

17- β -Estradiol Induces an Inhibitor of Active Caspases

Yan Zhang,^{1,3} Omar Tounekti,³ Beverly Akerman,³ Cynthia G. Goodyer,² and Andréa LeBlanc^{1,3}

Departments of ¹Neurology and Neurosurgery and ²Pediatrics, McGill University, Montréal, Québec, Canada H3A 2T5, and ³The Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Jewish General Hospital, Montréal, Québec, Canada H3T 1E2

We have shown previously that caspase-6 activity is lethal to human neurons (LeBlanc et al., 1999; Zhang et al., 2000). Here we find that 17- β -estradiol but not 17- α -estradiol prevents caspase-6-mediated neuronal cell death. 17- β -estradiol-treated neuronal extracts directly inhibit recombinant active caspase-6, caspase-3, caspase-7, and caspase-8 *in vitro*. We conclude that 17- β -estradiol induces a caspase inhibitory factor (CIF) that is preventing neuronal apoptosis. The induction of CIF occurs within 10 min of 17- β -estradiol exposure to neurons, does not require *de novo* protein synthesis, and involves mitogen-activated protein kinase activation. The effect is antagonized by the estrogen receptor antagonist tamoxifen. In

contrast, 17- β -estradiol does not induce CIF or prevent caspase-mediated cell death in cultured astrocytes. CIF does not act through oxidation of the caspase active site. CIF activity copurifies with proteins of between 12 and 14 kDa in size. Our results indicate that 17- β -estradiol induces an inhibitor of active caspases through a receptor-mediated nongenomic pathway and provide an additional mechanism for the neuroprotective action of 17- β -estradiol that is likely highly relevant to the understanding of the role of estrogen against Alzheimer's disease.

Key words: estrogen; caspase; human primary neurons; human primary astrocytes; caspase inhibitor; Alzheimer's disease

Caspase-6 (Mch2 α) is a member of the group of cysteine-dependent aspartate-specific proteases that are critically involved in apoptotic cell death (for review, see Nicholson, 1999). We have shown that serum deprivation-mediated neuronal cell death activates caspase-6 (LeBlanc et al., 1999). In addition, caspase-6 alters amyloid precursor protein metabolism and increases production of amyloid β peptide, a major component of senile plaques in Alzheimer's disease (LeBlanc et al., 1999; Pellegrini et al., 1999). In addition, caspase-6 but not caspase-3, caspase-7, and caspase-8 induce a protracted course of selective neuronal apoptosis in human neurons (Zhang et al., 2000). Caspase-6 p10 fragments generated through activation of caspase-6 are increased in Alzheimer's disease brains and suggest that caspase-6 may play an important role in the pathogenesis of Alzheimer's disease (LeBlanc et al., 1999). Therefore, it is of interest to determine whether natural inhibitors of caspase-6 exist in these human neurons.

Neuronal inhibitors of active caspase-6 are unknown at this time. The activity of other caspases is inhibited by six different groups of inhibitors: viral inhibitors, inhibitor of apoptosis proteins (IAPs), caspase-specific decoy molecules, oxidative agents, Bcl-2 proteins, and phosphorylation (for review, see Ekert et al., 1999). Bcl-2, decoy or mimic protein inhibitors such as FADD-like ICE inhibitory protein and apoptosis repressor with caspase

recruitment domain, truncated caspase-9, Mch2 β , IAPs, and phosphorylation of caspase-9 can prevent activation of the proenzyme form of caspases. Cowpox virus product cytokine response modifier A, baculoviral protein p35, IAPs, and nitric oxide nitrosylation (Kim et al., 1997) inhibit the active caspases.

To determine whether active caspase-6 leads to an obligatory neuronal cell death or can be inhibited, we assessed various known neuroprotective agents against caspase-6-mediated cell death. In the present manuscript we show that 17- β -estradiol prevents caspase-6-mediated apoptosis and induces a caspase inhibitory factor (CIF). Some studies, but not all, show that women on hormone replacement therapy are at a lower risk for Alzheimer's disease if taken prophylactically (Henderson, 2000; Mulnard et al., 2000). Our results introduce a novel regulatory mechanism of 17- β -estradiol on caspases that has important implications for the modulation of human neuronal cell death.

MATERIALS AND METHODS

Primary cultures of neurons and astrocytes and treatments

Primary cultures of neurons were established from 12- to 14-week-old fetal brains according to ethical regulations of the Medical Research

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Correspondence should be addressed to Dr. Andréa LeBlanc, The Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, The Sir Mortimer B. Davis Jewish General Hospital, 3755 ch. Côte Ste-Catherine, Montréal, Québec, Canada H3T 1E2. E-mail: andrea.leblanc@mcgill.ca.

