Introduction

Intracellular amyloid β peptide (iAβ_{1–42}) accumulates in the Alzheimer’s disease brain before plaque and tangle formation (Gouras et al., 2000) and is extremely toxic to human neurons (Zhang et al., 2002). Here, we investigated whether androgen and estrogen could prevent iAβ_{1–42} toxicity, because both these hormones have a wide range of neuroprotective actions. At physiological concentrations, 17-β-estradiol, testosterone, and methyl testosterone reduce iAβ_{1–42}-induced cell death by 50% in neurons treated after the injection and by 80–90% in neurons treated 1 hr before the injection. The neuroprotective action of the hormones is mediated by receptors, because the estrogen receptor (ER) antagonist tamoxifen and the androgen receptor (AR) antagonist flutamide completely block the estrogen- and androgen-mediated neuroprotection, respectively. Transcriptional activity is required for the neuroprotective action, because dominant negative forms of the receptors that block the transcriptional activity of the ER and AR prevent estrogen- and androgen-mediated neuroprotection. Proteomics followed by Western blot analyses identified increased levels of heat shock protein 70 (Hsp70) in testosterone- and estrogen-treated human neurons. Comicroinjection of Hsp70 with the iAβ_{1–42} neutralizes its neuroprotection. We conclude that estrogen and androgens protect human neurons against iAβ_{1–42} toxicity by increasing the levels of Hsp70 in the neurons.

Key words: intracellular amyloid; estrogen; androgen; neuroprotection; human neurons; Hsp70
1995, 1997). These steroid sex hormones can protect through activation of transcriptional activity or via signaling of survival pathways (Driggers and Segars, 2002; Heinlein and Chang, 2002; Segars and Driggers, 2002).

In the present study, we demonstrate that physiological concentrations of androgens or estrogens completely protect human neurons in primary cultures against microinjected iAβ_{1-42} toxicity. Using AR and estrogen receptor (ER) antagonists and dominant negative forms of these receptors, we show that the neuroprotection occurs in a receptor- and transcription-dependent manner. Proteomics studies followed by Western blotting identify an increase in heat shock protein 70 (Hsp70) levels in testosterone- and estrogen-treated neurons. Hsp70 co-microinjected with iAβ_{1-42} also completely prevents iAβ_{1-42} toxicity. We conclude that testosterone and estrogen can protect against iAβ_{1-42} through Hsp70.

Materials and Methods

**AB peptides, recombinant proteins, antibodies, and cDNAs**

Aβ peptides ( Bachem, King of Prussia, PA) were dissolved in sterile distilled water at 25 μM and incubated at 37°C for 5 d (Zhang et al., 2002). The peptide stock solutions were kept frozen in aliquots until use. The recombinant human Hsp70 protein and monoclonal anti-Hsp70 antibody were purchased from Stressgen Biotech (Victoria, British Columbia, Canada). The wild-type and mutant 12474 and 15579 human androgen receptor cDNAs have been described previously. The AR mutants, 12474 and 15579, contain a deletion of 579Val and 580Phe and of 614Cys and 615Arg in the DNA binding domain (DBD), respectively (Beitel et al., 1994). All cDNAs were cloned into the vector pcDNA3 under the cytomegalovirus promoter (Panet-Raymond et al., 2000). The human ER wild-type and mutant ΔDBD cDNAs were obtained from Dr. Sylvie Mader (University of Montreal, Montreal, Quebec, Canada). The ΔDBD ER construct contains a deletion of amino acids 185–251 (Mader et al., 1993). All ER cDNAs were cloned into the pSG5 vector under the simian virus 40 promoter (Rosenauer et al., 1998). The androgen responsive element (ARE) luciferase reporter vector pGL3-MMTV-(ARE)-Luc and the estrogen responsive element (ERE) luciferase reporter vector pGL3-(ERE)-Luc have been described previously (Panet-Raymond et al., 2000). The cDNAs were purified with the UltraClean-15 DNA Purification kit (MoBio Laboratories Inc., Solana Beach, CA).

**Human primary cell cultures and treatments**

**Primary cultures.** Primary cultures of human neurons were prepared from 12- to 16-week-old fetal brains collected as approved by the McGill University Institutional Review Board and according to Canadian Institute of Health Research regulations (LeBlanc, 1995). In brief, fetal cerebral brain tissues were dissected free of meninges and blood vessels in PBS (in mM: 0.14 NaCl, 0.003 KCl, 0.01 Na2HPO4, and 0.002 KH2PO4, pH 7.2) and 5% decomplemented FBS. Neurons attached to coverslips (33°C, 5 mm; Allied Chemical Inc., Minneapolis, MN) at a density of 3 × 10^5 cells/ml. The cultures were incubated at 37°C with 5% CO2 and the medium was changed every 48 hr. The neurons attached within 24 hr and developed extensive neuritic extensions within 3 d of plating. In general, the cultures contained 90–95% neurons and 5–10% astrocytes (LeBlanc, 1995).

