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17β-Estradiol – A New Modulator of Neuroglobin Levels in Neurons: Role in Neuroprotection against H_2O_2-Induced Toxicity

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Key Words
Neuroglobin · Estrogen receptor · H_2O_2 neurotoxicity · Neuroprotection · Apoptosis

Abstract
Although discovered in 2000, neuroglobin (Ngb) functions are still uncertain. A contribution to the role played by Ngb in neurons could certainly derive from the identification of Ngb endogenous modulators. Here, we evaluate the possibility that Ngb could be regulated by 17β-estradiol (E_2) signaling in both SK-N-BE human neuroblastoma cell line and mouse hippocampal neurons. 1 nM E_2 rapidly induced a 300% increase in Ngb levels in both models. The E_2 effect was specific, being not induced by testosterone or dihydrotestosterone. The E_2-induced Ngb increase requires estrogen receptor (ER) β, but not ERα, as evaluated by the mimetic effect of ERβ-specific agonist DPN and by the blockage of E_2 effect in ERβ-silenced SK-N-BE cells. Furthermore, both rapid (15 min) ERβ-dependent activation of p38/MAPK and transcriptional ERβ activity were required for the estrogenic regulation of Ngb. Finally, E_2 exerted a protective effect against H_2O_2-induced neuroblastoma cell death which was completely prevented in Ngb-silenced cells. Overall, these data suggest that Ngb is part of the E_2 signaling mechanism that is activated to exert protective effects against H_2O_2-induced neurotoxicity.

Introduction

Neuroglobin (Ngb), the third member of the globin family [1], is a monomeric hexa-coordinated heme protein of 17 kDa expressed not only in neurons of the central and peripheral nervous systems, but also in the gastrointestinal tract and in endocrine organs [1–18]. Recently, Ngb has also been detected in human glioblastoma cell lines [17] and in quiescent astrocytes of the healthy seal brain [15]. Although Ngb occurs at relatively low concentrations (μM) in a wide range of tissues, Ngb is found at relatively high concentrations in highly metabolically active cells and certain specialized cells, such as neurons of the hypothalamus, and particularly in retinal rod cells where its concentration has been estimated to be up to 100 μM [18–21]. Ngb binds several ligands, including diatomic gaseous ligands, and displays (pseudo-)enzymatic properties [1, 3, 6, 10, 22–33]. The P_{50} value for O_2 binding

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to Ngb has been reported to range between 2 and 10 Torr depending on pH, temperature, and the redox state of the cell [1, 22–24, 32].

Although discovered in 2000 [1], the cell function(s) of Ngb is still controversial. Indeed, the O_2 supply by Ngb to the mitochondria of the metabolically active neurons and retinal rod cells is highly debated [1, 15, 19, 33, 34]. In vitro, Ngb has been reported to scavenge nitrogen monoxide (NO) in the presence of high O_2 levels [25, 28, 35]; however, at low O_2 conditions Ngb may react with NO_2^- resulting in the formation of NO [36]. Therefore, the protective role of Ngb against NO in vivo is controversial [32, 33, 37]. Although in vitro Ngb does not react with hydrogen peroxide (H_2O_2) [25, 30] and the Ngb-NO_2 adduct reacts with H_2O_2 facilitating the nitration of aromatic substrates [30], the correlation between reactive oxygen species formation/decomposition and Ngb expression in vivo is debated [15, 38]. Moreover, Ngb has been reported to interact with several proteins (see [15]). In particular, Ngb binding to the Gα protein inhibits GDP dissociation, thereby protecting cells from apoptosis [39, 40]. Recently, in silico simulations indicate Ngb capability to reduce cytochrome *c* released from mitochondria suggesting its protective role against programmed cell death [19, 41–43].

Although Ngb properties are highly debated [12, 15, 19, 33, 44], it is unlikely that Ngb has so many distinct roles [15]; nevertheless, there is no doubt that Ngb is beneficial to neurons [15]. In vivo experiments, using transgenic rodents, have shown that increased levels of Ngb significantly protect both heart and brain tissues from hypoxic insult, whereas decreased Ngb levels lead to an exacerbation of tissue death [45–47]. In this way, Ngb could protect neurons from hypoxic insult by modulating the activation of the apoptotic cascade [19, 41]. A significant contribution to highlight the role played by Ngb in neuroprotection could derive from the identification of Ngb endogenous modulator(s) (e.g., hormones and neurotransmitters), but, as far as we know, no Ngb involvement in the hormone signal transduction pathways has been identified yet.

Female sex steroid hormones could represent good candidates as Ngb modulators. Indeed, in addition to their well-established role in reproductive organs, estrogens affect areas of the brain that are not primarily involved in reproduction [48]. Growing evidence documents profound effects of estrogens on learning, memory, and mood as well as neurodevelopmental and neurodegenerative processes [49, 50]. Although most studies have been conducted on females, there is mounting recognition that estrogens play important roles in the male brain, where they can be generated from circulating testosterone by local aromatase or synthesized de novo by neurons and glia [51]. Several sources of evidence confirm that estrogens serve as neurotrophic and neuroprotective agents. Notably, 17β-estradiol (E_2) attenuates the toxicity of the amyloid-β peptide and glutamate in a hippocampal cell line [52]. In addition, estrogen therapy in post-menopausal women is associated with decreased incidence and enhanced recovery from ischemic stroke [51]. The protective effects of estrogens have been widely reported in different types of neuronal cells against a variety of insults, including H_2O_2 [53, 54], serum deprivation [55], oxygen-glucose deprivation [56], and iron [57]. Due to myriad and often tissue-specific estrogen effects, the precise molecular events that mediate these protective actions are not fully understood. Here, we evaluate the possibility that Ngb could be part of 17β-estradiol-induced signals and effects in neuronal cells.

Materials and Methods

Reagents

E_2, testosterone (T), 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT), naringenin (Nar), insulin-like growth factor 1 (IGF-1), actinomycin D (Act), cycloheximide (Cxm), Pen-Strep solution, H_2O_2, RPMI-1640 media without phenol red, and charcoal-stripped fetal calf serum, the palmitoyl acyltransferase (PAT) inhibitor 2-bromohexadecanoid acid (2-Br-palmitate; 2-Br), the protease inhibitor cocktail, and the bovine serum albumin fraction V (BSA) were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Optimem, Hank's buffer salt solution (HBSS 1×), Neurobasal medium, B27 serum-free supplement, and GlutaMAX-I were purchased from Gibco-BRL (Gaithersburg, Md., USA). The p38 inhibitor SB 203 580 (SB), the AKT inhibitor, and the IGF-1 receptor (IGF-1R) inhibitor picropodophyllin (PPP) were obtained from Calbiochem (San Diego, Calif., USA). The E_2 antagonist fulvestrant (ICI 182,780, ICI), the estrogen receptor (ER) α-selective agonist 4,4′,4′′-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT), the ERβ-selective agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), and the ERβ-selective antagonist (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC) were obtained from Tocris (Ballwin, Mo., USA). Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, Calif., USA). The human recombinant ERα and ERβ were obtained by PanVera (Madison, Wisc., USA). The anti-phospho-ERK1/2, anti-AKT, anti-ERα (MC20), anti-ERβ (H150), anti-caspase-3, anti-poly(ADP-ribose)polymerase (PARP), and anti-ERK1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The polyclonal anti-phospho-AKT, anti-phospho-p38, and anti-p38 antibodies were purchased from New England Biolabs (Beverly, Mass., USA). The monoclonal anti-human Ngb (13C8) was purchased from Abcam (Cambridge, UK). The anti-β-tubulin was purchased from MP Biomedical (Solon, Ohio, USA). The chemiluminescence reagent for Western blot ECL was

obtained from GE Healthcare (Little Chalfont, UK). All the other products were from Sigma-Aldrich. Analytical or reagent grade products were used without further purification.