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Council of Canada that were approved by the McGill University Institutional Review Board. Neurons and astrocytes were cultured as described previously (LeBlanc, 1995; Zhang et al., 2000) except that cells were cultured in phenol-free MEM. In serum, testosterone is present at a final concentration of 9 pM and estrogen is present at 18 pM. All steroid hormones were obtained from Sigma (St. Louis, MO), dissolved as stock solutions in 100% ethanol, and diluted 1:1000 in culture media immediately before use. The medium was changed with fresh solution every 48 hr. Controls received an equivalent amount of ethanol.

Microinjection of recombinant caspase-6 in neurons or caspase-3 in astrocytes and measurement of cell death

Microinjections of recombinant caspases and a fluorescent marker dye, dextran Texas Red (DTR), were done as described previously (Zhang et al., 2000); neuronal cell death was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) using the Cell Death Kit I (Roche Molecular Biochemicals, Hertfordshire, UK) as described by the manufacturer. The percentage of neuronal cell death was determined by the ratio of the number of DTR-TUNEL double-positive neurons to the total number of DTR-positive neurons. The number of DTR-positive neurons did not decrease with time, indicating the retention of all apoptotic and nonapoptotic microinjected neurons on the coverslip.

Treatment of cells with cycloheximide

Cycloheximide (Sigma) was made at 1 mg/ml in distilled water and diluted at 20 μ g/ml in culture media before treatment. To test the effect of cycloheximide on the neuroprotective effect of 17- β -estradiol, neurons were microinjected with R-Csp-6 and incubated with 10 nM 17- β -estradiol in the absence or presence of cycloheximide for 48 hr. To test the effect of cycloheximide on 17- β -estradiol induction of CIF, neurons were incubated with 10 nM 17- β -estradiol in the absence or presence of cycloheximide for 6 hr.

Protein extracts of treated cells and measurement of caspase-6 inhibitory factor activity

After treatment, neuron proteins were extracted in caspase lysis buffer [50 mM HEPES, pH 7.4, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM DTT, and 0.1 mM EDTA] for 10 min on ice followed by microcentrifugation to remove insoluble material. Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Proteins (10 μ g per 100 μ l assay) were added to 10 ng of recombinant active caspase (PharMingen, San Diego, CA, or Biomol, Plymouth Meeting, PA) in caspase assay buffer (20 mM PIPES, 30 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose, pH 7.2) and 68.5 μ M acetylated (Ac)-valine-glutamic acid-isoleucine-aspartic acid (VEID)-7-amino-4-trifluoromethyl coumarin (AFC) for caspase-6, Ac-aspartic acid-glutamic acid-valine-aspartic acid (DEVD)-AFC for caspase-3 and caspase-7, and Ac-isoleucine-glutamic acid-threonine-aspartic acid (IETD)-7-amino-4-methyl-coumarin (AMC) for caspase-8 (Biomol). The caspase-6 activity was measured at 37°C every 2 min for 1 hr to determine the linear range of activity. Based on an AFC or AMC standard curve, the amount of released AFC or AMC was measured and the specific activity of the caspase was determined as nanomoles of released AFC or AMC per microgram of protein per minute. To determine whether 17- β -estradiol could induce CIF in cell-free extracts, untreated neuronal extracts were treated with 10 nM 17- β -estradiol or 17- α -estradiol or with the equivalent amount of ethanol and incubated for 0 and 1 hr at 37°C before testing CIF activity as described above.

Determination of estrogen receptors in neuron and astrocyte cultures

Neuron or astrocyte cultures were serum-deprived in phenol-free media for 2 hr and incubated for 2 additional hours with 5 nM 2,4,6,7-³H-estradiol (84.1 Ci/mmol) in the absence or presence of a 200-fold excess of cold 17- β -estradiol (to measure the nonspecific binding). The cells were collected and dissolved in 0.5N NaOH before calculating the protein concentration by the Lowry method and ³H-incorporation. Specific binding was calculated by removing nonspecific binding from total binding and dividing by the protein concentration.

Protein characteristics

RNase and DNase treatment. RNase A and DNase I were added to CIF-containing protein extracts at 2 μ g/ml and incubated for 1 hr at

37°C. The activity of RNase A and DNase I in the caspase lysis buffer was determined by adding exogenous RNA and DNA to the buffer and verifying the degradation of the RNA and DNA on agarose-ethidium bromide gels (data not shown).

Lipid extraction. Lipids from CIF-containing proteins were extracted with an equal volume of chloroform three times.