**Microinjection of AB peptides and cDNA constructs in primary cultures of human neurons.** Microinjections were performed 11 d after plating the neurons with the Eppendorf (Fishers, NY) Microinjector 5246 and the three-dimensional Burleigh (Fishers, NY) Micromanipulator MIS-5000 (Zhang et al., 2002). Microinjections were performed with a glass needle with a tip diameter of ~0.5 μm pulled from 1.0 mm outer diameter and 0.5 mm inner diameter thin-walled glass capillaries with microfilmation (borosilicate with filament MTW100F-4; World Precision Instruments, Sarasota, FL) with a Flaming/Brown micropipette puller (model P-87; Sutter Instruments, Novato, CA). Human neurons were injected at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 sec. The injected volume was 25 pl. AB (10 nM), cDNAs (30 ng/ml), and Hsp70 (5 μg/ml) were co-injected with 100 μg/ml fluorescent marker dye Dextran Texas Red (DTR) (MW3000; Molecular Probes, Eugene, OR) into the cytosolic area of the neuron (Zhang et al., 2002). Approximately 90% of neurons survived the injection. Microinjections were done in 200 neurons per preparation in three independent microscopic preparations, for a total of 600 injected neurons.

**Treatment of human neurons with estrogen, androgen, tamoxifen, or flutamide.** 17α-Estradiol, 17β-estradiol, BSA-17β-estradiol, testosterone enanthate, and epitestosterone were purchased from Sigma. Methyl testosterone was bought from Pharmacia (Rockville, MD). Hormones were dissolved in 100% ethanol at a 5 mM concentration and diluted to 2–10 nM with culture medium before use (Hammond et al., 2001). Tamoxifen (TMX; Sigma) and flutamide (Flut; Sigma) were dissolved in sterile distilled water at 10 and 20 mM and serially diluted to the indicated concentration (2–10 nM) with MEM before treatment of neurons (Hammond et al., 2001). For the postincubation experiments, cells were microinjected with peptides or cDNAs and incubated at 37°C with hormones in the presence or absence of hormone receptor antagonists for 24 hr. For the preincubation experiments, cells were incubated at 37°C with hormones and hormone receptor antagonists for 1 hr before the microinjection of peptide followed by incubation with hormones in the presence or absence of hormone receptor antagonists for 24 hr.

**Proteomics analyses and Western blot.** Neurons were treated with 10 nM testosterone for 1 hr. The cells were washed in 1× PBS and collected in 10 mM Tris-HCl, pH 7.4, and 5 mM MgCl2 for two-dimensional gel analysis. Cells were lysed with two freeze-thaw cycles in liquid nitrogen and extracts treated with 1000 U of DNase (Promega, Madison, WI) and 60 μg of RNase A for 10 min on ice. Proteins were precipitated with methanol-chloroform and solubilized in 9.5 mL urea, 2.8% 1–42 Toxicity

Cell line cultures and treatments

MCF-7 and AR24 cell cultures. Human breast cancer MCF-7 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Invitrogen) with 10% FBS. AR24 cells were created by stably expressing human androgen receptors in rat motor neuron hybrid cells and cultured in DMEM (Invitrogen) with 5% FBS (Brooks et al., 1998). Cells were incubated at 37°C with 5% CO2. The culture media was changed every 48 hr.

**Transfection of MCF-7 and AR24 cells.** Eighty percent confluent MCF-7 and AR24 cell cultures were washed and incubated at 37°C for 6 hr with 3 μg/ml CDNA and 6 μg/ml Lipofectamine-2000 (Invitrogen) in OPTIMEM (Invitrogen) without FBS. After the incubation, the cells were washed with DMEM–5% FBS and incubated at 37°C for 24 hr in the presence or absence of 10 nM estrogen or androgen.

**Luciferase reporter system is used to measure androgen and estrogen re-
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Receptor activity. The Dual-Luciferase Reporter Assay System kit (E1910; Promega) was used to investigate the dominant negative effects of the mutant AR and ER. The constructs with the firefly (Photinus pyralis) luciferase gene downstream of the estrogen (ERE) and androgen (ARE) promoters were cotransfected with the wild-type or mutant ER or AR constructs into estrogen- or androgen-receptor-positive MCF-7 or AR24 cells, respectively. After treatment with 10 nM estrogen or testosterone, firefly luminescence was detected as indicated by the manufacturer. To control for transfection efficiency, the Renilla reniformis luciferase construct was cotransfected into cells. The activity was corrected for the protein concentration of each sample and expressed as [(firefly/renilla luciferase) × 10,000]. The luminescence is expressed as relative light units.