Cells

The human SK-N-BE neuroblastoma cell line was routinely grown in air containing 5% CO_2 in modified, phenol red-free, RPMI-1640 medium containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2.0 mM), Pen-Strep solution (penicillin 100 U/ml, and streptomycin 100 mg/ml). Cells were passaged every 2 days. Cells were grown to approximately 70% confluence in 6-well plates before stimulation.

Hippocampal neurons were obtained from E18 mouse embryos after isolating the hippocampus in Ca^{2+}- and Mg^{2+}-free HBSS 1×. Mice were treated following the guidelines of the Council of Europe Convention ETS123, recently revised as indicated in the Directive 86/609/EEC. In addition, all protocols were approved by the Institutional Animal Care and Use Committee of CSIC-Cajal Institute (Madrid, Spain). Once 8–10 embryonic hippocampi were obtained, they were finely cut, washed twice in HBSS 1× buffer, and incubated in 0.1 mg/ml trypsin solution and 1 mg/ml DNAse (Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at 37°C. Trypsin and DNAse were then eliminated by washing 3 times, with HBSS 1×, and the cut tissue was then triturated using a siliconized pipette. Cells were counted and plated in polylysine-coated (1 mg/ml) 6-well plates containing phenol red-free neurobasal medium supplemented with 2% (v/v) B27 serum-free supplement, 0.25% (v/v) GlutaMAX-I, and 1% (v/v) penicillin/streptomycin solution. Neurons were maintained under these conditions for 3 days at 5% CO_2 and 37°C.

Cells were simultaneously treated with vehicle (ethanol/PBS 1:10, v/v) and/or E_2 (0.1–1,000 nM), PPT (0.1–100 nM), DPN (0.1–100 nM), T (0.1–1,000 nM), DHT (0.1–1,000 nM), IGF-1 (100 ng/ml), and H_2O_2 (50 μM). When indicated, the anti-estrogen ICI (1 μM), the PAT inhibitor 2-Br (10 μM), the AKT inhibitor (1 μM), the p38 inhibitor SB (5 μM), the ERβ inhibitor THC (1 μM), the IGF-1R inhibitor PPP (100 nM), and the transcription inhibitor Act (1 μg/ml) were added 30 min before E_2 or IGF-1 administration. The translational inhibitor Cxm (10 μg/ml), was added 1 h before E_2 administration.

Cell Viability

SK-N-BE cell lines were grown to 70% confluence in 6-well plates and stimulated either with vehicle or E_2 (1 nM) or THC (1 μM). After 24 h of stimulation, cells were treated either with vehicle or with H_2O_2 50 μM for 24 h. After treatment, cells were harvested with trypsin, and counted with Beckman Coulter Model ZM electronic particle (Palo Alto, Calif., USA).

Transfection of Short Interfering RNA

SK-N-BE cells, reaching 40–60% confluence, were transfected in a serum-free condition with either Stealth RNAi™ Ngb-silencing RNA or ERβ-silencing RNA (siRNA; Invitrogen) according to the manufacturer's instructions, using oligofectamine (Invitrogen) as the transfection reagent. The sequence used for Ngb oligonucleotides was 5′-CGUGAUUGAUGCUGCAGUGACCAAU-3′; the sequence used for ERβ oligonucleotides was 5′-GAAGAACUCUUUGCCCGGAAAUUUA-3′. The mismatch sequences used as a control were 5′-UGUGAUUUAUGGUGCAGUAACCAAC-3′ and 5′-GAAUCAUUCCGUGCCAAGUAGAUUA-3′ for Ngb and ERβ si-RNA, respectively. Briefly, oligofectamine and oligonucleotides (400 and 200 pM for Ngb siRNA and ERβ siRNA, respectively) were mixed with Optimem. The mixture was incubated for 20 min at room temperature, diluted with Optimem, and added to the cell medium for 4 h at 37°C. The medium was added to cells to reach the growing conditions (i.e., 10% (v/v) serum).

To evaluate the effective silencing of Ngb and ERβ, total proteins from cells transfected with MOCK (control), with scramble (mismatch sequence, data not shown), and with Ngb or ERβ oligonucleotides were extracted 48 h after transfection, and Ngb and ERβ expression was tested by Western blot analysis using anti-Ngb and anti-ERβ antibodies.

Western Blot Assays

After stimulation, the SK-N-BE cell line and hippocampal neurons were lysed and solubilized in 0.125 M Tris, pH 6.8, containing 10% (w/v) SDS and the protease inhibitor cocktail, then the cell lysates were boiled for 2 min. Total proteins were quantified using the Bradford protein assay. Solubilized proteins (20 μg) were resolved by 7 or 15% SDS-PAGE at 100 V for 1 h at 25°C and then electrophoretically transferred to nitrocellulose for 45 min at 100 V and 4°C. The nitrocellulose was treated with 3% (w/v) BSA in 138 mM NaCl, 25 mM Tris, pH 8.0, at 25°C for 1 h and then probed overnight at 4°C either with anti-Ngb (final dilution 1:1,000) or anti-ERα MC-20 (final dilution 1:500) or anti-ERβ H-150 (final dilution 1:3,000) or anti-caspase-3 (final dilution 1:1,000) or anti-PARP (final dilution 1:500) or anti-phospho-ERK1/2 (final dilution 1:200) or anti-phospho-AKT (final dilution 1:1,000) or anti-phospho-p38 (final dilution 1:1,000). The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, Ill., USA) for 10 min at room temperature and then probed with anti-β-tubulin (final dilution 1:1,000) to normalize total lysate. Moreover, the nitrocellulose incubated with either anti-phospho-ERK1/2 or anti-phospho-AKT or anti-phospho-p38 was stripped and probed with anti-ERK1/2 (final dilution 1:200), anti-AKT (final dilution 1:100) and anti-p38 (final dilution 1:1,000), respectively. To evidence ERα and ERβ levels, electrophoresis was performed in the presence of 5 ng of recombinant ERα and ERβ. Antibody reaction was visualized with chemiluminescence Western blot detection reagent.

Densitometric analyses were performed by ImageJ software for Windows. The densitometry quantification of protein was normalized to tubulin.

Statistical Analysis

A statistical analysis was performed by using ANOVA followed by Tukey-Kramer post-test with the GraphPad InStat3 software system for Windows. In all cases, $p < 0.05$ was considered significant.

