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Seladin-1 Is a Fundamental Mediator of the Neuroprotective Effects of Estrogen in Human Neuroblast Long-Term Cell Cultures

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Estrogen exerts neuroprotective effects and reduces β-amyloid accumulation in models of Alzheimer's disease (AD). A few years ago, a new neuroprotective gene, *i.e. seladin-1* (for selective AD indicator-1), was identified and found to be down-regulated in AD vulnerable brain regions. Seladin-1 inhibits the activation of caspase-3, a key modulator of apoptosis. In addition, it has been demonstrated that the *seladin-1* gene encodes 3β-hydroxysterol Δ24-reductase, which catalyzes the synthesis of cholesterol from desmosterol. We have demonstrated previously that in fetal neuroepithelial cells, 17β-estradiol ($17\beta E_2$), raloxifene, and tamoxifen exert neuroprotective effects and increase the expression of seladin-1. The aim of the present study was to elucidate whether seladin-1 is directly involved in estrogen-mediated neuroprotection. Using the small interfering RNA methodology, significantly reduced levels of seladin-1 mRNA and protein were obtained in fetal neuroepithelial cells. Seladin-1 silencing determined the loss of the protective effect of $17\beta E_2$ against β-amyloid and oxidative stress toxicity and caspase-3 activation. A computer-assisted analysis revealed the presence of half-palindromic estrogen responsive elements upstream from the coding region of the *seladin-1* gene. A 1490-bp region was cloned in a luciferase reporter vector, which was transiently cotransfected with the estrogen receptor α in Chinese hamster ovarian cells. The exposure to $17\beta E_2$, raloxifene, tamoxifen, and the soy isoflavones genistein and zearalenone increased luciferase activity, thus suggesting a functional role for the half-estrogen responsive elements of the *seladin-1* gene. Our data provide for the first time a direct demonstration that seladin-1 may be considered a fundamental mediator of the neuroprotective effects of estrogen. (*Endocrinology* 149: 4256–4266, 2008)

EXPERIMENTAL EVIDENCE strongly supports a neurotrophic and neuroprotective role of estrogen both *in vitro* and *in vivo* (1–3), and suggests a possible role of this family of hormones in the prevention and/or treatment of neurodegenerative diseases. Alzheimer's disease (AD) is the most common neurodegenerative disease associated with aging, affecting over 18 million people worldwide, representing 50–70% of all causes of dementia, and constituting a major public health problem (4). In fact, although specific drugs that may help to improve patients' quality and expectancy of life are currently available, to date there is no way to prevent, reverse, or even stop the course of AD (5).

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Abbreviations: AD, Alzheimer's disease; CHO, Chinese hamster ovarian; CNS, central nervous system; C-silFNC, FNC treated with control small interference RNA; $17\beta E_2$, estradiol; ER, estrogen receptor; ERE, estrogen responsive element; FCS, fetal calf serum; FNC, fetal neuroepithelial cells; HT, hormone therapy; IFN, interferon; INDO, indoleamine-pyrrole 2,3 dioxygenase; NCBI, National Center for Biotechnology Information; nt, nucleotide; SERM, selective estrogen receptor modulator; silFNC, FNC treated with seladin-1 targeting small interference RNA; siRNA, small interference RNA; WHIMS, Women's Health Initiative Memory Study.

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A number of studies indicated the presence of an evident sex dependence in AD prevalence (2:1 female to male), symptomatology, and prognosis (6). Sex steroids have been potent neuroprotective agents in a variety of *in vivo* models of AD (7), and a decreased incidence and delay in the onset of AD has been described in women on hormone therapy (HT) (8). HT in postmenopausal women has improved cognitive and visual-spatial functions (6), and appears to protect against AD; however, it is of little or no value once the disease has progressed so as to display clinical signs. In addition, a number of critical factors must be preliminarily considered, which may determine the efficacy of HT in the central nervous system (CNS) (*i.e.* age of initiation of the therapy, type of estrogen and progestin used, route of delivery and dose, genetic background) (9). These critical issues may have significantly contributed to the negative conclusions of the Women's Health Initiative Memory Study (WHIMS) about the protective effect of estrogen against dementia in postmenopausal women (10–13). As a matter of fact, the role of estrogen against AD in the clinical practice is still an open issue.

A few years ago, a novel gene, named *seladin-1* (for SElective AD INdicator-1), has been isolated and found to be down-regulated in brain regions affected by AD (14). Remarkably, overexpression of seladin-1 in neuroglioma H4

cells conferred protection against β-amyloid-mediated toxicity and oxidative stress. The neuroprotective effect of seladin-1 appears to be mediated, at least in part, by its inhibitory effect on the activation of caspase-3, a key mediator of apoptosis (14). A subsequent study demonstrated that seladin-1 is the gene encoding 3β-hydroxysterol Δ24-reductase, which catalyzes the reduction of the Δ24 double bond of desmosterol to produce cholesterol. Mutations of this gene have been found in desmosterolosis, a rare severe multiple-congenital-anomaly syndrome, including developmental and growth retardation (15). We have demonstrated previously that in neuroblast long-term cell cultures from human fetal olfactory epithelium [fetal neuroepithelial cells (FNC)] (16), 17β-estradiol ($17\beta E_2$) and the selective estrogen receptor modulators (SERMs) raloxifene and tamoxifen effectively protect against β-amyloid toxicity, oxidative stress, and apoptosis, and up-regulate the expression of *seladin-1* (17). The estrogen receptor (ER) α-selective agonist propylpyrazole-triol exerted a much stronger stimulatory effect on *seladin-1* expression than the ERβ-selective agonist diarylpropionitrile in FNCs, which express both ERα and ERβ (18), thus indicating that the expression of this neuroprotective factor is mainly mediated by ERα activation (17). These data suggested that seladin-1 may be a mediator of the protective effects of estrogen in neuronal cells.

The aim of the present study was to provide for the first time a direct demonstration that this is the case. To this purpose, seladin-1 expression was silenced in FNCs using the small interference RNA (siRNA) technology. In "silenced" cells the effect of $17\beta E_2$ against β-amyloid toxicity, oxidative stress, and apoptosis was evaluated. Furthermore, the presence and functionality of estrogen-responsive elements (EREs) upstream from the promoter region of the *seladin-1* gene were assessed.

Materials and Methods

Materials

Media and sera for cell cultures were purchased from Life Technologies (Grand Island, NY), and tissue plasticware was obtained from Falcon (Oxnard, CA). $17\beta E_2$ was purchased from Sigma (Milan, Italy) and dissolved in absolute ethanol to a final concentration of 0.1 mM. Human β-amyloid peptide was obtained from Calbiochem (San Diego, CA), solubilized in 5% acetic acid, and stored at −20 C. Rabbit anti-seladin-1 N-terminal antibody, tamoxifen, genistein, quercetin, and zearalenone were from Sigma. Raloxifene was kindly provided by Eli Lilly and Co. (Indianapolis, IN). Interferon (IFN)-γ was from R&D Systems, Inc. (Minneapolis, MN). Stigmasterol was from Steraloids Inc. (Newport, RI). N,O-Bis(trimethylsilyl)-trifluoroacetamide at 10% in trimethyl-chlorosilane was from Sigma.