Heat denaturation. Protein extracts were boiled for 30 min and cooled.

DTT. The CIF assay was conducted in the presence of 10 or 20 mM DTT as described previously (Kim et al., 1997).

Dialysis. Protein extracts were dialyzed in Snakeskin (Pierce) dialysis tubing of molecular mass cutoff 3.5, 7, 10 and 12–14 kDa against a \times 5000-fold volume excess of caspase lysis buffer overnight at 4°C.

Chromatography. Total protein extracts were fractionated on a Sepharose Q anion exchange column (Amersham Pharmacia Biotech, Arlington Heights, IL) and CIF activity was eluted with a gradient of 0–0.8 M NaCl. The activity was then separated on a Superdex G75 HR10/30 column (Amersham Pharmacia Biotech). Fractions with activity were separated by 15% PAGE and silver-stained. CIF units were defined as follows: 100 U = 50% inhibition of R-Csp-6 activity.

PD98059 inhibition of CIF and mitogen-activated protein kinase activity

We pretreated neurons with a 100 μ M concentration of the mitogen-activated protein/extracellular signal-regulated kinase (MEK)-specific inhibitor PD98059 (Calbiochem, La Jolla, CA) (stock solution of 100 mM in 100% DMSO) for 5 hr in the absence of serum and then treated neurons for 1 hr with either 10 nM 17- β -estradiol or 10 nM 17- α -estradiol in the presence of PD98059. The control cells were pretreated with the equivalent amount of DMSO before stimulation of CIF with 17- β -estradiol. Proteins were extracted in caspase lysis buffer containing 500 μ M sodium vanadate and 50 mM sodium fluoride. Western blots of mitogen-activated protein kinase (MAPK) were done with anti-phospho extracellular signal-regulated kinase 1 (ERK1) and ERK2 (Cell Signaling Technologies) or with a polyclonal antisera against total MAPK (a kind gift from Dr Paudel, McGill University).

Statistics

Statistical evaluations of the difference between untreated and estrogen-treated samples were done by using one-way or two-way ANOVAs as specifically indicated in the figure legends. *Post hoc* analysis was done by Dunnett's test to compare each treatment with one control or by Scheffe's test to compare between treatments. ANOVA and *post hoc* results of $p < 0.05$ were taken as statistically significant.

RESULTS

17- β -estradiol prevents caspase-6-mediated neuronal cell death

We treated neurons microinjected with a lethal dose of 5 pg of exogenously synthesized active R-Csp-6 per cell with 0.01–100 nM 17- β -estradiol (Fig. 1A). In 48 hr, R-Csp-6 induces apoptosis in 50% of microinjected neurons. A 1–100 nM concentration of 17- β -estradiol decreases the level of apoptosis to 20%. In contrast, a 1–100 nM concentration of the transcriptionally inactive estrogen 17- α -estradiol or androgens, testosterone, and epitestosterone (data not shown) does not protect significantly against R-Csp-6. 17- β -estradiol also inhibits endogenous caspase-6 activity (Fig. 1B). Because the normal premenopausal level of estrogen in plasma is 2 nM, our results show that physiological concentrations of 17- β -estradiol can protect neurons against active caspases.

17- β -estradiol induces an inhibitory factor of active caspase in human neurons

To determine whether the 17- β -estradiol-mediated neuroprotective effect against caspase-6 is directly preventing R-Csp-6 activity or activating a survival pathway that interferes downstream of caspase-6, we tested 17- β -estradiol-treated neuronal extracts on R-Csp-6 activity *in vitro*. Neuronal extracts from 17- β -estradiol-treated neurons inhibit the activity of R-Csp-6