Results

E_2 Specifically Increases Ngb Levels in Neurons

Figure 1 shows that E_2 stimulation induced a time- and dose-dependent increase in Ngb levels in SK-N-BE cells (fig. 1A, B). The E_2 (10 nM) effect on Ngb levels started 30

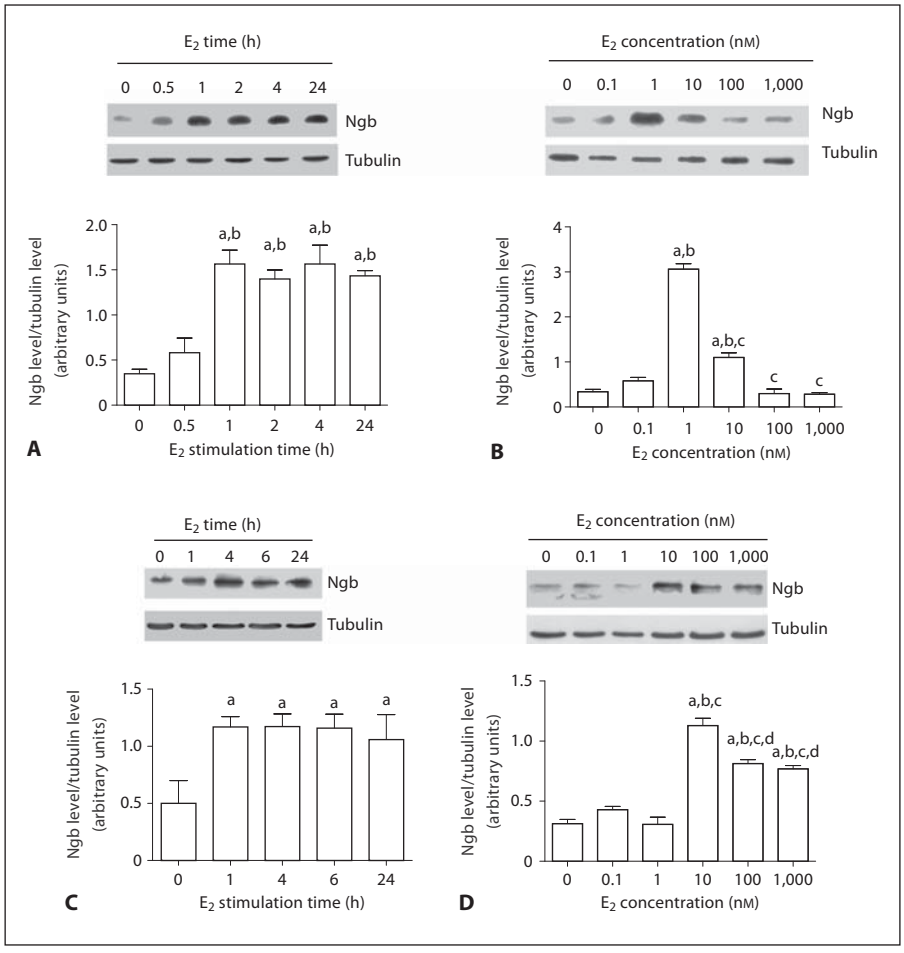

Fig. 1. Effect of 17β-estradiol (E_2) on Ngb protein levels in SK-N-BE human neuroblastoma cell line (**A**, **B**) and in mouse hippocampal primary neurons (**C**, **D**). **A**, **C** Time-course analysis of E_2 treatment (10 nM) on Ngb levels. **B**, **D** E_2 dose-dependent (0.1–1,000 nM) effect on Ngb levels (24 h of stimulation). The amount of protein was normalized by comparison with tubulin levels. The data are typical Western blots of five independent experiments (top panels); densitometric analysis related to E_2 dose- and time-dependent experiments (bottom panels). Data are means ± SD of five different experiments. $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test. **A**, **C** a significant vs. 0 h and b vs. 0.5 h; **B**, **D** a vs. 0, b vs. 0.1, c vs. 1, and d vs. 10 nM.

min after stimulation being significant 1 h after stimulation and remained constant 24 h after hormone stimulation. The E_2 dose-response curve was bell-shaped with a maximum effect at physiological E_2 concentrations (i.e., 1–10 nM; 24 h stimulation).

In contrast, the male sex steroid hormone DHT and the common precursor of E_2 and DHT, T, did not modify Ngb levels at any tested concentration (data not shown), suggesting the specificity of the E_2 effect. These data were confirmed in freshly isolated mouse hippocampal neurons (fig. 1C, D). Indeed, in these primary neurons, the E_2 effect was rapid (1 h), persistent (24 h) (fig. 1C), and specific in that neither DHT nor T were able to increase Ngb levels at any tested concentration (data not shown). In addition, even in mouse hippocampal neurons, 10 nM E_2 increased Ngb levels which remained significantly higher than control cells even at higher E_2 concentrations (fig. 1D). In line with the slight differences found in the E_2 concentration to obtain the maximum effect in both cell types, 1 and 10 nM were used in the consecutive experiments to stimulate the SK-N-BE cell line and hippocampal neurons, respectively.

ERβ was necessary for E_2-induced increase in Ngb levels. The pretreatment of SK-N-BE cells with the pure E_2 antagonist, ICI, completely prevented the E_2 effect on Ngb levels (fig. 2A), suggesting an ER-mediated mechanism. As SK-N-BE cells contain high ERβ and low ERα levels (fig. 2B), cells were stimulated with either the specific ERα agonist PPT or the specific ERβ agonist DPN to discriminate the role of each ER isoform in the E_2-induced Ngb level increase. Only 1 and 10 nM DPN mimicked the E_2 effect on Ngb levels (fig. 2C), whereas PPT was unable to increase Ngb levels, at any concentration investigated (fig. 2D). This result was confirmed by cell pretreatment with the specific ERβ inhibitor THC, which completely prevented the E_2 effect (fig. 2C). Furthermore, the decrease of ERβ protein level by ERβ SiRNA transfection caused an impairment of the E_2 ability to increase

