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RESEARCH PAPER

The selective β_1-adrenoceptor antagonist nebivolol is a potential oestrogen receptor agonist with neuroprotective abilities

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Background and purpose: Nebivolol, a selective β_1-adrenoceptor antagonist mediating rapid vasodilating effects, is used clinically to treat hypertension. Recently, it was reported that nebivolol also acts as an oestrogen receptor (ER) agonist. To investigate the neuroprotective potential of oestrogens, we assessed the oestrogenic effects of nebivolol in several *in vitro* neuronal models.
Experimental approach: Human neuroepithelioma SK-N-MC cells stably transfected with human ER α and β, and mouse N2A neuroblastoma cells expressing human $APP695_{SWE}$ [N2Aswe, stably transfected with the Swedish mutation form of the Alzheimer-associated amyloid precursor protein (APPswe, K670M/N671L)] were incubated with different concentrations of nebivolol and 17β-oestradiol (E2) for 24–48 h. ER activation was detected in a specific reporter assay, and ER-dependent gene expression was measured by quantitative real-time PCR (qRT PCR). Furthermore, cell survival rates were determined, and oxidative stress was induced by hydrogen peroxide and paraquat. Amyloid β protein precursor (APP) processing was investigated, and the cleavage fragments sAPPα and Aβ were quantified via α-, β- and γ-secretase activity assays. Alterations of secretase expression levels were determined by qRT PCR.
Key results: Nebivolol induces oestrogen-dependent gene transcription, and protects neuronal cells against oxidative stress even at low and physiological concentrations (10^{-8} M). Moreover, nebivolol modulates processing of APP in mouse neuronal N2Aswe cells by increasing α-secretase activity, ultimately leading to enhanced release of soluble non-amyloidogenic sAPPα.
Conclusions and implications: We showed that nebivolol acts as ER agonist in neuronal cell lines, and suggest oestrogen-like neuroprotective effects mediated by nebivolol.
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Keywords: oestrogen receptors; oxidative stress; neuroprotection; amyloid precursor protein; Alzheimer's disease; selective oestrogen receptor modulator

Abbreviations: AD, Alzheimer's disease; APP, amyloid β protein precursor; E2, 17-β oestradiol; eNOS, endothelial NOS; ER, oestrogen receptor; ERE, oestrogen response element; GRE, glucocorticoid response element

Introduction

Nebivolol is known to be a selective β_1-adrenoceptor antagonist (Van de Water *et al.*, 1988) with endothelium-dependent vasodilating properties mediated through stimulation of endothelial NOS (eNOS) (Broeders *et al.*, 2000; Garban *et al.*, 2004). Interestingly, at high concentrations, nebivolol also has antioxidant actions (Troost *et al.*, 2000; De Groot *et al.*, 2004; Mason *et al.*, 2006; Wagenfeld *et al.*, 2008), although the exact underlying mechanism is not well understood. In addition, it has been reported that nebivolol has oestrogen receptor (ER) agonistic properties (Garban *et al.*, 2004; Grundt *et al.*, 2007). These interesting activities of nebivolol combined with the fact that one central effect of the female sex hormone oestrogen (17β-oestradiol, E2) in endothelial cells is the activation of eNOS (Thomson *et al.*, 1997; Nuedling *et al.*, 1999; Su *et al.*, 2002), suggest that some of the beneficial features of nebivolol as an anti-inflammatory compound (Garbin *et al.*, 2008), reducing atherosclerosis (De Nigris *et al.*, 2008) and osteoporosis (Toker *et al.*, 2008), are based on its oestrogenic properties.

In the past few decades, it has become apparent that, in addition to their effects in sexual differentiation and behaviour, oestrogens modulate a plethora of physiological processes from bone formation, lipid metabolism, cardiovascular function and inflammation to synaptic plasticity (Turgeon

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et al., 2006). Several molecular mechanisms are known to be involved in the effects of oestrogen. First, a direct genomic mechanism, where nuclear forms of ER, ERα or ERβ activate or repress transcription of oestrogen-responsive genes (McEwen, 2001; Behl, 2002). Second, an indirect genomic effect mediated by oestrogen through an ER-linked second messenger system via ERs, G protein-coupled receptors or insulin-like growth factor-I receptor, which leads to a modulation of cell homeostasis and transcriptional activity (Cardona-Gomez *et al*., 2001; Mendez *et al*., 2005). Third, non-genomic effects at high concentrations involve antioxidant effects independently of known ERs and are based on the phenolic structure of oestrogen (Behl *et al*., 1995; 1997). Various studies focusing on the neuroprotective capacity of oestrogens have shown that oestrogens provide some protective effects in acute and chronic neurodegenerative diseases like cerebral ischaemia, Alzheimer's disease (AD) and Parkinson's disease (Garcia-Segura *et al*., 2001; Behl, 2002; Brann *et al*., 2007).