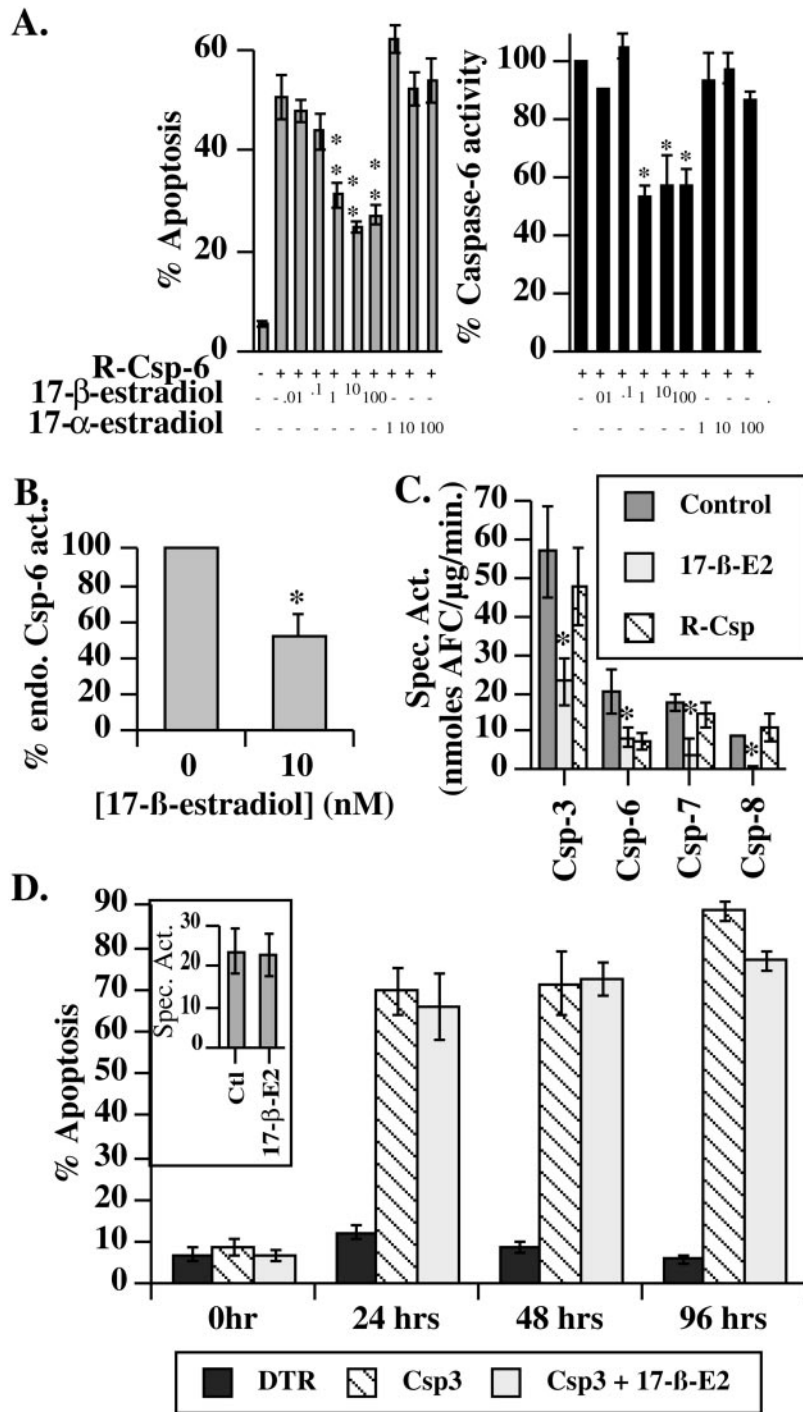


Figure 1. 17-β-estradiol inhibits R-Csp-6-mediated apoptosis and induces a CIF. *A*, Neurons were microinjected with 5 pg/cell R-Csp-6 and treated with varying concentrations of 17-β-estradiol and 17-α-estradiol; neuronal cell death was measured 48 hr after treatment (*left panel*). The *right panel* indicates that neuronal extracts from 17-β-estradiol-treated neurons inhibit R-Csp-6 activity *in vitro*. The control represents an untreated neuronal extract and was arbitrarily placed at 100%. Data represent the mean and SEM ($n = 4$). A one-way ANOVA ($df = 9$ for apoptosis and $df = 8$ for CIF activity); $p < 0.05$. A *post hoc* Dunnett's test compared hormone treatment with controls. * $0.01 < p < 0.05$; † $p < 0.01$. *B*, Inhibition of endogenous neuronal caspase-6 in 17-β-estradiol-treated neurons for 48 hr. Data represent the mean and SEM ($n = 3$); * $p < 0.01$ by a two-tailed unpaired *t* test. *C*, Neuronal extracts containing CIF activity against R-Csp-6 were tested for inhibitory activity of caspase-3, caspase-7, and caspase-8. Results show the mean and SEM ($n = 3$). The control represents neuronal extracts from untreated neurons. The third column represents the activity of the recombinant caspase in the absence of neuronal protein extract. * $p < 0.01$ by a two-tailed unpaired *t* test. *D*, Human astrocytes were microinjected with caspase-3 and incubated in the presence or absence of 10 nM 17-β-estradiol. Cell death was measured by TUNEL. No significant difference was observed between untreated and 17-β-estradiol-treated astrocytes. A two-way ANOVA ($df_{time} = 3$, $df_{injection\ treatment} = 2$) followed by a Scheffe's test indicated a p value of < 0.01 for caspase-3 injections but no difference with 17-β-estradiol treatments. *Inset*, Human astrocytic extracts were tested for CIF activity after a 6 hr treatment with 17-β-estradiol.