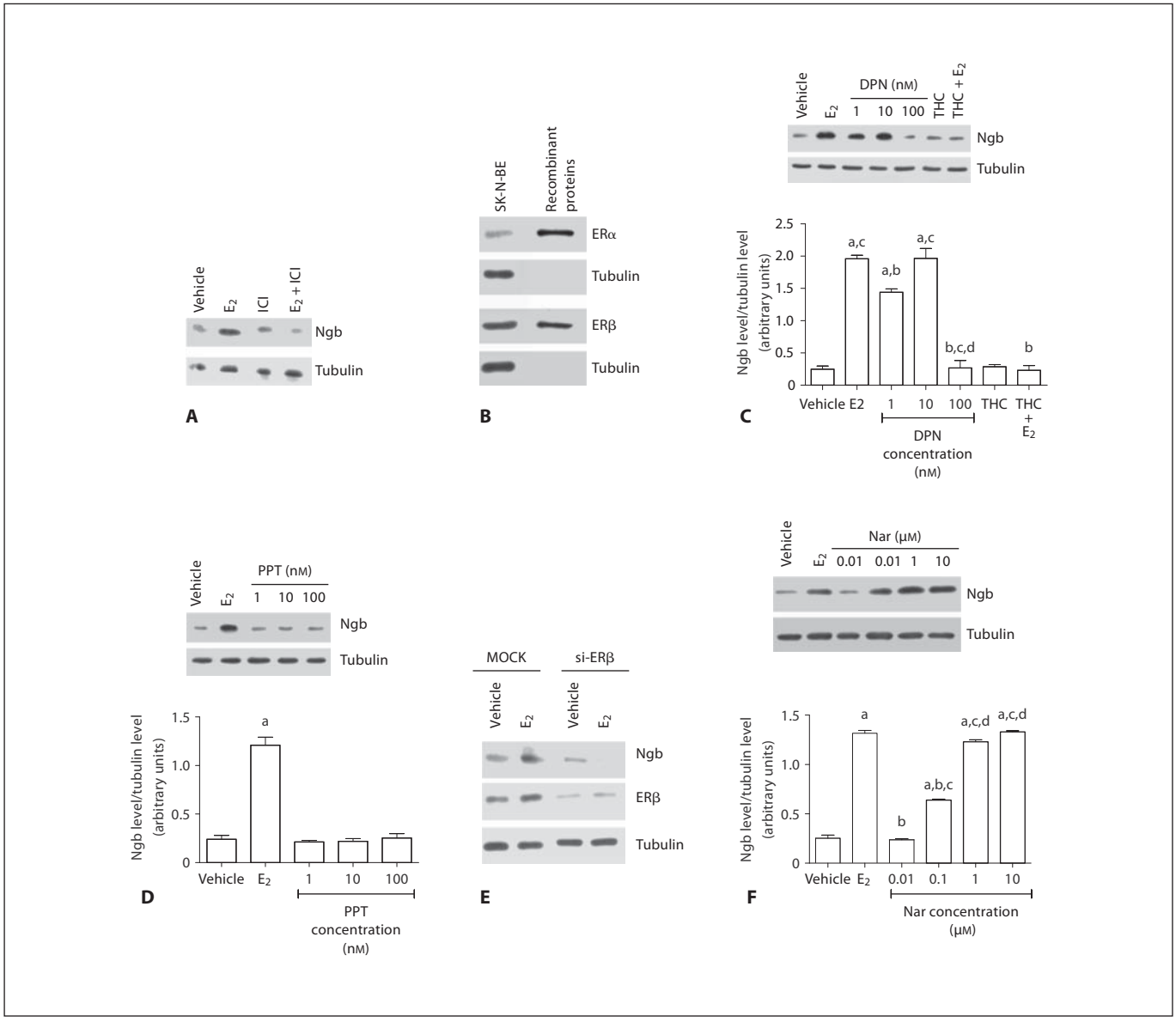

Fig. 2. Impact of estrogen receptors (ER) α and β on Ngb protein expression in SK-N-BE human neuroblastoma cell line. **A** Western blot analysis of Ngb levels in cells stimulated for 24 h with either vehicle or E_2 (1 nM) and/or the ER inhibitor ICI 182,870 (ICI; 1 μM). **B** ER isoform levels in non-stimulated cells compared to recombinant proteins (5 ng). **C** Analysis of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM), the ERβ agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN; 1–100 nM) or the ERβ-selective antagonist (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC; 1 μM). **D** Analysis of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM) or the ERα agonist 4,4′,4′′-(4-propyl [^{1}H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1–100 nM). **E** Analysis of Ngb and ERβ levels in cells transfected with either MOCK (control) or ERβ small interference mRNA (si-ERβ) in the absence or presence of E_2 (1 nM). **F** Analysis of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM) or naringenin (Nar; 0.01–10 μM). The amount of proteins was normalized by comparison with tubulin levels. The data are typical Western blots of four independent experiments; densitometric analyses related to DPN, PPT, and Nar dose-dependent experiments (**C**, **D** and **F** bottom panels). Data are means ± SD of four different experiments. $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test. **C**, **D** a significant vs. vehicle, b vs. E_2, c vs. 1, and d vs. 10 nM. **F** a significant vs. vehicle, b vs. E_2, c vs. 0.01, and d vs. 0.1 μM.