Measurement of cell death. Cells were fixed in fresh 4% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature and permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 2 min on ice. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was performed using the in Situ Cell Death Detection Kit as described by the manufacturer (Roche, Quebec, Canada). The TUNEL-stained coverslips were then washed once in distilled water for 5 min and mounted on glass slides. The percentage of cell death was determined by the number of DTR–TUNEL double-positive cells to the total number of DTR-positive cells. All of the coverslips were counted blindly.

Statistical analysis

One-way ANOVA was used with post hoc tests (Statview v.5.01, SAS Institute, Cary, NC) to determine the statistical significance of the differences between treatments. The Sheffé’s test was applied as the post hoc analysis comparing data between each treatment group. A p value of <0.05 was taken as the criteria for statistical significance.

Results

Physiological concentrations of estrogen and androgen protect human neurons in primary culture against intracellular Aβ_{1–42}-induced cell death

Human fetal brain expresses estrogen receptor α and β subtypes and androgen receptor β subtype (Wilson and McPhaul, 1996; Takeyama et al., 2001). Microinjection of Aβ_{1–42} into the cytosol of neurons induces ~60% cell death within 24 hr (Fig. 1A,B) (Zhang et al., 2002). Immediate injection of the injected neurons with 2, 4, or 10 nM 17β-estradiol, testosterone, or methyl testosterone significantly decreases cell death by 50% as measured by TUNEL and morphological assessments (Fig. 1A,B,D). A 1 hr preincubation with these hormones before the microinjection of Aβ_{1–42} into neurons completely protects against cell death (Fig. 1C). In contrast, the transcriptionally inactive isoforms 17α-estradiol, membrane-impermeable BSA-conjugated 17β-estradiol, and the testosterone antagonist epitestosterone do not have any protective effects (Fig. 1A,B). These results show that physiological concentrations of estrogen and androgens (Wilson et al., 1978) protect human neurons against iAβ_{1–42}. The increased protection with preincubation indicates that the hormones alter the state of neuronal susceptibility to the iAβ_{1–42}.

Estrogen and androgen protect against intracellular Aβ through their respective receptors

Both estrogen and androgen receptors exist in the primary cultures of human neurons (Hammond et al., 2001; Zhang et al., 2001). However, androgens can also be aromatized into estrogen (Finkelstein et al., 1981). To determine whether each hormone is neuroprotective through its respective receptor, the effect of antagonists on hormone-mediated neuroprotection was evaluated. The estrogen receptor antagonist tamoxifen and the androgen receptor antagonist flutamide completely blocked the protection induced by estrogen and androgen, respectively (Fig. 2). Tamoxifen and flutamide treatments alone do not induce cell death in human neurons. These results show that both hormones are neuroprotective through their respective receptors.

Mass spectrometric analysis of proteins from neurons incubated with testosterone identified Hsp70 with an expectation value of 4 × 10^−9. Because Hsp70 has been found to interact with both Aβ_{1–42} (Fonte et al., 2002) and p53 (King et al., 2001), which is activated by iAβ_{1–42} in neurons (Zhang et al., 2002), we pursued the potential role of Hsp70 in hormone-mediated neuroprotection. Western blot analyses confirm the increased levels of Hsp70 in estrogen- and androgen-treated neurons (Fig. 5A). Microinjection of recombinant human Hsp70 with iAβ_{1–42} into primary neurons abolishes iAβ_{1–42}–mediated neurotoxicity to the same extent as with testosterone and estrogen treatments (Fig. 5B). We conclude from these experiments that androgen- and estrogen-mediated increases in Hsp70 cellular levels are sufficient to modulate estrogen- and androgen-mediated protection against iAβ_{1–42}.

Discussion

In this study, we show that: (1) physiological concentrations of both estrogen and androgen completely protect human primary neurons against iAβ_{1–42} toxicity, (2) estrogen and androgens are...
neuroprotective through their respective receptors, (3) estrogen- and androgen-mediated protection against iAβ1–42 depends on transcriptional activation, and (4) Hsp70, which is increased in estrogen- and testosterone-treated neurons, completely protects against iAβ1–42-mediated toxicity.