by ~40–60% ($p < 0.05$) (Fig. 1*A*). The caspase inhibitory activity is induced with physiological 1 nM concentrations of 17-β-estradiol but not 17-α-estradiol and does not change significantly with 10 or 100 nM concentrations. Hormones added directly to the R-Csp-6 assay in the absence of neuronal extracts do not alter the activity of caspase-6 (data not shown). Hormones added to neuronal extract and incubated for 1 hr at 37°C also do not induce CIF activity (control, 54 ± 49 ; 17-β-estradiol, 55.95 ± 0.1 ; 17-α-estradiol, 58.35 ± 1 nmol/μg protein per minute). The profile of caspase inhibition at different doses of 17-β-estradiol parallels that of the inhibition of neuronal apoptosis. These results indicate that physiological levels

of 17-β-estradiol induce a neuronal CIF that acts directly on the active caspase-6.

CIF also inhibits caspase-3, caspase-7, and caspase-8

To determine whether CIF activity is specific to caspase-6, we tested the 17-β-estradiol-treated neuronal extracts for CIF activity on recombinant caspase-3, caspase-7, and caspase-8. All of these caspases are inhibited by CIF (Fig. 1*C*). The inhibitory effect is stronger on caspase-7 (70%) and caspase-8 (90%) and similar for caspase-3 and caspase-6 (~50%). These results show that CIF is not specific to caspase-6 and can inhibit other active caspases.

Cell-type specificity of 17- β -estradiol mediated CIF activity

To determine whether CIF can be activated in other cell types of the CNS, we treated astrocytes with 10 nM 17- β -estradiol for 6 hr and tested CIF activity *in vitro* (Fig. 1D, inset). In contrast to neurons, 17- β -estradiol does not protect astrocytes against caspase-3 (Zhang et al., 2000) or induce CIF (Fig. 1D). Astrocytes contain only 3 fmol of estrogen receptors per milligram of total protein, whereas neurons contain 14 fmol/mg. The amount of estrogen receptor in astrocytes is therefore very low and could be responsible for the lack of CIF induction and neuroprotection in astrocytes. These results support the hypothesis that CIF is required for 17- β -estradiol inhibition of caspase-mediated cell death.

De novo protein synthesis is not required for 17- β -estradiol induction of CIF in neurons

A time response curve of CIF activity shows that 17- β -estradiol induces CIF activity within 10 min of exposure to neurons and is sufficient to prevent neuronal cell death (Fig. 2A). The rapid induction of CIF suggests that CIF activity does not require *de novo* protein synthesis. To conclusively determine whether 17- β -estradiol can induce CIF without protein translation, we treated the neurons with the translation inhibitor cycloheximide (20 μ g/ml) in the presence of 10 nM 17- β -estradiol. The efficiency of cycloheximide as an inhibitor of translation was assessed by metabolic labeling with 35 S-methionine. Although these doses of cycloheximide inhibit protein translation in neurons (results not shown), cycloheximide has no effect on 17- β -estradiol-mediated neuroprotection or induction of CIF (Fig. 2B). These results show that the activation of CIF does not require *de novo* protein synthesis.

Anti-estrogen, tamoxifen, inhibits CIF induction by 17- β -estradiol

To determine whether CIF activity is induced through estrogen receptors, we assessed the ability of the estrogen receptor antagonist tamoxifen to block 17- β -estradiol-induced CIF activity. Tamoxifen efficiently blocks both the neuroprotective function of 17- β -estradiol against caspase-6 and CIF activation (Fig. 2C). These results indicate that estrogen receptors mediate CIF induction.

CIF is a protein

To determine whether CIF is a protein, neuronal extracts containing CIF activity were submitted to boiling, RNase A, DNase I, and chloroform lipid extraction; size was assessed by dialysis. CIF activity was abolished by boiling (Fig. 3A) and proteinase K digestion (results not shown). The decrease in caspase-6 activity observed in boiled control neuronal extract is attributable to the fact that protein (BSA or neuronal extract) enhances the recombinant caspase-6 activity *in vitro*. Neither RNase A nor DNase I destroyed CIF activity, indicating that nucleic acids are not part of CIF activity (Fig. 3A). Chloroform extraction of lipids did not eliminate CIF activity (Fig. 3A). Increasing the amount of DTT to 20 mM (Kim et al., 1997) could not reverse the caspase inhibitory activity, indicating that CIF is not acting through an oxidative mechanism (Fig. 3A). Dialysis of CIF-containing extracts in 3.5, 7, 10, and 12–14 kDa molecular mass cutoff dialysis membranes resulted in the retention of CIF activity in the 3.5–10 kDa molecular mass cutoff membranes but not in the 12–14 kDa molecular mass cutoff membranes (Fig. 3B). These sizes were also con-