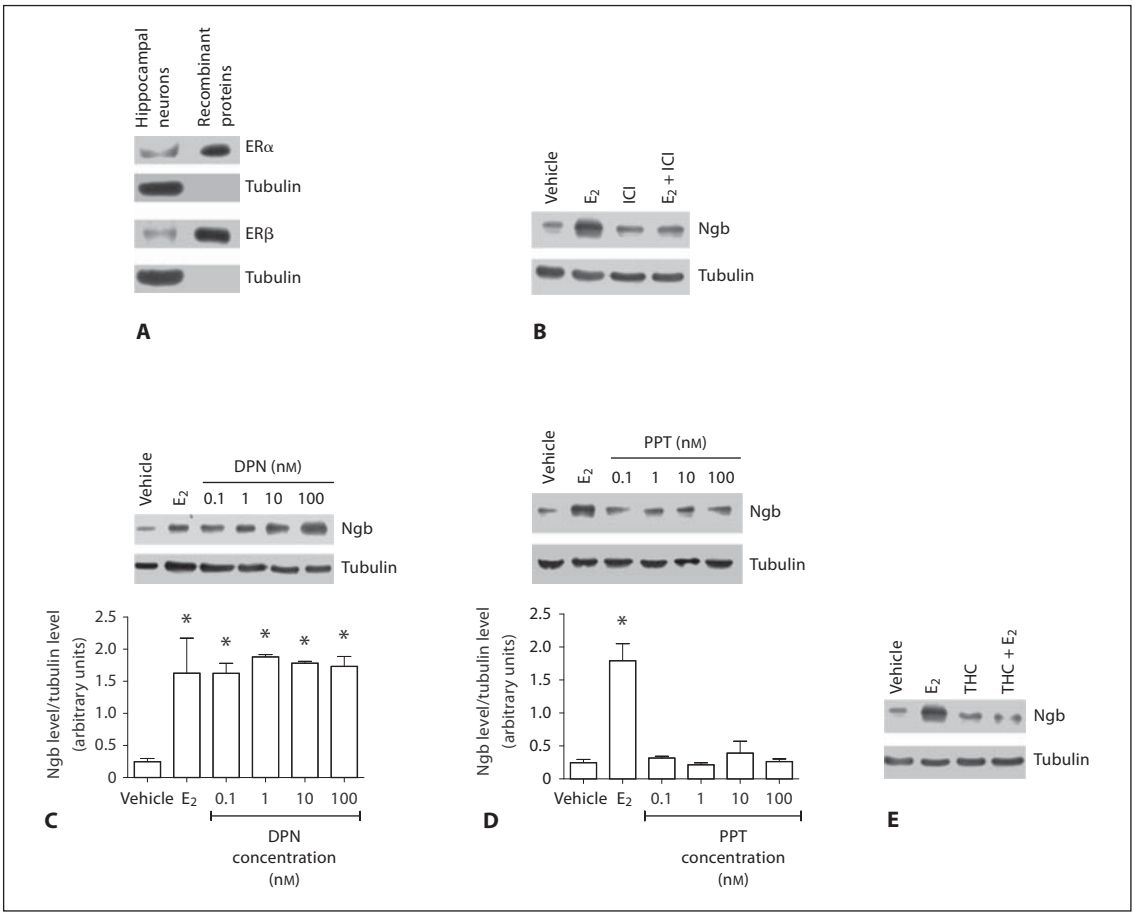

Fig. 3. Impact of estrogen receptors (ER) α and β on Ngb protein expression in mouse hippocampal primary neurons. **A** ER isoform levels in non-stimulated cells compared to recombinant proteins (5 ng). **B** Western blot analyses of Ngb levels in cells stimulated for 24 h with either vehicle or E_2 (10 nM) and/or the ER inhibitor ICI 182,870 (ICI; 1 μM). **C** Analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (10 nM) or the ERβ agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN; 0.1–100 nM). **D** Analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (10 nM) or the ERα agonist 4,4′,4′′-(4-propyl [^{1}H]-pyrazole-1,3,5-tri-yl)trisphenol (PPT; 0.1–100 nM). **E** Analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (10 nM) and/or the ERβ-selective antagonist (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC; 1 μM). The amount of proteins was normalized by comparison with tubulin levels. Typical blots of five independent experiments are shown; densitometric analyses related to DPN and PPT dose-dependent experiments (**C**, **D** bottom panels). Data are means ± SD of five different experiments. * $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test with respect to vehicle-treated samples.

Ngb levels (fig. 2E). A further confirmation of the ERβ involvement in the effect of E_2 effects derives from the results obtained using the flavonoid Nar (fig. 2F). Indeed, we previously reported that this flavonoid is a partial antagonist of E_2 in the presence of ERα [58] and an E_2 mimetic in the presence of ERβ [59]. Figure 2F shows that, like E_2, 0.1 μM Nar was sufficient to increase Ngb levels. This effect persisted at Nar high concentrations (i.e., 1 and 10 μM). ERβ was also necessary for the E_2-induced Ngb increase in mouse primary neurons (fig. 3). In these cells, containing a similar amount of ERα and ERβ (fig. 3A), ICI prevented the E_2 effect (fig. 3B), DPN mimicked the E_2 effect (fig. 3C), whereas PPT was unable to increase Ngb levels (fig. 3D). Cell pretreatment with the specific ERβ inhibitor THC further confirmed these results (fig. 3E).

Mechanism Involved in the E_2-Induced Increase of Ngb Levels in Neurons

ERs are ligand-activated transcription factors which possess both transcriptional and extranuclear activities [48]. Thus, we evaluated the impact on the E_2-induced

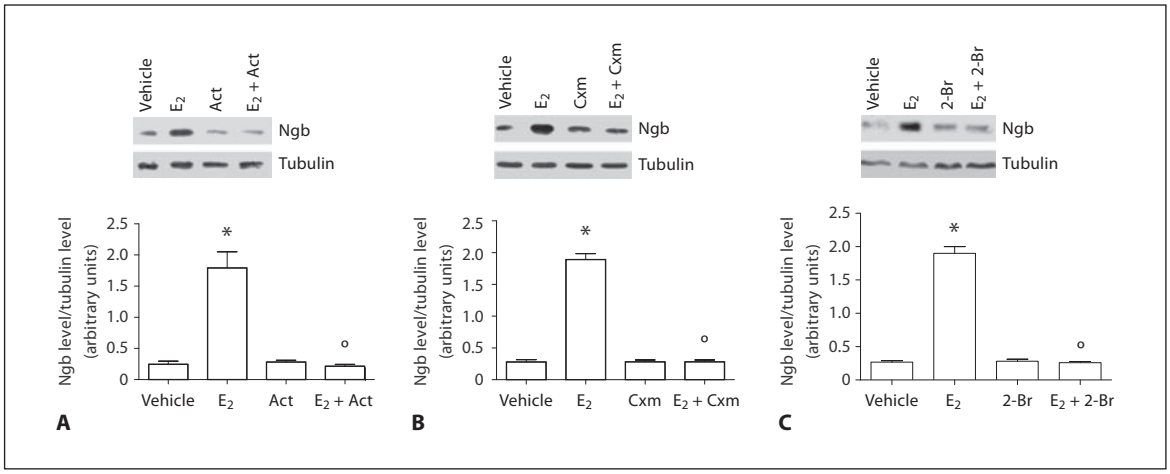

Fig. 4. Mechanisms underlying E_2 effects on Ngb protein expression in SK-N-BE human neuroblastoma cells. **A** Western blot analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM) and/or the transcription inhibitor actinomycin D (Act, 1 μg/ml). **B** Analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM) and/or the translation inhibitor cycloheximide (Cxm, 10 μg/ml). **C** Analyses of Ngb levels in cells stimulated for 24 h with either vehicle, E_2 (1 nM) and/or the palmitoylacyl transferase inhibitor 2-bromohexadecanoic acid (2-Br; 10 μM). The amount of proteins was normalized by comparison with tubulin levels. Data are representative Western blots of independent experiments (**A–C** top panels); densitometric analyses (**A–C** bottom panels). Data are means ± SD of four different experiments. $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test with respect to (*) vehicle- or (°) E_2-treated samples.

Ngb levels of the transcription inhibitor Act, the translation inhibitor Cxm, and the ER membrane localization inhibitor 2-Br [60]. Although the Ngb promoter sequence analysis (accession No. 12,581 from Transcriptional Regulatory Element Database http://rulai.cshl.edu/cgibin/TRED/tred.cgi?process=home) indicates that no canonical estrogen-responsive element (ERE) is present, SK-N-BE cell pretreatment for 24 h with Act completely prevented the E_2 effect on Ngb levels (fig. 4A). Similarly, Cxm (fig. 4B) and 2-Br (fig. 4C) impaired the increase of Ngb levels induced by E_2, suggesting that both transcriptional and extranuclear mechanisms contribute to E_2 effects. This prompted us to evaluate which signal transduction cascade was activated by E_2 in neurons. After 15 min of stimulation with 1 nM E_2, an increase of AKT and p38 phosphorylation in SK-N-BE cells was observed (fig. 5A, B). The E_2-induced activation of these kinases was still present 1 h after E_2 stimulation (fig. 5A, B), but only the E_2-induced p38 phosphorylation persisted 24 h after hormone stimulation (data not shown). By 30 min after 1 nM E_2 stimulation, the ERK1/2 phosphorylation status decreased (fig. 5A, B), in agreement with previous data obtained in cortical neurons [61]. The cell pretreatment with either AKT or p38 or ERK1/2 inhibitors suggested that neither AKT nor ERK1/2 are involved in the E_2-induced increase of Ngb levels (data not shown), whereas p38 activation was required for both rapid (i.e., 1 h) and long term (i.e., 24 h) E_2 effects on Ngb levels (fig. 5C, D). Similarly, only p38 inhibitor prevents an E_2-induced Ngb level increase in hippocampal neurons (data not shown). Notably, the SK-N-BE cell transfection with ERβ SiRNA reduced both ERβ levels and the E_2 ability to induce p38 phosphorylation (fig. 5E). On the other hand, ERβ SiRNA did not impair the E_2-induced AKT activation (data not shown), suggesting that ERα could be the molecular mediator of AKT activation in these cells.

