried out. It has to be recognized that occasionally, neuroprotection by 17β-E_2 requires a longer incubation time. However, neuroprotection by 17β-E_2 is known to involve a rapid response to 17β-E_2, *i.e.* activation of the MAPK cascade (32) and, specifically, reduction of βAP-induced neuronal death by 17β-E_2 in HT22 cells transfected with ERα and/or ERβ has been related to activation of this intracellular pathway (11). Interestingly, in hippocampal and cortical neurons as well as in neuronal cell lines (33, 34), βAP has been shown to activate MAPK, and we have recently shown that in SH-SY5Y neuroblastoma cells, activation of MAPK is critical for induction of cell cycle and ensuing neuronal death by $\beta AP_{(25–35)}$ (35). Thus, in pure cortical neurons challenged with βAP, the protective activity of estrogen may be masked by the concurrent activation of a common intracellular pathway by $\beta AP_{(25–35)}$ in its attempt to initiate a mitotic cell cycle that precedes neuronal death.

Interestingly, the lack a of neuroprotective effect by 17β-E_2 has been reported in pure cortical neurons challenged with different toxic insults (16). More defined is the protective effect of estrogen against toxic insults in neuronal cultures grown in the presence of glial cells (16). In support of this, we demonstrate in this study that astrocytes can, in fact, mediate the protective effect of 17β-E_2 against $\beta AP_{(25–35)}$. As a soluble factor released by astrocytes seemed to be involved in this effect, attention has been focused on TGF-β1, because it might represent the ideal link among estrogen, glial cells, and neuroprotection against $\beta AP_{(25–35)}$-induced neuronal death. The evidence indicates that 1) 17β-E_2 increases the expression of this cytokine in different tissues (36, 37) and also in astrocytes (17); 2) TGF-β1 is primarily involved in the cross-talk between astrocytes and neurons (38–40); 3) TGF-β1 protects neurons from death (21) and may signifi-

cantly contribute to astrocyte-mediated neuroprotection (41); and 4) TGF-β1 is specifically involved in the neuroprotective effect of estrogen (16). With regard to the specific role of TGF-β1 in AD, conflicting data are available. Despite the protective activity demonstrated for this cytokine (42), the ability of TGF-β1 to enhance amyloid deposits *in vivo* and *in vitro* has also been reported (43). We now demonstrate that in pure cortical neurons, TGF-β1 is primarily involved in the astrocyte-mediated protection of 17β-E_2 against neuronal death induced by $\beta AP_{(25–35)}$. Interestingly, analysis of the intracellular content of TGF-β1, carried out by Western blot and flow cytometry, revealed conflicting results. The slight increase in TGF-β1 expression detected by Western blot analysis contrasted with the reduction in the MFI of TGF-β1 labeling that is indicative of the mean content of the cytokine in immunopositive cells. Thus, it seems that 17β-E_2 treatment induced an increased synthesis of TGF-β1, but, more importantly, it favored the release of the cytokine, as suggested by the reduced intracellular content of the cytokine in labeled cells. This finds support in the increased levels of TGF-β1 measured in the incubation medium. The enhanced TGF-β1 released by astrocytes exposed to 17β-E_2 may thus contribute to the reduction of cell death and the induction of cell cycle induced by $\beta AP_{(25–35)}$ in pure neuronal cultures. A key supportive element in this context is the fact that TGF-β1, added directly to neurons, mimicked the action of 17β-E_2 astrocyte-conditioned medium, as found in the present study (not shown) and our previous results (21). Furthermore, treatment with a neutralizing anti-TGF-β1 antibody significantly reduced the ability of conditioned medium from 17β-E_2-treated astrocytes to reduce $\beta AP_{(25–35)}$-induced neuronal death. The fact that both conditioned medium from 17β-E_2-treated astrocytes and TGF-β1 induce only partial protection against $\beta AP_{(25–35)}$-induced neuronal death, and treatment with the neutralizing anti-TGF-β1 prevents only in part $\beta AP_{(25–35)}$ toxicity suggests that other molecules may be involved in this protective effect, but certainly identifies TGF-β1 as a key factor in this regard.

In conclusion, glial cells are necessary for 17β-E_2 to exert neuroprotective activity in cortical neurons challenged with $\beta AP_{(25–35)}$. This effect may be mediated by soluble factors released by glia in response to 17β-E_2, and TGF-β1 seems to play a major role in this regard. Intervention of glia in neuroprotection of estrogen against βAP toxicity gives additional significance to the reported overexpression of ERs in hippocampal astrocytes in AD (19, 20), indicating astrocytes as the potential specific target for estrogen action in AD.

Acknowledgments

Received July 27, 2004. Accepted August 3, 2004.

Address all correspondence and requests for reprints to: Dr. M. A. Sortino, Department of Experimental and Clinical Pharmacology, University of Catania, Viale A. Doria 6, 95125 Catania, Italy. E-mail: msortino@unict.it.

This work was supported by a grant from Italian Ministry of Health (NICS 140.1/RF00; to M.A.S.) and in part by a grant from the Alzheimer's Association (to A.C.).

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***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**