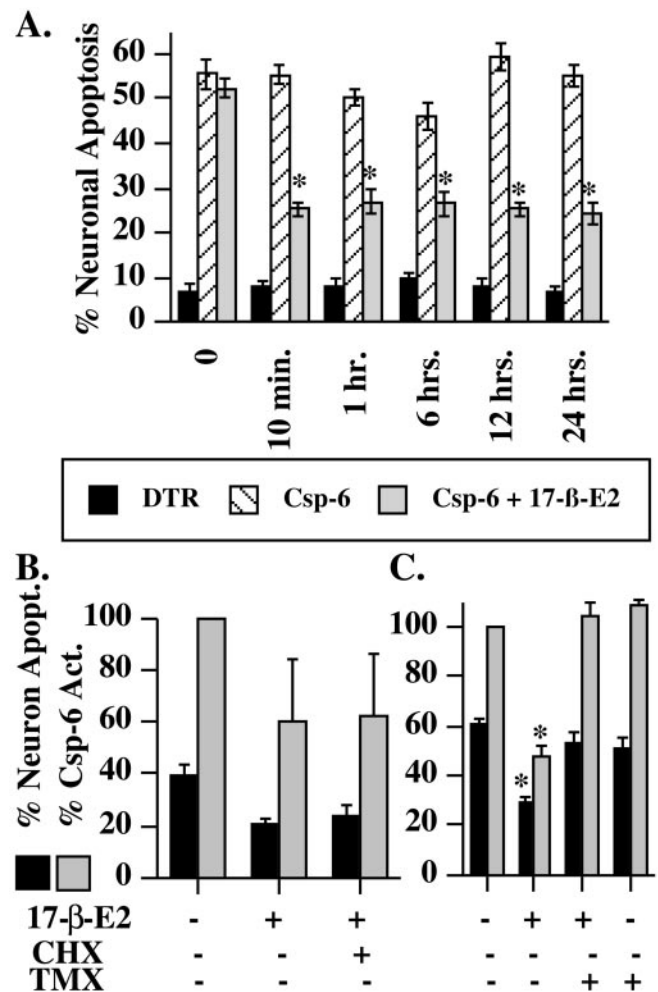


Figure 2. Induction of CIF by 17- β -estradiol is rapid and does not require *de novo* protein synthesis but acts through the estrogen receptor. **A**, Neurons were treated with 17- β -estradiol for the time indicated, the hormone was washed away, and cells were incubated further until 48 hr before the measurement of neuronal cell death. Data represent the mean and SEM ($n = 3$). A two-way ANOVA ($df_{\text{time}} = 5$, $df_{\text{injection treatment}} = 2$) indicated a p value of <0.05 . A *post hoc* Dunnett's test compared estrogen treatments at various times with untreated neurons; $*p < 0.01$. **B**, Neuroprotective effect and CIF-induced activity of 17- β -estradiol in the absence or presence of cycloheximide (CHX). Data represent the mean and SD ($n = 4$). A one-way ANOVA ($df = 2$) followed by a Scheffé's test showed no significant difference ($p > 0.5$). **C**, Neurons were treated with 10 nM 17- β -estradiol in the absence or presence of 10 μ M tamoxifen (TMX). Neuronal extracts were assayed for CIF activity. Data represent the mean and SEM ($n = 4$). A one-way ANOVA ($df = 3$) indicated a p value of <0.05 , as did a *post hoc* Scheffé's test. $*p < 0.01$ for TMX versus no TMX.

firmed using microcon centrifugal devices (results not shown). Fractionation and purification of CIF activity by ion exchange chromatography and gel filtration increased the specific activity of CIF by 1.4- and 500-fold, respectively (12.33 U/ μ g of protein in the initial material and 6255 U/ μ g of protein in the final gel filtration fraction; 80% recovery). PAGE resolved two small proteins of 12 and 14 kDa in the gel filtration-purified fraction containing most of the CIF activity (Fig. 3C). Therefore, the size of the proteins obtained by purification of CIF activity is consistent with the size determined by dialysis, indicating that CIF is possibly a small protein.