FNC cultures

FNC were isolated from human fetal olfactory neuroepithelium, cloned, and long-term cell cultures were established and propagated in Coon's modified Ham's F12, supplemented with 10% fetal calf serum (FCS) and antibiotics (growth medium), as described previously (16). The B4 clone, showing the highest levels of expression of neuronal and olfactory markers, was used in this study. In addition, the B4 clone stained positively for both the ERα and ERβ (18). When the cells were treated with $17\beta E_2$, the growth medium was without phenol red and supplemented with 1% charcoal-stripped FCS.

Seladin-1 siRNA design

The design of siRNA sequences was based on current recommendations for siRNA oligonucleotide design, such as a 19 nucleotide (nt) double-stranded complementary region, a GC ratio between 45 and 55%, and exclusion of specific motifs such as G or C triplets (19). In addition to current recommendations, siRNAs were designed also on the basis of structural characteristics of the target RNA. For prediction of RNA secondary structures, a computer-aided structure analysis was performed as described (20). The computer program mfold 2.0, which is included in the Heidelberg Unix Sequence Analysis Resources (21), was used to predict secondary structures of the seladin-1 RNA. Based on this computer analysis, favorable target elements for short-chain inhibitory nucleic acids, such as large loops, bulges, joints, and free ends (20), were selected on the basis of semiempirical data. The alignment of the sequences of the siRNAs with sequences of the National Center for Biotechnology Information (NCBI) nt databases was performed using the Basic Local Alignment Search Tool (BLAST) program (NCBI, Bethesda, MD). The sequences showed no homology with any cDNA from the database. siRNAs against seladin-1 (target sequence: 5′-AAGAAGTACGTCAAGCTGCGT-3′) and negative control (siCONTROL nontargeting pool no. 2) were synthesized by a commercial supplier (Dharmacon, Lafayette, CO). All siRNAs were High-Performance Purity-Grade and analyzed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. The lyophilized RNA was dissolved in ribonuclease-free deoxyribonuclease-free water (Sigma) resulting in a 20 μM stock solution.

Seladin-1 siRNA transfection and IFN induction test

Cells were grown to confluence between 70 and 80% in six-well culture plates and washed with prewarmed (37 C) PBS. siRNAs targeting seladin-1 or control siRNAs (100 nM) were mixed with Lipofectamine 2000 (Invitrogen s.r.l., Milan, Italy) and administered to the cells (24 h), following the manufacturer's instructions. For seladin-1 expression studies, treatment with $17\beta E_2$ (10 nM) or IFN-γ (1000 U/ml) was performed for an additional 24 h after silencing. Experiments (n = 3) were performed in triplicate.

Quantitative RT-PCR analysis

Total RNA was isolated using NucleoSpinRNAII (Macherey-Nagel, Duren, Germany) with deoxyribonuclease treatment according to the manufacturer's instructions, the concentration determined spectrophotometrically with Nanodrop ND-1000, and the integrity of RNA was verified by measuring expression of β-actin gene by real-time RT-PCR (Hs00242273_m1; Applied Biosystems, Foster City, CA). The amount of seladin-1 mRNA was determined as described previously (16). The IFN induction test was performed by real-time RT-PCR measuring the expression of the following genes: chemokine C-X-C motif ligand 10 (CXCL10) (Hs99999049_m1; Applied Biosystems); chemokine C-X-C motif ligand 11 (CXCL11) (Hs00171138_m1; Applied Biosystems); and indoleamine-pyrrole 2,3 dioxygenase (INDO) (Hs00158027_m1; Applied Biosystems). According to the comparative threshold cycle (Ct) method, the amount of target mRNA normalized to an endogenous reference (18S rRNA) and relative to an internal control was calculated by $2^{-\Delta\Delta Ct}$. The results (mean ± SE) were expressed as fold mRNA variations compared with control. All measurements were performed in triplicate, and three experiments were performed.

Western blot analysis

Samples were kept in lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 0.2 mM EDTA, 0.3% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin (Sigma)] for 2 h at 0 C. Protein concentration was measured using a Coomassie Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). SDS-PAGE and Western blot analysis for the detection of seladin-1 were performed as described previously (22). The intensities of the immunoreactive bands were quantified by Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories). Each band was normalized with respect to its corresponding signal stained with ponceau to verify the degree of protein

loading, and the values were expressed as an intensity ratio (OD units). Results are the mean ± SE of three experiments.

Cell cholesterol measurement

The amount of cell cholesterol was determined by gas chromatography-mass spectrometry as described previously (23), with minor modifications. Briefly, cells were maintained in medium without fetal bovine serum for at least 6 h, then lysed in 1 N NaOH, and the cell lysate was used for cholesterol quantification or frozen for storage at −80 C until cholesterol measurement. Aliquots corresponding to 5.0 μg protein, as determined by Coomassie staining, were used for cholesterol determination. After the addition of stigmasterol (1000 ng) as an internal standard, sterols were extracted with n-exane, derivatized with N,O-Bis(trimethylsilyl)-trifluoroacetamide at 10% in trimethyl-chlorosilane, and automatically injected in a Hewlett-Packard gas chromatography-mass spectrometry system (Hewlett-Packard Co., Palo Alto, CA). A six-point calibration curve in the 50- to 2000-ng cholesterol range was used for cholesterol quantification. The peak area ratios were calculated using the signals at 458 and 484 m/z (mass-to-charge ratio) for cholesterol and stigmasterol, respectively. Each point was performed in duplicate, and the results represent the mean ± SE of three experiments.

Viability assays

Cell viability after β-amyloid exposure was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, as described previously (17). Briefly, the cells were seeded in 96-well plates for 24 h in medium without antibiotics. Thereafter, the cells were subjected to seladin-1 silencing or to control siRNA for 24 h, and 17βE_2 (10 nM) was added for a further 48 h in selected wells. During the last 18 h, 100 nM β-amyloid was added to each well, and afterwards the assay was performed according to the manufacturer's instructions. Multiwell dishes were analyzed by an ELISA plate reader (Seac-Radim, Moncalieri, Italy), and absorbance at 490 nm was considered directly proportional to the number of living cells. The results were expressed in terms of mean ± SE viable cells per well in three different experiments.

To assess the resistance against H_2O_2-mediated oxidative stress, viable cells were determined by Trypan blue dye exclusion test, as described previously (17). Briefly, the cells were cultured in 25 cm^2 flasks in medium without antibiotics. Thereafter, seladin-1 silencing or negative control silencing was performed for 24 h, and 17βE_2 (10 nM) was added for 48 h to selected cell samples. During the last 20 h, 200 μM H_2O_2 was added. Subsequently, cells were stained with Trypan blue dye for 1 min. Blue-positive (*i.e.* dead) and white-negative (*i.e.* living) cells were counted in 10 fields (20×), and the results were expressed as the mean ± SE of viable (over total) cells per field in three different experiments.

Immunostaining for cleaved caspase-3

The amount of caspase-3-immunoreactive cells was determined as described previously (17). Briefly, the cells were seeded in chamber slides and incubated for 24 h in medium without antibiotics. Thereafter, seladin-1 silencing was performed, 17βE_2 (10 nM) was added to selected chamber slides, and the slides were incubated at 37 C in a humidified atmosphere for 48 h (5% CO_2/95% air). During the last 20 h, 200 μM H_2O_2 was added to each chamber slide. The cells were then fixed in paraformaldehyde, incubated with a polyclonal antibody against cleaved caspase-3 (Asp 175) (Cell Signaling Technology, Inc., Beverly, MA), and subsequently with a biotinylated secondary antibody. The reaction product was visualized by ABC peroxidase-based detection protocol and AEC kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Finally, the cells were counterstained with hematoxylin according to the manufacturer's instructions. Apoptotic cells per field were counted in 10 fields (40×), and the results were expressed as the number of apoptotic cells per field (mean ± SE). Experiments (n = 3) were performed in triplicate.