The neuroprotective role of physiological concentrations of androgens and estrogens against iAβ1–42 neurotoxicity is an important finding, because iAβ1–42 accumulation in AD neurons precedes other pathological hallmarks and is an early event that likely leads to neuronal dysfunction and cell death (Gouras et al., 2000; Takahashi et al., 2002; Zhang et al., 2002). Except for inhibitors of γ-secretase to prevent the production of amyloid, most efforts to inhibit or remove amyloid toxicity, including vaccines, protease treatments, and anti-fibril agents, are directed at the extracellular amyloid but not intracellular amyloid toxicity. Our results indicate that sex steroids could play an important role in the early treatment of AD by preventing potential intracellular amyloid toxicity.

There is presently much controversial evidence on the use of sex steroids against neurodegeneration. However, the strong neuroprotective role of these hormones shown in this study justifies additional investigations on their potential use in neurodegenerative diseases. Our results indicate that potential problems incurred in clinical trials may be avoided. First, the use of physiological levels of hormones would likely be sufficient for experiments. *p < 0.002 compared with Aβ1–42. C, TUNEL-positive cell death in human neurons preincubated with 10 μM estrogen or androgens for 1 hr before microinjections of iAβ1–42 and further incubated with hormones for 24 hr after the injection. Data represent the mean ± SEM of three independent experiments. *p < 0.001 compared with Aβ1–42 alone. D, Immunofluorescence micrographs of neurons injected with Aβ1–42 and DTR and either left untreated (control) or treated with 2 and 4 μM 17βE2 or testosterone (Test), respectively. The panels show the DTR-injected neurons stained with the DNA stain 4',6'-diamidino-2-phenylindole (DAPI) or the cell death marker TUNEL. Ctl, Control.
the protective action and may avoid undue complications caused by pharmacological doses of the hormones. Second, because both estrogens and androgens protect equally well through their respective receptors, both men and women can be treated with lower side effects. Third, understanding the underlying molecular mechanism of the action of these hormones may help target a pathway that is involved in neuroprotection against iAβ1-42 toxicity while avoiding the multiple potentially detrimental actions of these hormones.

Increased levels of Hsp70 are not unique to estrogen- and androgen-treated neurons. Several studies have found increased expression of Hsp70 in various hormone-treated cell types (Tang et al., 1995; Jones et al., 2000; Lu et al., 2002; Gehring, 2004). The fact that Hsp70 can completely prevent iAβ1-42-mediated toxicity indicates only that Hsp70 is sufficient for neuroprotection against iAβ1-42 toxicity but does not rule out the possibility that other effects of the steroid hormones on neurons could also be neuroprotective. However, our results corroborate those showing that Hsp70 can protect against polyglutamine toxicity (Warwick et al., 1998; Jana et al., 2000; Cummings et al., 2001), α-synuclein toxicity (Auluck et al., 2002), and iAβ1-42 toxicity (Fonte et al., 2002; Magrane et al., 2004) in flies, cell cultures, and mice, respectively. Most importantly, prevention by Hsp70 of a progressive paralysis phenotype caused by overexpression of iAβ1-42 in muscle cells of Caenorhabditis elegans indicates a functional protection against iAβ1-42 (Fonte et al., 2002).

The molecular mechanism underlying Hsp70 neuroprotection against neurodegenerative conditions is not clear. In our system, we have demonstrated previously that iAβ1-42 induces human neuronal apoptosis through the activation of p53 and Bax
(Zhang et al., 2002). Hsp70 could sequester p53 in the cytosol and prevent its translocation to the nucleus and activation of apoptosis (King et al., 2001; Zyllicz et al., 2001). An alternative possibility is that Hsp70 interacts directly with iAβ1–42. Fonte et al. (2002) have shown interaction between iAβ1–42 and Hsp70 in C. elegans that is consistent with the ability of Hsp70 to bind peptides and denatured proteins. Finally, Hsp70 has been implicated recently in steroid-mediated transcriptional activation by forming a complex with Bag-1 and steroids (Gehring, 2004). Hsp70 may enhance steroid-mediated transcriptional activation of prosurvival genes. These are issues that will be difficult to resolve in human neurons considering the natural predisposition of Aβ1–42 to form aggregates that can interact nonspecifically with proteins and the resistance of human neurons in culture to infections or transfections, thereby limiting our studies to single-cell analyses (our unpublished observations).

In summary, we have shown that physiological concentrations of estrogen and androgens protect human neurons from iAβ1–42-mediated toxicity and increase Hsp70 in these neurons. Hsp70 completely protects against iAβ1–42-mediated toxicity. This study therefore identifies in Hsp70 a common downstream target of estrogen and androgen that is involved in neuroprotection.

References