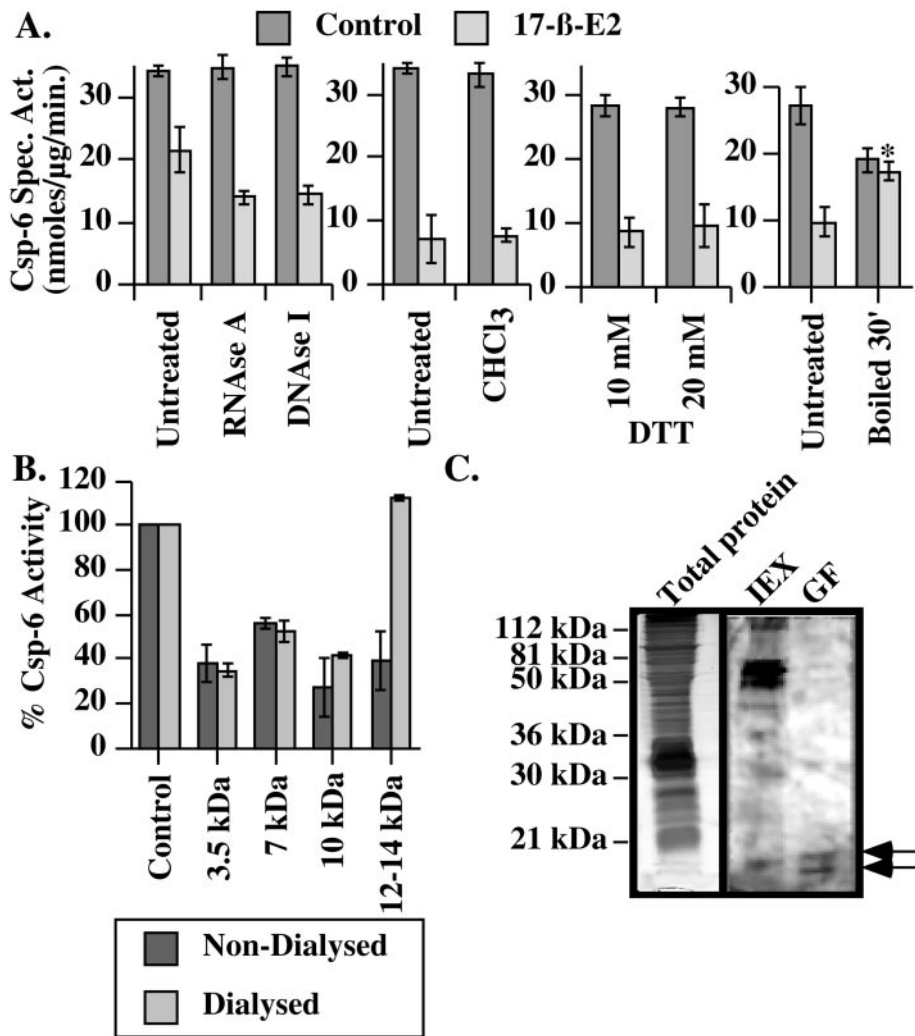


Figure 3. Biochemical characterization of CIF activity. *A*, Effect of RNase A, DNase I, lipid extraction in CHCl₃, DTT, and boiling on CIF activity; **p* < 0.04 by a two-tailed unpaired *t* test. *B*, Dialysis of CIF-containing neuronal extracts through various molecular mass cutoff membranes. CIF-containing extracts were dialyzed against caspase lysis buffer overnight, and 10 μg of protein was remeasured for CIF activity. Data represent the mean and SEM (*n* = 3). A two-way ANOVA (*df*_{size} = 4, *df*_{dialysis} = 1) (*p* < 0.05) followed by a Scheffe's test shows that significance is lost in the 12–14 kDa dialysis. *C*, Silver-stained PAGE analysis of total neuronal protein extracts, ion exchange (IEX), and gel filtration (GF) fractions containing CIF activity.

MAPK is necessary but not sufficient to induce CIF activity

We investigated whether MAPK activation was involved in 17-β-estradiol induction of CIF. The results show that the MEK1-specific inhibitor PD98059 effectively inhibits the 17-β-estradiol-mediated induction of CIF (Fig. 4). Immunoblotting shows that 17-β-estradiol induces MAPK phosphorylation in neurons and that PD98059 completely inhibits the phosphorylation of ERK1 and ERK2 (Fig. 4). However, as shown previously (Singh et al., 1999, 2000), 17-α-estradiol also induces MAPK phosphorylation despite its inability to induce CIF. These results suggest that MAPK activation is necessary but not sufficient to induce CIF activity.