In silico analysis and vector design

The gene sequence on chromosome 1 was localized using the database NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi). A 6-kb region upstream seladin-1 open reading frame was analyzed using eukaryotic promoter identification programs (http://www.softberry.com/berry.phtml? topic=fprom&group=programs&subgroup=promoter; http://www.softberry.com/berry.phtml?topic=tssw&group=programs&subgroup=promoter; and http://www.softberry.com/berry.phtml?topic=tssg&group=programs&subgroup=promoter), and a promoter sequence was identified between −383 and −112 bp from the translational start site. A further analysis was performed using the GRAIL software (http://compbio.ornl.gov/grailexp/), which detects CpG islands, and TFPROM software (http://www.cbrc.jp/research/db/TFSEARCH.html) to identify transcription factor binding sites. The 1477-bp region detected (promosel) was amplified from human genomic DNA by PCR using the primers 5′ F2(kpn)-ggtaccTCTTGGTCAATCTGCATTCG-3′; 5′R2(nhe)-gctagcCTGGAGTCAAAGCAGCTTCC-3′, containing flanking *KpnI* and *NheI* recognition sequences, respectively. Primer sequences were selected using the software Primer Express (Applied Biosystems). The PCR products were gel purified and ligated upstream of the firefly luciferase coding region of the ptata-luc plasmid (24) after subcloning in pCRII-TOPO (Invitrogen), thus obtaining the ptataluc-sel plasmid. The ptata-luc vector backbone contains a minimal TATA E1b-promoter without enhancers; therefore, changes in luciferase activity can be attributed to the effect of the promosel insert. As a positive control, a 13-bp ERE sequence from the human complement C3 gene promoter (25) was inserted in the ptata-luc plasmid using the method reported by Hall *et al.* (26) to obtain the ptataluc-C3 ERE plasmid.

Cell transfection experiments

Chinese hamster ovarian (CHO) cells were cultured in Ham's/F12 medium supplemented with 10% FCS and maintained at 37 C in a humidified atmosphere containing 5% CO_2. The cells were plated at 80–90% confluence in 96-well dishes, in DMEM/F12 medium supplemented with FCS-charcoal-stripped 10% without phenol red or antibiotics 24 h before transfection. The following vectors were cotransfected into the cells: 1) ptataluc-sel plasmid, containing the promosel region, or positive control ptataluc-C3 ERE plasmid upstream from the firefly luciferase coding sequence (100 ng/well); 2) pCMV-ER overexpression plasmid containing the ERα coding sequence (18 ng/well) (27); and 3) pGL4.75 normalization plasmid containing the renilla luciferase coding sequence under the control of the CMV promoter (2 ng/well). The cells were transfected using Lipofectamine 2000 according to the manufacturer's recommendations. Five hours after transfection, the cells were treated with ER ligands 20 h before luciferase assays were performed. Luciferase reporter assays were performed using the Dual-Glo Luciferase Reporter Assay System (Promega Corp., Madison, WI). Passive lysis buffer was added to each well, and the dishes were incubated for 15 min at room temperature. An equal volume of Dual-Glo Luciferase Reagent was placed in every well and incubated for 10 min. Firefly luciferase luminescence was measured. Before measurement of renilla luciferase, 100 μl of the Stop and Glo reagent (Promega) was added to each well to quench the firefly luciferase reaction. Renilla luciferase luminescence was measured after an incubation of 10 min. All luciferase measurements performed on a Victor3 multilabel reader (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) represent an average of readings obtained in triplicate. Relative firefly luciferase light output was normalized by renilla luciferase output after appropriate subtraction of background light output. In most cases, data points represent the mean ± SE of two or three iterations of three to six independent experiments.

Statistical analysis

Data were expressed as mean ± SE. Statistical differences were analyzed using the unpaired Student's *t* test. Significance was adjusted for multiple comparisons of means using Bonferroni's approximation.

Results

Seladin-1 silencing in FNC

Seladin-1 siRNA and control siRNA were transfected into FNC by lipofection. Real-time RT-PCR analysis for the determination of seladin-1 mRNA showed that the amount of