Since it has been reported that several actions of E_2 in the nervous system involve cross-talk between ERα and the IGF-1 receptor [62], we evaluated the possibility that the ERβ-dependent E_2-induced p38 phosphorylation and the Ngb-increased levels are dependent on the ERβ-IGF-1 receptor cross-talk. IGF-1 is more efficient than E_2 to activate AKT phosphorylation and cell pretreatment with PPP, the IGF-1 receptor inhibitor, strongly prevented both IGF-1 and E_2 effects on AKT activation (fig. 5F), confirming that cross-talk between the IGF-1 receptor and ERs is important for AKT activation. However, IGF-1 was unable to increase p38 phosphorylation and PPP did not prevent E_2-induced p38 activation (fig. 5F). In addition, IGF-1 did not modify Ngb levels in SK-N-BE cells (fig. 5G) further sustaining the high specificity of the E_2 effect.

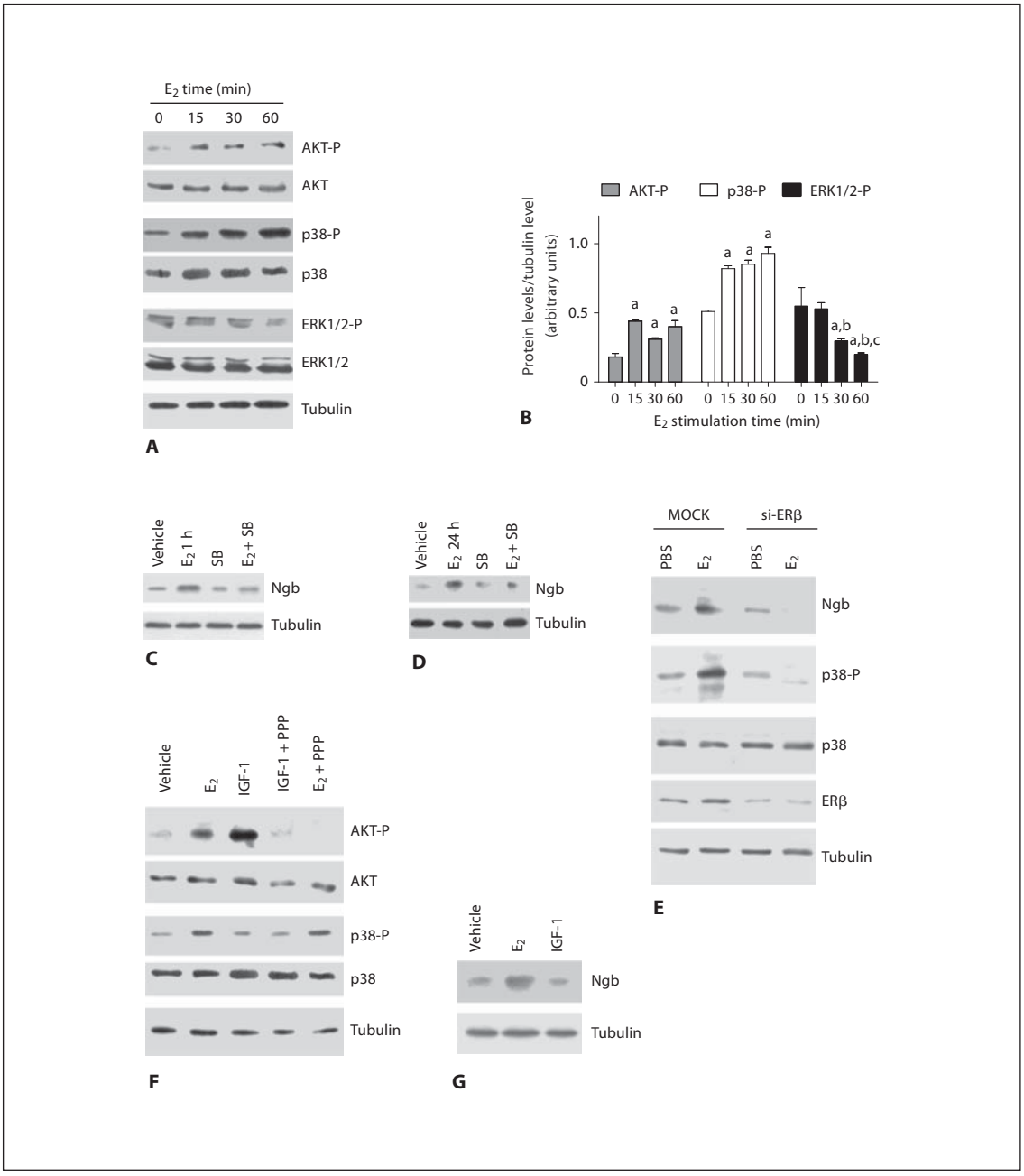

Fig. 5. Rapid signal transduction pathways activated by E_2 and impact of E_2-dependent rapid signal inhibitors on Ngb protein levels in SK-N-BE human neuroblastoma cell line. **A** Time-course analyses of phosphorylated (P) and unphosphorylated AKT, p38, and ERK1/2 in cells stimulated for 0, 15, 30, and 60 min with E_2 (1 nM). **B** Densitometric analysis related to E_2-induced AKT, p38, and ERK1/2 phosphorylation experiments. Data are means ± SD of four different experiments. * $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test: a significant vs. 0, b vs. 15, and c vs. 30 min. **C**, **D** Analyses of Ngb levels in cells stimulated for 1 h (**C**) or 24 h (**D**) with either vehicle, E_2 (1 nM) and/or the p38 inhibitor SB-203580 (SB; 5 μM). **E** Analysis of Ngb, ERβ, and phosphorylated (P) and unphosphorylated p38 levels in cells transfected with either MOCK (control) or ERβ small interference mRNA (si-ERβ) in the absence or presence of E_2 (1 nM). The amount of proteins was normalized by comparison with tubulin levels. Data are representative Western blots of three independent experiments. **F** Western blot analyses of phosphorylated (P) and unphosphorylated AKT and p38 levels in cells treated for 24 h with either vehicle, E_2 (1 nM) or IGF-1 (100 ng/ml). When indicated, cells were pretreated with the IGF-1 receptor inhibitor picropodophyllin (PPP; 100 μM). **G** Analyses of Ngb protein levels in cells treated for 24 h with either vehicle, E_2 (1 nM) or IGF-1 (100 ng/ml). The amount of proteins was normalized by comparison with tubulin levels. Data are representative Western blots of three independent experiments.