Here, we describe the oestrogenic effects and potential neuroprotective activities of nebivolol by employing two neuronal *in vitro* models: the human neuroepithelioma SK-N-MC and mouse N2A neuroblastoma cells expressing human $APP695_{SWE}$ ($N2A_{swe}$) (Thinakaran *et al*., 1996). The stable transfection of the patient-derived so-called Swedish mutation form of the AD-associated APP (APPswe, K670M/N671L) leads to an enhanced neurotoxic amyloid β-protein (Aβ) 40 and 42 production (Pietrzik and Behl, 2005). To determine whether ERs and if so, which subtype, α or β, can be activated by nebivolol, we used SK-N-MC-cell clones stably overexpressing ERα (SK-ERα) or ERβ (SK-ERβ) (Manthey *et al*., 2001; Zschocke *et al*., 2002). To analyse the neuroprotective activities that may be relevant to AD, we used $N2A_{swe}$ cells, which express both ER subtypes endogenously.

Methods

Cells and culture conditions

Experiments were performed with mouse N2A neuroblastoma cell lines expressing human $APP695_{SWE}$ (Thinakaran *et al*., 1996) and human neuroepithelioma SK-N-MC cells (ATCC no. HTB-10) stably transfected with human ERα and β cDNA (Manthey *et al*., 2001; Zschocke *et al*., 2003). Cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen Inc., San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS) under standard culture conditions as described previously (Manthey *et al*., 2001). For oestrogen-free conditions, the medium was replaced 24 h before experiments with phenol red-free DMEM with 1% charcoal/dextran-treated FBS supplemented with 10^{-4} M formestane to suppress endogenous oestrogen synthesis.

Experimental procedures

ER binding assay

The specific binding of nebivolol to the ER was analysed by radioligand binding assay as described by Garban *et al*. (2004). The ER-expressing cell line N2A was cultured 48 h prior to cell harvesting under oestrogen-free conditions using phenol red-free DMEM medium supplemented with 1% dextran-coated charcoal-treated FBS. The cells were harvested by incubating cells at 37°C for 5 min in trypsin EDTA (0.05% trypsin/53 mM EDTA). The cells were washed twice and finally resuspended in ice-cold PBS; 2×10^6 cells were incubated in microcentrifuge assay tubes at RT in the presence of 2×10^{-9} M (K_D: $0.1–1 \times 10^{-9}$ M) [2,4,6,7,16,17-^{3}H]-E2 and different concentrations of nebivolol, from 2×10^{-2} M to 1×10^{-6} M. A 100-fold molar excess of unlabelled E2 was used to determine the specific displaceable binding in a separate tube. After incubation for 1 h, cells were washed twice in 1 mL of ice-cold PBS, and dissolved in 600 μL of 0.1 N NaOH; 10 μL aliquots from each sample were used for determination of total protein concentration by bicinchoninic acid (BCA)-based assay, and 250 μL was used in combination with 4 mL of scintillation fluid and counted in a Packard Tri-Carb LS Counter (PerkinElmer, Waltham, MA, USA). Results are expressed as the mean ± SEM of three independent measurements.

Luciferase reporter assays

For the functional quantification of nuclear receptor activity, we transiently transfected cells with plasmid DNA containing an oestrogen response element (ERE) (D-MTV-ERE-LUC; Moosmann and Behl, 1999), glucocorticoid response element (GRE) or mouse mammary tumour virus promoter containing four androgen response element (ARE) sequences (Yeh and Chang, 1996) linked to the firefly luciferase gene and cotransfected with a *Renilla* luciferase expressing plasmid (pRL) for control and normalization of the transfection efficiency. Twelve hours before transfection, cells were seeded at approximately 500 000 cells per well in six-well tissue culture dishes in phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS. The transfection was carried out with FuGene HD as described by the supplier; 12 h after transfection, 8000 cells per well were plated on 96-well white microtitre plates, and treated immediately. After 24 h, cells were harvested and analysed with the Dual Luciferase Assay System. The luminescence readings were performed in an automatic counter (Wallac Victor3, PerkinElmer).