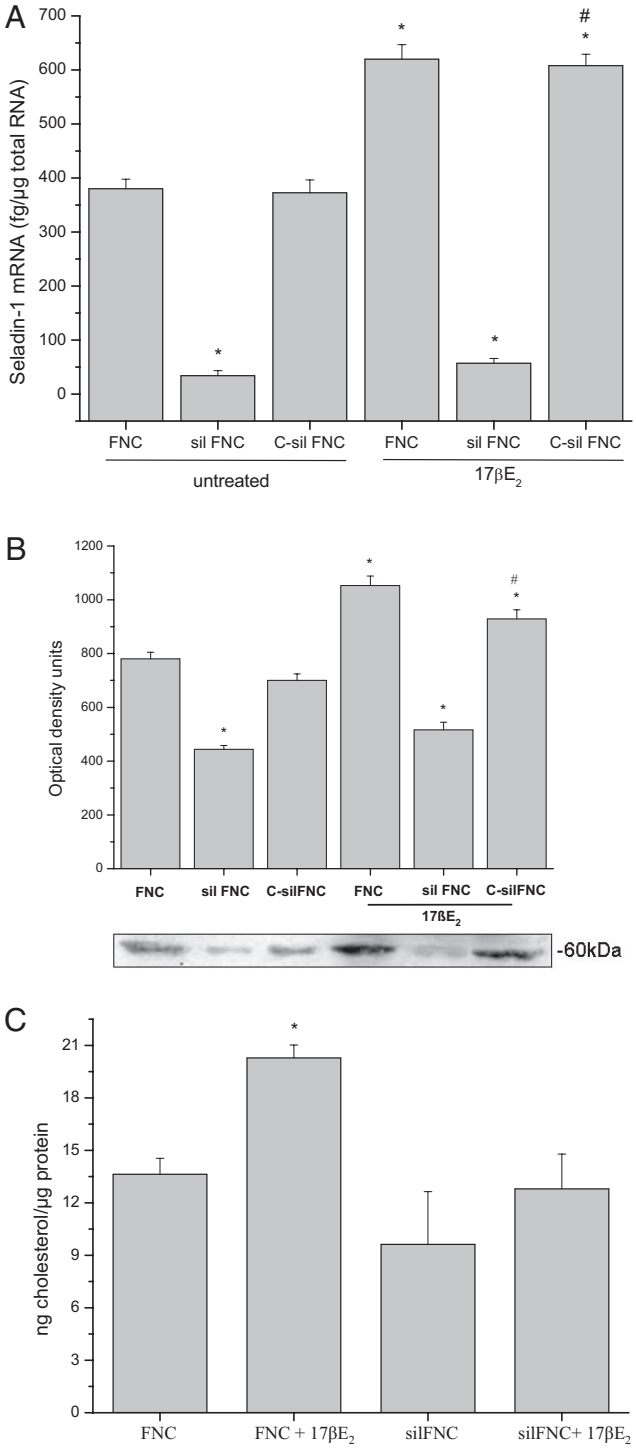


FIG. 1. A, Amount of seladin-1 mRNA in FNC and in FNC treated with seladin-1 targeting siRNA (silFNC) or with control siRNA (C-silFNC) for 24 h, with or without exposure to 10 nM $17\beta E_2$ for a further 24 h, as assessed by real-time RT-PCR. Experiments (n = 3) were performed in triplicate. B, Densitometric analysis (values are reported as OD units) of Western blot experiments (n = 3) for the expression of seladin-1 protein detected in FNC, silFNC, or C-silFNC (24 h treatments), with or without exposure to 10 nM $17\beta E_2$ for a further 24 h. A representative immunoblot is shown at the *bottom of the panel*. *, $P < 0.05$ *vs* untreated. FNC. #, $P < 0.05$ *vs*. the corresponding untreated cells (A and B). C, Amount of cell cholesterol in FNC and silFNC (siRNA administration for 24 h), with or without exposure to 10 nM $17\beta E_2$ for a further 48 h. Each point was performed in duplicate, and the results represent the mean ± SE of three experiments. *, $P < 0.05$ *vs*. untreated FNC.

TABLE 1. IFN-γ-induced gene expression (fold increase *vs*. FNC)

	INDO	CXCL11	CXCL10
FNC	1 ± 0.2	1 ± 0.095	1 ± 0.09
silFNC	0.39 ± 0.05[a]	0.2 ± 0.05[a]	1.39 ± 0.2
+IFN-γ	3373.4 ± 21[a]	359.5 ± 2.5[a]	77.17 ± 6.2[a]

The values represent the mean ± SE of three independent experiments, performed in triplicate.
[a] $P < 0.05$.

the transcript was markedly decreased in FNC after 48 h from administration of seladin-1 targeting siRNA (silFNC), but not in cells transfected with control siRNA(C-silFNC), compared with untreated cells (Fig. 1A). Treatment with $17\beta E_2$ (10 nM) increased the expression level of seladin-1 in FNC, confirming previously reported data (17), and in C-silFNC, but not in silFNC. Because it has been shown that siRNA may activate the innate immune response and trigger the expression of IFN-responsive genes (28, 29), the transcription level of the IFN-responsive genes INDO, CXCL10, and CXCL11 was also assessed by real-time RT-PCR (Table 1). No significant increase was observed in silFNC compared with FNC, thus indicating that the introduction of the seladin-1 targeting siRNA, selected for our experimental design, does not have an IFN-mediated off-target effect.

In agreement with the mRNA data, the amount of seladin-1 protein was also significantly decreased in silFNC, but not in C-silFNC, as assessed by immunoblot analysis (Fig. 1B). A significant reduction of seladin-1 protein was maintained also after 72 h from silencing (data not shown). In agreement with mRNA data, $17\beta E_2$ determined a significant increase of seladin-1 protein in FNC and in C-silFNC, but not in silFNC. The measurement of cell cholesterol in silFNC revealed a moderate, yet not statistically significant, decrease *vs*. FNC. The exposure to 10 nM $17\beta E_2$ determined a significant increase in the amount of cell cholesterol in FNC, but not in silFNC (Fig. 1C).

Loss of the protective effect of $17\beta E_2$ on cell survival in silFNC

We have previously demonstrated that $17\beta E_2$ effectively counteracts β-amyloid-induced toxicity in FNC (17). Here, we confirmed those findings in both FNC and C-silFNC, whereas we found that in silFNC $17\beta E_2$ (10 nM) fails to protect the cells from the toxic insult of β-amyloid, as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Fig. 2A). It has to be said that these results may not be affected by differences in cell proliferation because we have previously shown that 10 nM $17\beta E_2$ does not have any effect on the rate of FNC proliferation (17).

Furthermore, the effect of $17\beta E_2$ against H_2O_2-mediated oxidative stress in silFNC was assessed by the Trypan blue dye exclusion test. $17\beta E_2$ exposure prevented H_2O_2-induced cell death in FNC, as demonstrated previously (17), and in C-silFNC. Conversely, the pro-survival effect of this hormone was lost in silFNC (Fig. 2B). Overall, these results

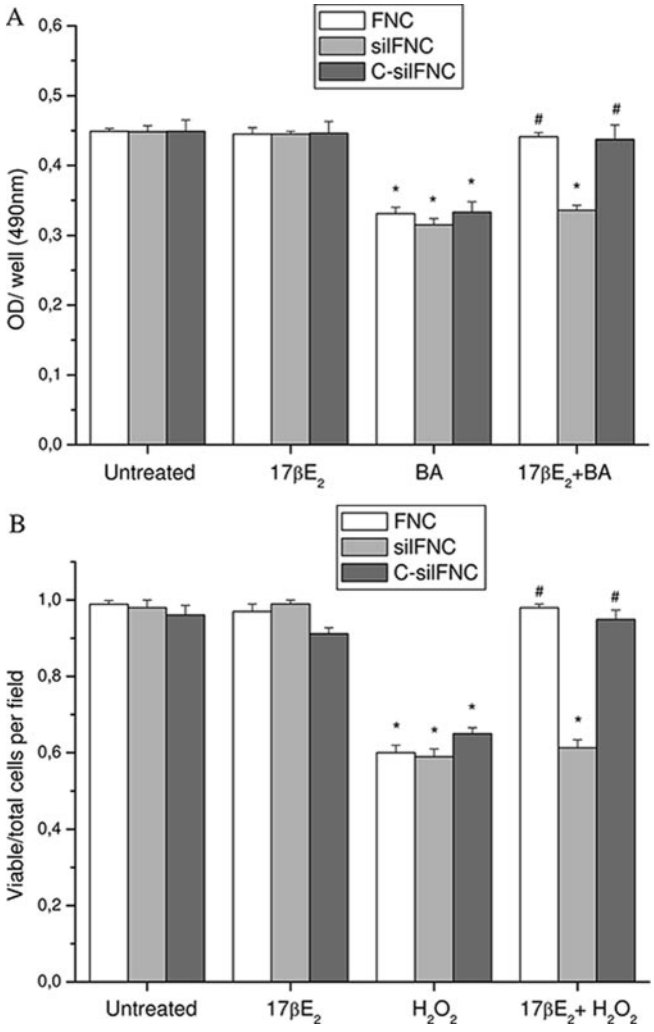


FIG. 2. A, Effect of $17\beta E_2$ (10 nM for 48 h) against β-amyloid (BA) (100 nM for 18 h) toxicity in FNC, silFNC, or C-silFNC. B, Effect of $17\beta E_2$ (10 nM for 48 h) against oxidative stress (200 μM H_2O_2 for 20 h) in FNC, silFNC, or C-silFNC. The results were expressed as mean percentage ± SE of viable cells per well in three different experiments. *, $P < 0.05$ *vs.* the corresponding untreated control cells. #, $P < 0.05$ *vs.* the corresponding cells exposed to β-amyloid (A) or H_2O_2 (B).