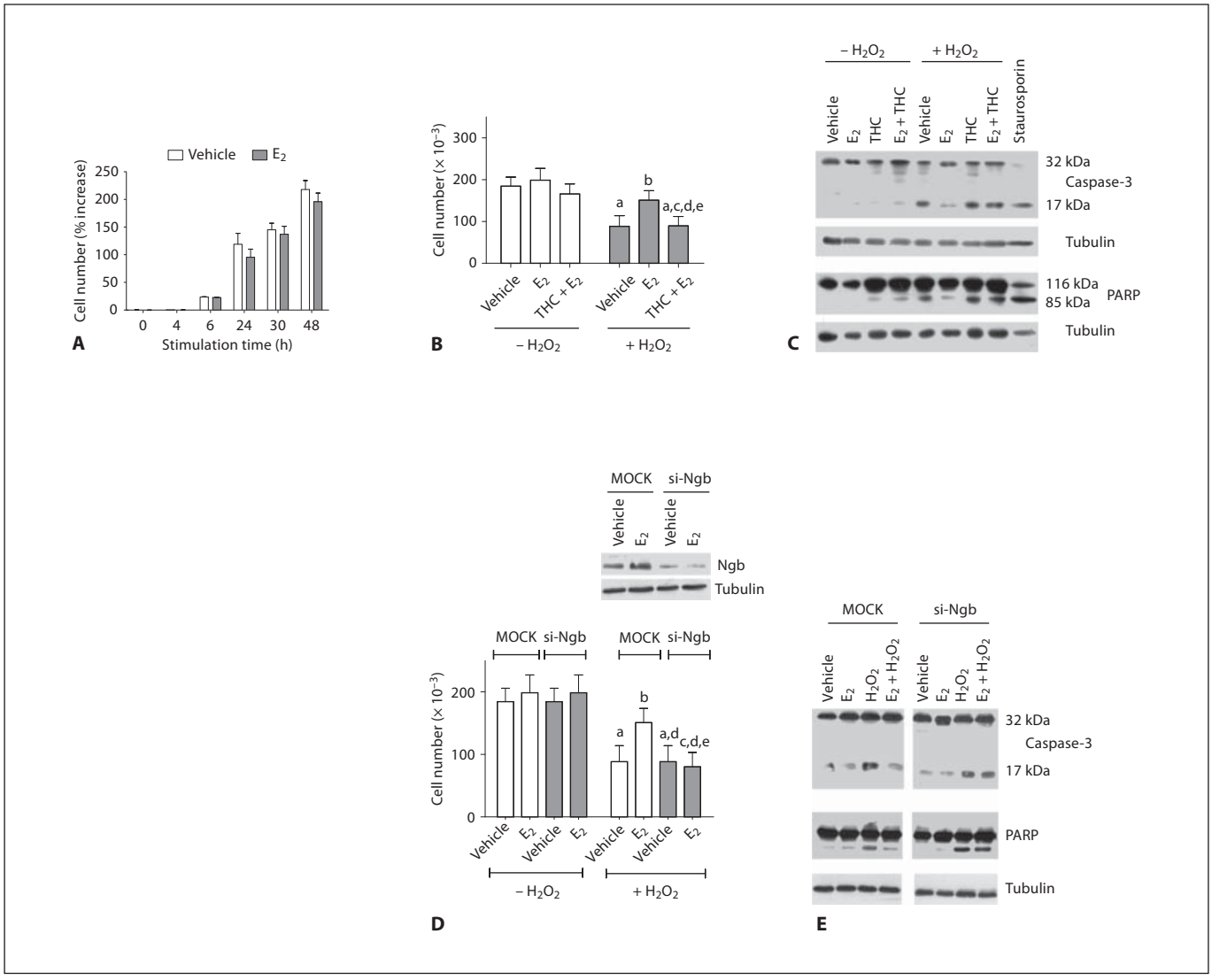

Fig. 6. E_2 effect on SK-N-BE human neuroblastoma cell line viability. **A** Cells were grown in the presence of either vehicle or E_2 (1 nM) and counted at the indicated time. Data are means ± SD of five independent experiments carried out in duplicate. **B** Cells were grown for 24 h in the presence of either vehicle, E_2 (1 nM) or E_2 in the presence of the ERβ-selective antagonist (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC; 1 μM). After 24 h, cells were stimulated with H_2O_2 50 μM (24 h of stimulation), and counted. Data are means ± SD of five independent experiments carried out in duplicate. $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test: a significant vs. Vehicle – H_2O_2, b vs. vehicle + H_2O_2, c vs. E_2 – H_2O_2, d vs. E_2 + H_2O_2, and e vs. THC + E_2 – H_2O_2. **C** Western blot analyses of caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage were performed on cells stimulated with either the vehicle or pretreated with E_2 (1 nM) for 24 h in the presence or absence of THC pretreatment, and then treated with H_2O_2 50 μM (24 h of stimulation). Staurosporin (2 μM for 24 h) was used as positive control of caspase activation. The amount of proteins was normalized by comparison with tubulin level. Data are representative Western blots of three independent experiments. **D** Western blot analysis of Ngb protein levels in cells transfected with either MOCK (control) or with Ngb small interference mRNA (si-Ngb) in the absence or presence of E_2 (1 nM). The amount of proteins was normalized by comparison with tubulin levels. Data are representative Western blots of three independent experiments. Cells transfected with either MOCK (empty bars) or si-Ngb (filled bars) were grown for 24 h in the presence of either the vehicle or E_2 (1 nM), stimulated with H_2O_2 50 μM (24 h of stimulation), and counted. Data are means ± SD of three independent experiments carried out in duplicate. $p < 0.001$ was calculated with ANOVA followed by Tukey-Kramer post-test: a significant vs. vehicle – H_2O_2, b vs. vehicle + H_2O_2, c vs. E_2 + H_2O_2, d vs. vehicle – H_2O_2 si-Ngb, and e vs. E_2 – H_2O_2 si-Ngb. **E** Western blot analyses of caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage were performed on cells transfected with either MOCK or si-Ngb and stimulated with either vehicle or 50 μM H_2O_2 in the presence or absence of E_2 (1 nM) (24 h pretreatment). The amount of proteins was normalized by comparison with tubulin level. Data are representative Western blots of three independent experiments.

Ngb Involvement in E_2-Induced Protection against H_2O_2-Induced Oxidative Stress

Finally we evaluated the role played by increased levels of Ngb on E_2 effects in SK-N-BE cells. E_2 stimulation did not modify SK-N-BE cell number (fig. 6A) but, as expected [61], reduced by 50% the H_2O_2-induced decrease in cell number (fig. 6B) as well as the increase of the 17-kDa active caspase-3 subunit and the cleavage of the caspase-3 substrate PARP (fig. 6C). Staurosporin (2 μM for 24 h) was used as positive control of caspase activation (fig. 6C). Notably, the E_2-protective effect against H_2O_2-induced neuron toxicity seems to require ERβ, since the cell pretreatment with the specific ERβ inhibitor THC completely prevented E_2 effects (fig. 6B, C). In line with this result, E_2 was unable to counteract the H_2O_2-induced decrease in cell number (fig. 6D) and the activation of the pro-apoptotic cascade (i.e., caspase-3 activation and PARP cleavage) (fig. 6E) in Ngb-silenced SK-N-BE cells.