DISCUSSION

Caspases are implicated in a broad range of CNS diseases, such as neurodegeneration, trauma, and stroke (Thornberry, 1999). Once activated, caspases induce irreversible molecular proteolytic cascades that result in cell death. Considerable evidence supports a role for caspases in the pathogenesis of Alzheimer's disease (Masliah et al., 1998; Yang et al., 1998; Gervais et al., 1999; LeBlanc et al., 1999; Selznick et al., 1999; Stadelmann et al., 1999; Lu et al., 2000). Therefore, there is a strong interest in preventing caspase activation to avoid the loss of indispensable

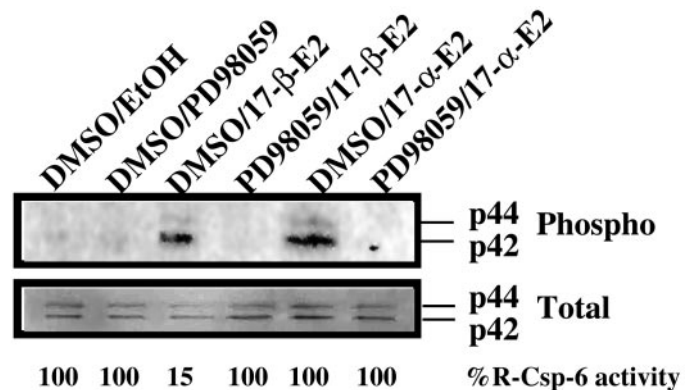


Figure 4. MAPK is necessary but not sufficient for CIF activation in neurons. An immunoblot of phosphorylated and total MAPK ERK1 and ERK2 in neurons pretreated for 5 hr with control DMSO equivalents or 100 μM PD98059 and then incubated for 1 hr with an ethanol-equivalent control or 10 nM 17-β-estradiol and 17-α-estradiol is shown. CIF activity was measured against R-Csp-6 as before and expressed as the percentage of inhibition of R-Csp-6.

neurons and in the hope that survival of this cell type will allow treatment of the disease.

It has been known for some time that 17-β-estradiol is a neuroprotective agent. Evaluation of the potential role of estro-

gen on neurons has indicated previously that estrogen enhances neuritic outgrowth and survival; upregulates brain-derived neurotrophic factor, nerve growth factor, epidermal growth factor, and Bcl-2; and reverses the behavioral and biochemical changes in ovariectomized rats (for review, see Dubal et al., 1999; Woolley, 1999). Others propose that estrogen acts as an anti-oxidant, although it is unlikely that physiological levels of estrogen will have antioxidant activity (Moosmann and Behl, 1999). In addition, 17- β -estradiol decreases amyloidogenic processing of the amyloid precursor protein (Xu et al., 1998). In the present manuscript, we show that physiological concentrations of 17- β -estradiol specifically induce a CIF in primary cultures of human neurons.

We have yet to identify CIF, but our results show that CIF is a small protein of 12–14 kDa. We believe that CIF represents a novel caspase inhibitor. Natural endogenous inhibitors of caspase-6 are unknown at this time. Within the proteins known to prevent active caspases (for review, see Ekert et al., 1999), p35 can inhibit caspase-6 but is absent in our system. Members of the IAP family (X-IAP, c-IAP-1, and c-IAP-2) inhibit caspase-3, caspase-7, and caspase-9 but cannot inhibit caspase-6 (Deveraux et al., 1997; Roy et al., 1997). We ruled out oxidation as CIF activity. Caspase-6 lacks the Akt motifs that contribute to inhibition of caspase-9 (Cardone et al., 1998). However, it is possible that other kinases are activated and phosphorylate caspase-6. Much more work will be required to sequence and confirm CIF identity. However, our results indicate several important new findings. First, caspase inhibitory proteins can be activated through hormones. Second, CIF activation does not require *de novo* protein expression and involves MAPK signal transduction, thereby providing a rapid mechanism to inhibit active caspases. Third, CIF is a broad-spectrum inhibitor and inhibits caspase-3, caspase-6, caspase-7, and caspase-8. Fourth, the activation of CIF occurs through the estrogen receptors. Lastly, the induction of CIF in neurons but not in astrocytes provides a selective target to prevent neuronal loss without risk of inducing tumorigenicity in dividing cell types. However, some astrocytes do express estrogen receptor β in the hippocampus and dentate gyrus (Azcoitia et al., 1999).

In conclusion, we have identified a novel and unsuspected mechanism by which estrogen protects human neurons against cell death by inducing a neuronal CIF. We believe that CIF could prevent caspase-mediated cell death in neurodegenerative diseases. It will also be of considerable interest to determine whether CIF may be involved in the protective role of estrogen against osteoporosis or in the promotion of cancer by estrogen in estrogen-responsive tissues. CIF may provide a molecular handle to better develop therapeutic interventions against these diseases.

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