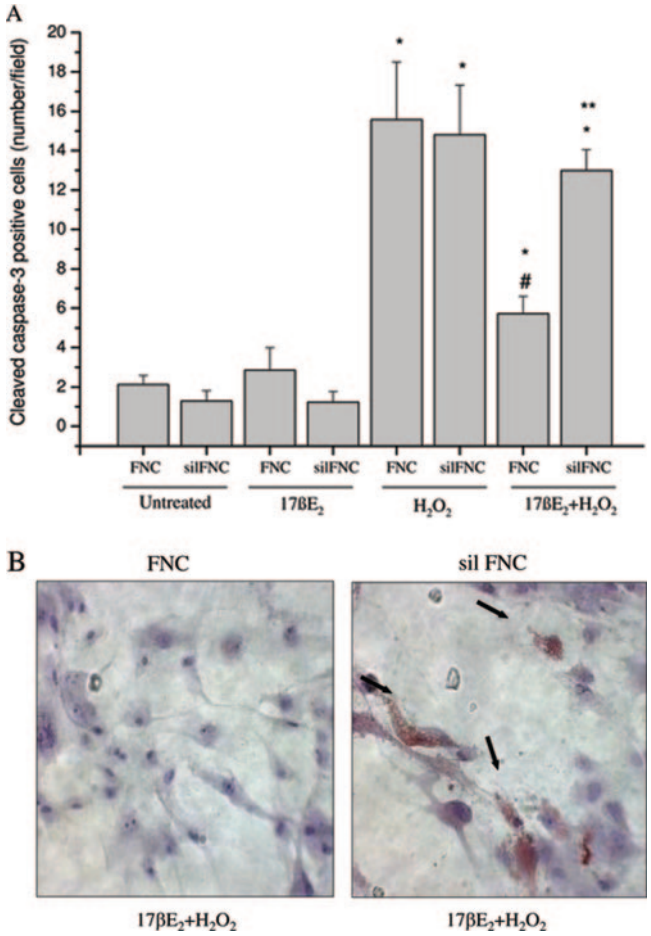


FIG. 3. A, Assessment of cleaved caspase-3 positive cells by immunocytochemistry. The cells were treated with $17\beta E_2$ (10 nM for 48 h), H_2O_2 (200 μM for 20 h), or with $17\beta E_2$ (10 nM for 48 h) plus H_2O_2 (200 μM, added during the last 20 h of $17\beta E_2$ exposure). B, Representative example showing FNC (*left*) and silFNC (*right*) pretreated with $17\beta E_2$ before exposure to H_2O_2. The *arrows* indicate cells showing a positive immunostaining for cleaved caspase-3. *, $P < 0.05$ *vs.* the corresponding untreated cells and cells treated with 17βE2. #, $P < 0.05$ *vs.* the corresponding cells treated with H_2O_2. **, $P < 0.05$ *vs.* FNC treated with 17βE2 plus H_2O_2. Experiments (n = 3) were performed in triplicate.

indicate for the first time that seladin-1 plays a crucial role in mediating the protective effects of $17\beta E_2$ against β-amyloid toxicity and oxidative stress.

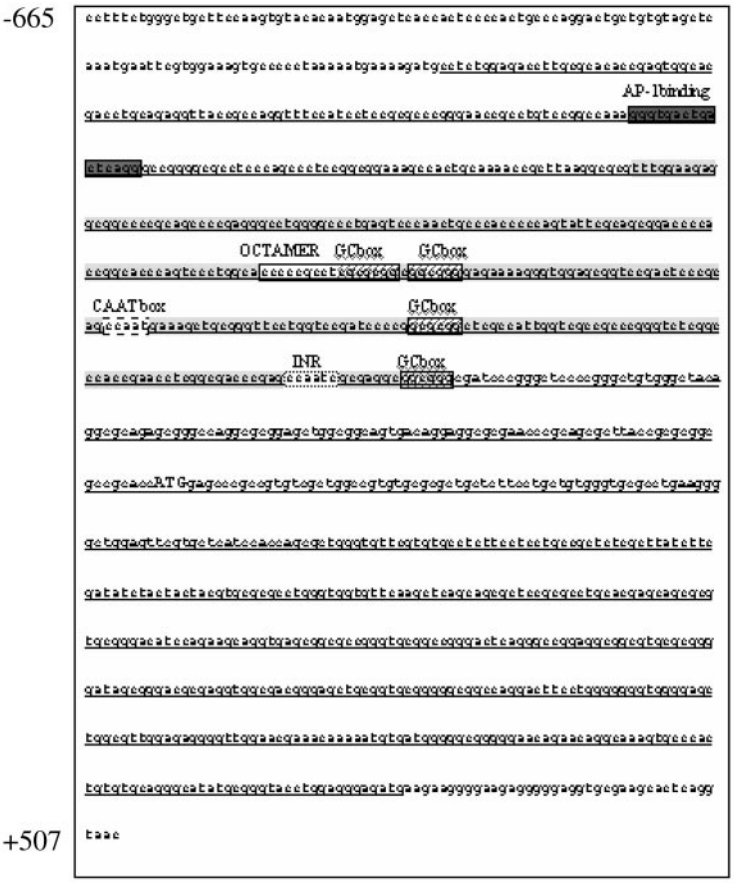
Loss of the effect of $17\beta E_2$ in preventing H_2O_2-induced caspase-3 activation in silFNC

There is evidence that seladin-1 prevents caspase-3 activation (14). We have previously shown that $17\beta E_2$ counteracts H_2O_2-induced caspase-3 activation in FNC (17). To determine whether seladin-1 is a mediator of the antiapoptotic effect of $17\beta E_2$ in these cells, we evaluated whether this hormone retains its ability to prevent H_2O_2-induced caspase-3 activation in silFNC. The results, shown in Fig. 3A, indicate that a few cleaved caspase-3 positive cells per field were detectable by immunocytochemistry in untreated or in $17\beta E_2$-treated FNC and silFNC. Upon exposure to 200 μM H_2O_2, a significant increase in the amount of cleaved caspase-3 positive cells was observed, which was counteracted by $17\beta E_2$ in FNC, as demonstrated previously, but not in silFNC. A representative example is shown in Fig. 3B.

Characterization of the seladin-1 gene promoter and identification of ERE sequences

The *seladin-1* gene promoter sequence (−383/−112 bp from the translation start site) was identified for the presence of several general transcription factor binding sites and of a wide CpG island going from −551 to +467 (Fig. 4). A 6-kb region upstream from the identified gene promoter was analyzed to identify regulatory sequences that confer estrogen responsiveness acting as transcriptional enhancers. This region revealed no perfectly palindromic ERE sequences, although a large number of half-palindromic EREs were found from −5000 to −3000, as shown in Fig. 5A. Here, two short

A



B

Binding site	Distance from ATG
Inr	-131
CAAT-box	-225
Octamer	-280
GC-box	-118, -190, -264, -271
Ap-1	-456

FIG. 4. A, Characterization of the *seladin-1* gene promoter (marked in *gray*). The translational start site (ATG) is indicated in *capital letters*; the CpG island is *underlined*. The major transcription factor binding sites are *boxed*. B, General transcription factor binding sites and their localization on the *seladin-1* gene promoter sequence.

sequences showing a very high homology percentage with ERE-like sequences, whose transactivational ability had already been observed for other gene promoters, were also detected:

1. A 29-bp region TGGCCA(N)3TGGTC(N)9TGACCN (−4148) (seq-A, *gray square*), which shows a 97% homology with a sequence detected in the *BRCA1* gene promoter, whose estrogenic responsiveness has already