Discussion

The aim of this paper was to identify a possible endogenous modulator of Ngb. Thus, we investigated the effect of E_2, a well-known neurotrophic and neuroprotective hormone [52–57, 63–66], on Ngb expression. Our results indicated that E_2 increases Ngb levels of about 300% in both the human neuroblastoma cell line and mouse primary hippocampal neurons. Although it has been obtained with a qualitative technique (i.e., Western blot), this effect is conspicuous in that the well-known E_2 effect on cyclin D1 expression, playing a relevant role in E_2-induced cell proliferation, is only of about 50–70% [67]. In addition, the E_2-induced Ngb increase is rapid (1 h), persistent (24 h), and specific, being not mimicked by either the male sex steroid hormone DHT or by the common precursor T or by IGF-1, another well-known neuroprotective hormone. These results represent the first evidence for steroid hormone modulation of globin levels in cells. Recently, it has been reported that hemoglobin is specifically expressed in neurons, its expression being upregulated by erythropoietin and accompanied by enhanced brain oxygenation under physiologic and hypoxic conditions [68]. At the present, the relationship between hemoglobin and Ngb in neurons is still unclear. Although hemoglobin α-chains and Ngb are expressed in the same nerve cells, Ngb levels are not increased by erythropoietin [68]. It is therefore unlikely that they have a tightly linked function, e.g. in facilitated oxygen transport; however, hemoglobin and Ngb could fulfill independent tasks in neurons.

It is now well known that sex steroid hormones have numerous effects on the brain throughout the lifespan, beginning during gestation and continuing on into senescence [69]. However, the inability of Ngb to react with androgens renders Ngb a new E_2 target that should be added to the variety of E_2-specific actions on the brain which include mood, locomotor activity, pain sensitivity, vulnerability to epilepsy, attentional mechanisms, and cognition [64].

The E_2 effect on Ngb levels is rapid and dose-dependent with the maximum effect at E_2 physiological concentration (i.e., 1–10 nM). Notably, the E_2 dose-response curve results in being bell-shaped. This is typical for E_2 [70], whose effects are mediated by two receptor isoforms. Accordingly, the plant-derived flavonoid Nar, which partially blocks the rapid activities of ERα [58], increased Ngb levels with a plateau at 1 μM concentration. This result suggests a functional antagonism between the activities of ERα and ERβ in neurons, as has been reported in other cell types [71].

Although human neuroblastoma cell line and mouse primary hippocampal neurons express different levels of both ER isoforms, the effect of E_2 on Ngb levels specifically requires all ERβ activities. In fact, ERβ extranuclear and genomic signals cross-talk each other to guarantee both the rapid (1 h) and the persistent (24 h) E_2 effects. In particular, the rapid (15 min) and persistent (24 h) ERβ-mediated p38 activation is required for E_2-induced Ngb increase. The E_2-dependent activation of p38, a mitogen-activated protein kinase (MAPK) family component, seems to represent a conserved pathway in ERβ-based E_2 rapid signals. Indeed, the E_2-induced ERβ-mediated activation of the p38/MAPK occurs in ERβ-transfected HeLa cells and in ERβ-containing rat myoblasts and colon adenocarcinoma cells [70, 72, 73]. This signaling pathway transduces different E_2 effects depending on the cell context. In fact, p38 activation is required for E_2-induced apoptosis of cancer cells [72, 73], for E_2-induced gene transcription [73], and for E_2-induced protection against oxidative stress in neuroblastoma cell line (present results) and in rat myoblasts [Marino, unpubl. results]. Thus, although Ngb promoter does not contain any canonical ERE, it is not surprising that the transcription inhibitor Act completely prevents the increase of the E_2-induced Ngb levels. The sequence analysis (accession No. 12,581 from Transcriptional Regulatory Element Database http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) indicates that several non-canonical half

ERE sites are present in the Ngb promoter along with a responsive element for other transcription factors. These results suggest that the E_2-induced Ngb transcription could be mediated by tethered interactions of ER with other transcription factors to activate gene expression (i.e., indirect genomic mechanism) [48]. Thus, the integration between extranuclear and genomic events may be required to provide plasticity for the neuronal response to E_2. The hormone rapidly induces AKT activation and ERK dephosphorylation in neuroblastoma cells. These effects are still detected in ERα-containing but ERβ-silenced cells. Although these kinases are not directly involved in an E_2-induced Ngb increase, they could contribute to the E_2 effects in neurons, since AKT activation has been associated with the increase of the anti-apoptotic protein Bcl-2 and to the E_2-induced cell survival [72], while E_2 protected cortical neurons against oxidative stress by reducing H_2O_2-induced activation of ERK1/2 [61].

The main result reported here is that Ngb is part of the E_2 response to H_2O_2-induced toxicity. In fact, exposure to 50 μM H_2O_2 induces neuroblastoma cell death (about 50%) which is accompanied by a dramatic increase in caspase-3 activation. The cell pretreatment with E_2 (1 nM) decreases cell death and reduces caspase-3 activation triggered by exposure to H_2O_2 in good accordance with the literature [52–54, 61, 74]. This E_2 effect against H_2O_2 toxicity is completely prevented by treatment with THC, ERβ inhibitor, and by knocking out Ngb using small interfering RNA.

Exposure to H_2O_2 induces a robust increase of reactive oxygen species in cells (followed by oxidation of lipids, proteins, and DNA), intracellular calcium increase, glutathione depletion, mitochondria dysfunction, and caspase-3 activation followed by apoptotic cell death [74]. It has been demonstrated that E_2 exerts protective effects on several of these cellular events including potent attenuation of lipid peroxidation, attenuated ATP depletion, alleviated intracellular calcium elevation, ablated mitochondrial calcium loading (with the subsequent mitochondrial membrane potential maintenance), reduced caspase-3 activation, and enhanced cell survival ([74] and present results). At present, it is difficult to discriminate the role played by Ngb in each of these E_2-induced cellular outcomes because it is not yet clear if they require ERα or ERβ or both receptors. Moreover, most of the E_2-protective effects have been obtained at pharmacological E_2 concentrations (0.1–10 μM) [74]. However, it has recently shown that the elevation of human Ngb expression in neurons prior to insult with H_2O_2 enhances cell viability and results in a significant decrease in oxidative stress and an increased intracellular ATP concentration [75]. In addition, a linkage of Ngb to oxidative metabolism has been proposed [76]. These data are strongly suggestive of the involvement of Ngb in E_2-induced attenuated ATP depletion. Furthermore, in the presence of Ngb the initially released cytochrome *c* may be sequestered by the Ngb [42, 43] and the apoptotic cascade avoided [19].

In the future, different strategies (e.g., E_2-induced Ngb localization, association between Ngb and ERs, and E_2-induced ER-mediated Ngb promoter activity) will substantiate Ngb action in neurons, our laboratories being active in this field. However, from now, Ngb can be regarded as part of signals activated by E_2 to exert neuroprotective effects definitely validating the role played by Ngb as an anti-apoptotic neuroprotective globin.

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