A

B

SeqA 5'-*T*GGCCA*GGC*TGGTC*TCGAACTCC*TGACC*T*-3'
BRCA1 5'-*T*GGTCA*GGC*TGGTC*TGGAACTCC*TGACC*T*-3'
Alu ERE 5'-*T*GGTCA*GGC*TGGTC*TCAAACTCC*TGACC*T*-3'

SeqB 5'-*T*AGCCA*GGA*TGGTC*T*GA*T*CTCC*TGACC*T*-3'
BRCA1 5'-*T*GGTCA*GGC*TGGTC*TGGAACTCC*TGACC*T*-3'
Alu ERE 5'-*T*GGTCA*GGC*TGGTC*TCAAACTCC*TGACC*T*-3'

C

Match	BRCA1	*Alu* ERE
SeqA	97%	86.4%
SeqB	82.7%	79.3%

FIG. 5. A, Half-ERE-rich regions (*gray boxes*) upstream from the *seladin-1* gene promoter. SeqA, *gray square*; SeqB, *black square*. Primer sequences are *underlined*. B, Sequences found upstream from the *seladin-1* gene promoter, compared with those found in the *BRCA1* gene promoter and to *Alu* EREs. The mismatches are marked in *light gray*. The half-palindromic sequences are *underlined*; the spacer regions are in *italics*. C, Homology percentage of the aligned sequences.

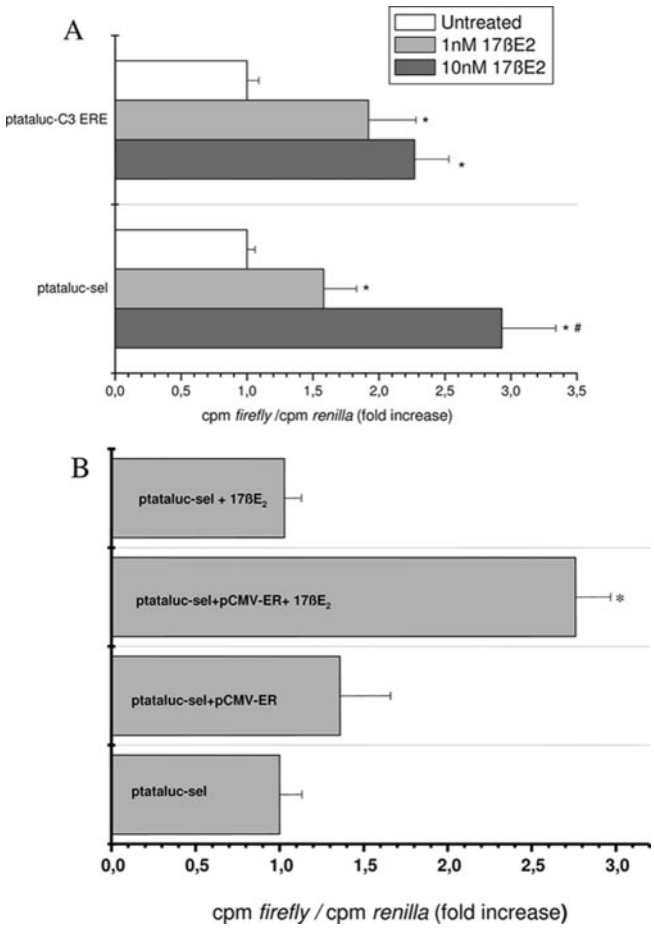


FIG. 6. A, Normalized luciferase activity elicited by $17\beta E_2$ (1 or 10 nM for 20 h) in CHO cells cotransfected with a plasmid containing the *C3* gene promoter (ptataluc-C3 ERE) (positive control) or the promosel sequence (ptataluc-sel) and an ERα-overexpressing plasmid (pCMV-ER). *, $P < 0.05$ *vs.* untreated cells. #, $P < 0.05$ *vs.* cells transfected with ptataluc-sel and treated with 1 nM $17\beta E_2$. Experiments (n = 6) were performed in triplicate. B, Specificity assay showing normalized luciferase activity of ptataluc-sel transfected cells in the presence or not of $17\beta E_2$ and/or pCMV-ER transfection.*, $P < 0.001$ *vs.* cells only transfected with ptataluc-sel. Experiments (n = 3) were performed in triplicate.

been proven (30). Seq-A also shows a high homology (86.4%) with *Alu* ERE sequences (31) (Fig. 5, B and C).

2. A 28-bp sequence (seq-B, black square), located at −3789 (82.7 and 79.3% homology with *BRCA1* and *Alu* ERE, respectively, Fig. 5, B and C).

Only two of the five mismatches found are located in the responsive sequences (Fig. 5B).

Evaluation of the estrogen responsiveness of the seladin-1 gene by luciferase assays

To verify the estrogen responsiveness of the detected half-palindromic EREs upstream from the promoter region of the *seladin-1* gene, the sequence from −4384 to −2892 was cloned into ptata-luc plasmid (ptataluc-sel) and transfected into CHO cells, to perform transactivational assays. The cells were cotransfected with an ERα overexpressing vector (pCMV-ER). Treatment with $17\beta E_2$ (1 and 10 nM) induced a dose-dependent significant increase in luciferase transcription, as assessed by light output measurement (1.6- and 2.9-fold *vs.* control, respectively). The results were comparable to those observed in CHO cells transfected with the same plasmid containing ERE sequences within the *C3* gene promoter (ptataluc-C3 ERE), as a positive control (Fig. 6A). Luciferase activity in ptataluc-sel transfected cells increased only in the presence of both ERα overexpression and $17\beta E_2$ stimulation, thus indicating the specificity of the assay (Fig. 6B).

We had previously demonstrated that the SERMs raloxifene and tamoxifen are able to up-regulate the expression of seladin-1 (17). Therefore, in the present study, the responsiveness of the promoter region of the *seladin-1* gene to these molecules was also tested. As shown in Fig. 7, treatment with 10 nM raloxifene or tamoxifen induced a significant increase in luciferase activity. Again, the results were similar to those observed in CHO cells transfected with ptataluc-C3 ERE. Finally, the responsiveness of the ptataluc-sel to the phytoestrogens genistein, zearalenone, and quercetin, which bind ERs with different affinity, was assessed. Preliminary results obtained with genistein indicated that this soy isoflavone also up-regulates the expression of seladin-1 (Luciani, P., and C. Deledda, un-

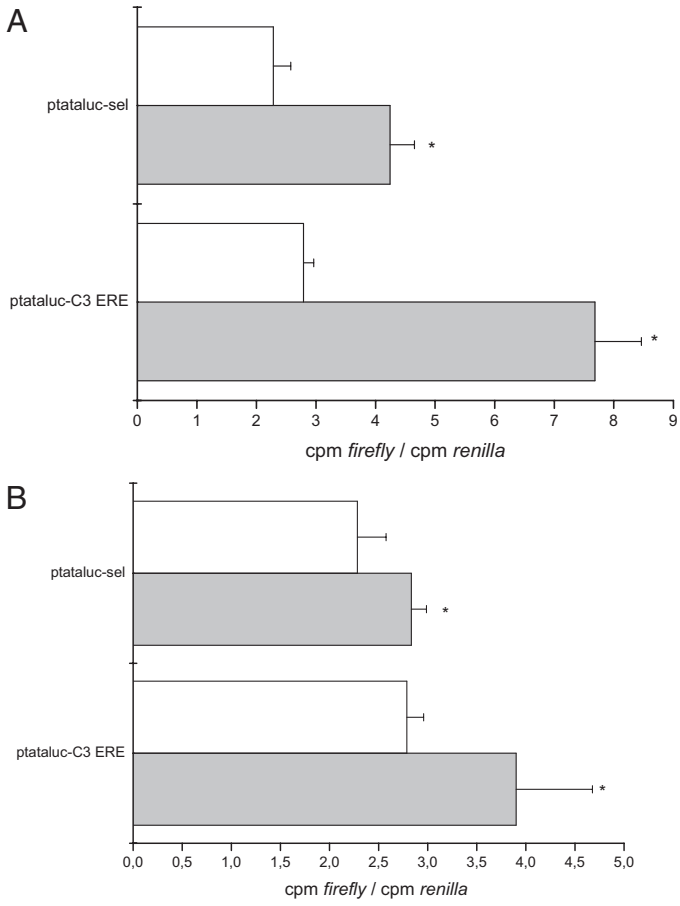


FIG. 7. Normalized luciferase activity detected in CHO cells cotransfected with pCMV-ER and the ptataluc-C3 ERE vector or the ptataluc-sel vector before (*white bars*) and after (*gray bars*) treatment with 10 nM raloxifene for 20 h (A) or 10 nM tamoxifen for 20 h (B). *, $P < 0.03$ *vs.* untreated cells. Experiments (n = 3) were performed in triplicate.

published data). We found that 1 μM genistein and zearalenone, but not quercetin, was able to induce luciferase activity, as shown in Fig. 8.

Discussion

Both *in vitro* and *in vivo* evidence supports a protective role of estrogen in the CNS (1–3); however, the efficacy of this family of hormones against AD has not been completely ascertained yet (9), and the molecular mechanisms of their action remain to be definitely elucidated. The results presented in this study demonstrate that the AD-related gene *seladin-1*, whose expression is up-regulated by 17βE_2 and SERMs (17), is one of the genes mediating the neuroprotective effects of estrogen. In fact, in our neuronal precursor cell model, the silencing of the *seladin-1* gene completely abrogated the protective effect of 17βE_2 against the toxic insults of β-amyloid and H_2O_2. In silFNC a significantly reduced expression of seladin-1 was obtained, and it is noteworthy that 17βE_2 was no more able to up-regulate the expression of this gene. Because siRNA may activate the innate immune response and trigger the expression of IFN-responsive genes (28, 29), we demonstrated that the introduction of siRNAs targeting seladin-1 in FNC did not stimulate the expression of three of these genes (*i.e.* INDO, CXCL11, and CXCL10). The reduction of the protein amount of seladin-1 obtained by gene silencing was less marked, although statistically significant, compared with the mRNA level. This result is probably due to a slow turnover of this protein. Moreover, the amount of cell cholesterol, the product of the seladin-1 enzymatic activity, did not significantly decrease 72 h after silencing. This is likely ascribable to the activity of the residual protein, the complex homeostatic system for cholesterol, and its extremely low turnover in the CNS (32–35). The moderate reduction of the amount of cell cholesterol in silFNC may explain the finding that seladin-1 silencing did not increase *per se* the detrimental effect of β-amyloid and oxidative stress on cell viability. Conversely, we demonstrated that the exposure to 17βE_2, which significantly increased both seladin-1 expression and cell cholesterol amount in FNC, prevented the toxic effect of β-amyloid and oxidative stress in these cells, yet not in silFNC. This finding is in agreement with our very recent demonstration that the 3β-hydroxysterol Δ24-reductase activity of seladin-1 plays a fundamental role in preventing β-amyloid-mediated toxicity (23). The specificity of our results was validated by the observation that the effects of 17βE_2 on both seladin-1 expression and on neuro-

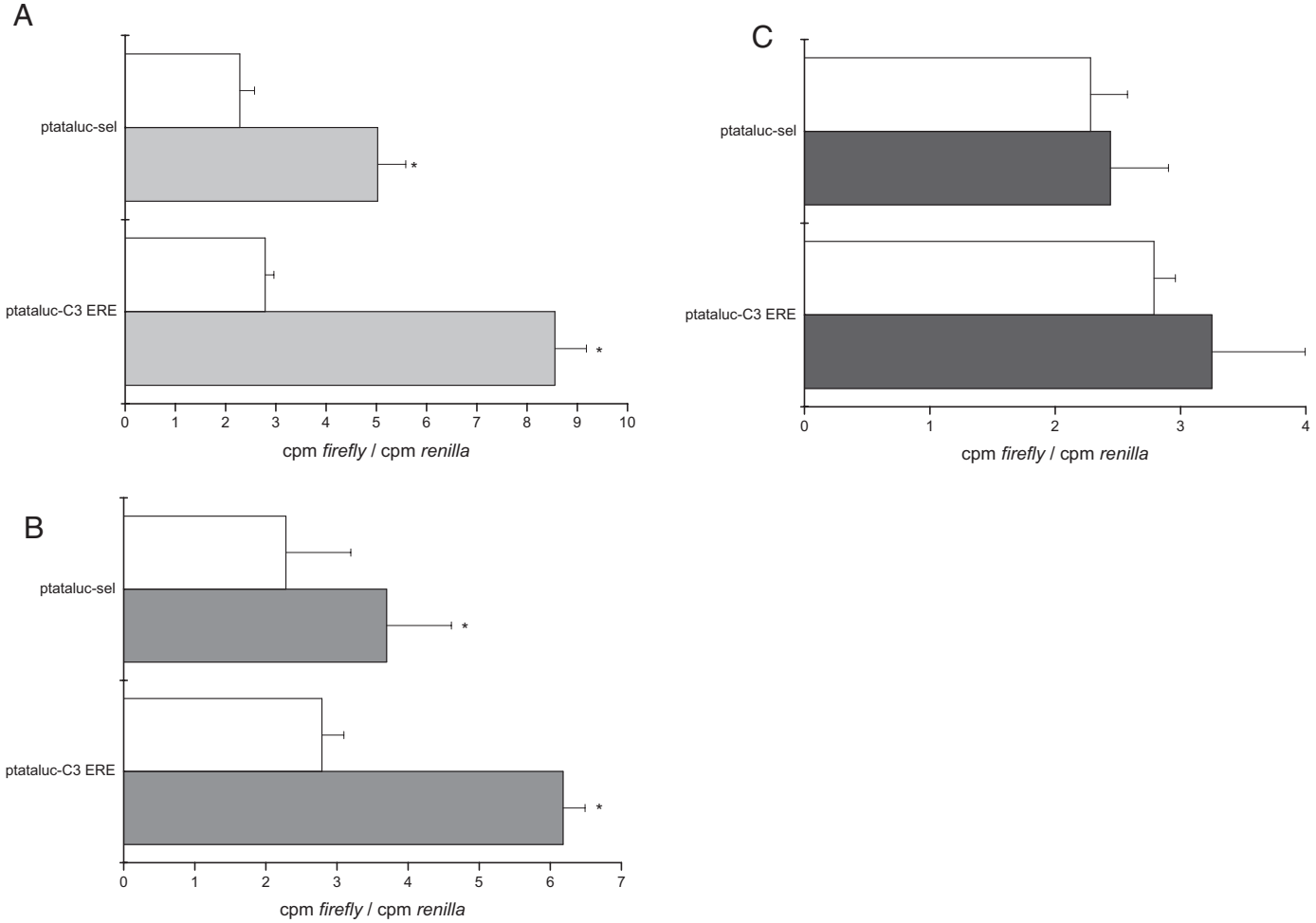


FIG. 8. Normalized luciferase activity detected in CHO cells cotransfected with pCMV-ER and the ptataluc-C3 ERE vector or the ptataluc-sel vector before (*white bars*) and after treatment with: 1 μM genistein for 20 h (A, *light gray bars*); 1 μM zearalenone for 20 h (B, *gray bars*); and 1 μM quercetin for 20 h (C, *black bars*). *, $P < 0.03$ *vs.* untreated cells. Experiments (n = 3) were performed in triplicate.

protection in cells treated with control siRNA were not different from those found in FNC. The neuroprotective properties of seladin-1 have also been associated with its inhibitory activity on the activation of the pro-apoptotic enzyme caspase-3 (14), and we have demonstrated previously that $17\beta E_2$ prevents H_2O_2-induced caspase-3 activation (17). Therefore, in the present study, we also evaluated whether seladin-1 is a mediator of the inhibitory effect of estrogen on the apoptotic cascade. In basal conditions, a few cleaved caspase-3 positive cells were detected both in FNC and silFNC. This finding is in agreement with previous observations in pituitary tumors. In fact, although lower expression levels of seladin-1 were found in GH-secreting adenomas compared with nonfunctioning adenomas, the amount of activated caspase-3 was similar in the two groups of tumors (36). It is conceivable that, in basal conditions, caspase-3 is minimally activated, independent of the amount of expression of seladin-1. In agreement with cell viability experiments, we found a similar increase in the number of apoptotic cells in FNC and silFNC upon exposure to H_2O_2. However, $17\beta E_2$ was able to counteract caspase-3 activation in FNC, yet not in silFNC, again suggesting the presence of a critical threshold for the expression and activity of seladin-1, to warrant effective protection against cell degeneration and apoptosis. Overall, these results indicate that seladin-1 is a pivotal mediator of the effects of $17\beta E_2$ against β-amyloid- and oxidative stress-induced toxicity, and against caspase-3 activation by H_2O_2 in human fetal neuroblasts. It is noteworthy that an important role of caspase-3 in determining β-amyloid production has been demonstrated very recently. In fact, it has been shown that caspase-3, by cleaving the adaptor protein GGA3 involved in β-secretase trafficking, prevents the degradation of this enzyme, thus increasing β-amyloid generation (37). These findings address caspase-3, together with its negative modulator seladin-1, as a possible target for pharmacological interventions against AD and, therefore, further support a proper role of estrogen in neuroprotective strategies. Of course, future trials designed to test the efficacy of estrogen against dementia should consider several critical issues that may have heavily influenced the negative results of the WHIMS, the only randomized clinical trial focused on the effects of HT on dementia risk, to date (10–13). For instance, in the WHIMS, women older than 65 yr were enrolled. Recent data suggest that HT confers neuroprotection when initiated closely to the menopausal transition (9), in agreement with the recent observation that $17\beta E_2$ exerts profound neuroprotective effects in mice when administered immediately after ovariectomy, but not after a delayed period of time (38). Another important issue is the choice of progestins. In the WHIMS the combined effect of medroxyprogesterone acetate and estrogen, but not estrogen alone, had a significant negative impact on dementia risk (9). Other studies supported a negative effect of medroxyprogesterone acetate, whereas a beneficial effect of natural progesterone in the CNS was addressed (39–41).

Furthermore, in the present study, the *seladin-1* promoter region was characterized to determine whether the estrogen responsiveness of this gene is mediated by EREs. The *seladin-1* gene promoter was localized at −383 bp from the translation start site. The sequence was analyzed by transcription factor binding motif identification softwares (42, 43), which allowed the identification of general transcription factor binding sites. The estrogen responsiveness results from the presence of ERE sequences that can act as enhancers and that are usually located many kilobase pairs upstream from the transcription start site (44). In the 6-kb region upstream from the *seladin-1* promoter that was analyzed, several half-palindromic ERE sequences were detected. These sequences are able to activate the transcription of the downstream genes even more when they are spaced by 15–20 nts (45). Some of these half-EREs have an AT-rich flanking region, which increases their affinity for ERs (46). The higher frequency of these sequences was in the 1.5-kb region, going from −4384 to −2887, in which two interesting motifs (seqA and seqB) were detected; these motifs show high similarity with two sequences previously described in: 1) the *BRCA1* gene promoter, a gene known to be involved in the development of breast and ovarian cancer (47); and 2) inside mutated *Alu* repeated elements (31). The estrogen responsivity of these sequences has been demonstrated (30, 31).

To verify the functionality of the half-palindromic ERE sequences upstream from the promoter of the *seladin-1* gene, transcriptional transactivation assays in response to administration of $17\beta E_2$, SERMs, and phytoestrogen molecules were performed in CHO cells cotransfected with vectors containing the putative enhancer ERE sequences of *seladin-1* and ERα. The choice to use ERα was based on the fact that we had demonstrated previously that the expression of seladin-1 is significantly increased by an ERα-selective agonist, but not by an ERβ-selective agonist (17). Transactivation assays demonstrated the responsivity of the promoter of *seladin-1* to $17\beta E_2$. It is noteworthy that luciferase activity increased only in the presence of both ERα and $17\beta E_2$, thus validating the specificity of the assay. These results indicate that the transcriptional activation occurs specifically as a consequence of the interaction of the DNA with the ERα-hormone complex. Similarly, tamoxifen and raloxifene increased the luciferase output signal, in agreement with the up-regulation of seladin-1 expression after SERMs treatment observed previously in FNC (17). Finally, we evaluated whether phytoestrogens were also able to elicit promosel-induced transactivation of the reporter gene. Phytoestrogens are naturally occurring nonsteroidal chemicals derived from plant sources that can bind ERs, and induce ER-dependent DNA binding and activation of estrogen responsive promoters in many cell types (48). Many of these compounds, particularly isoflavanones, have been proposed as natural SERMs, and the use of phytoestrogens, such as those obtained from soy, has been proposed as an alternative therapy after menopause. Although the efficacy of such compounds has not been rigorously examined yet, there is evidence that phytoestrogens may be neuroprotective, and positively affect mood, cognitive function, and behavior (48, 49). According to these observations, a phase II clinical trial is currently ongoing to assess the effects of soy isoflavones on cognitive function in women and men with AD (www.clinicaltrials.gov; trial no. NCT00205179). We found that genistein (an isoflavone) and zearalenone (a mycoestrogen produced by the fungi *Fusarium* spp, which plays an important role as a plant pathogen) (50) significantly increased the light signal, whereas quercetin (a flavonol) did not. In agreement with these observations, Mueller *et al.* (51) reported that genistein and

zearalenone that bind, yet with different affinity, ERα and ERβ are able to transactivate the estrogen-responsive gene expression. On the other hand, quercetin has weak affinity for both ERα and ERβ, and its estrogen antagonistic property has been postulated (52). Together, these findings indicate that the *seladin-1* gene transcription is activated, upon exposure to $17\beta E_2$, SERMs, or phytoestrogens, through a direct interaction of ERα with the half-palindromic EREs located upstream from the gene promoter. In summary, our results indicate that seladin-1 is a direct mediator of the neuroprotective effects of $17\beta E_2$, and support a role for estrogen and estrogen-related molecules in pharmacological interventions against cognitive impairment and dementia.

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