RNA extraction and quantification of mRNA expression

Total RNA was extracted 18 h after treatment of the cells using Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Böblingen, Germany) according to the manufacturer's instructions. RNA samples were reversely transcribed by Verso cDNA Kit. Quantitative RT-PCR was performed on an iCycler PCR Thermocycler (Bio-Rad, Munich, Germany) using Absolute SYBRGreen Mix according to the manufacturer's recommendations with primer pairs and conditions listed in Table 1. Relative expression levels were calculated using REST-MCS (relative expression software tool) (Pfaffl *et al*., 2002), whereas glyceraldehyde-3-phosphate dehydrogenase and RPL19 were used as housekeeping and reference genes.

Cell survival assay

Survival assays were performed with cells seeded into 96-well microtitre plates at a density of 4000 cells per well in a medium

Table 1 PCR primers and conditions

Gene	*Symbol*	*Species*	*GenBank accession no.*	*Primer sequence*	*Annealing temp. (°C) Elongation time (s) Product size (bp)*
Androgen receptor	AR	Human	BC132975	5′- gtcaactccaggatgctctac-3′	62,
		Mouse	M37890	5′-ccaggagcttggtgagctggta-3′	25, 321
ADAM metallopeptidase domain 9	ADAM 9	Human	BC126406	5′- cagaatggatatccttgccag-3′	62,
		Mouse	BC047156	5′- tcacagtgacaattcttattgc-3′	40, 496
ADAM metallopeptidase domain 10	ADAM 10	Human	BC066207	5′- ggaacacgagaagctgtgattg-3′	62,
		Mouse	AF009615	5′- cagtagtcatcatgattctgctc-3′	40, 229
ADAM metallopeptidase domain 17	ADAM 17	Human	BC146658	5′- ggattcctttcagcattcttgtc-3′	62,
		Mouse	BC136783	5′- gtgagtctgtgctggggtcttc-3′	40, 260
Beta-site APP-cleaving enzyme 1	BACE 1	Human	NM_012104	5′-cgcagacgctcaacatcc-3′	62,
				5′- gggcacatacacacccttcc-3′	35, 139
		Mouse	NM_011792	5′- cagtgggaccaccaaccttc-3′	60,
				5′-gctgccttgatggacttgac-3′	30, 70
Baculoviral IAP repeat-containing 3	Birc3	Human	AF070674	5′-tactacataggacctggaga-3′	62,
		Mouse	NM_007464	5′-caagtactcacaccttggaaac-3′	30, 383
Heat shock 27 kDa protein 1	Hsp B1	Human	NM_001540	5′-ggacgagctgacggtcaag-3′	60
				5′-cgcgactcgaaggtgactg-3′	30, 245
		Mouse	L27560	5′-atcccctgagggcacactta-3′	58,
				5′-ccagactgttcagagttcccag-3′	35 137
Trefoilfactor 1	pS2	Human	NM_003225	5′-acaaggtgatctgcg-3′	58,
				5′-gaagcaccaggggac-3′	35 190
		Mouse	NM_009362	5′-cactcgtggtcttcccgtga-3′	60,
				5′-ctgtgtcaccagccagatcca-3′	40, 411
Presenilin 1	PSEN1	Human	NM_000021	5′-ctctgcaacagtgtcttgtg-3′	62,
				5′-gttgctgtggactacattac-3′	30 400
		Mouse	NM_008943	5′-acaatggtgtggttggtgaat-3′	62,
				5′-agcaggctatggttgtgttcc-3′	30 360
Progesterone receptor	PGR	Human	M15716	5′-acaccttgcctgaagtttcg-3′	62,
				5′-ctgtccttttctgggggact-3′	20, 195
		Mouse	M68915	5′-gattcagaagccagccagagc-3′	62,
				5′-gacctccaaggaccatgccagc-3′	20 267
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Human	NM_002046	5′-cctgcaccaccaactgcttagc-3′	62
		Mouse	NM_008084	5′-caccaccttcttgatgtcatc-3′	30 334
Ribosomal protein L19	RPL19	Human	NM_000981	5′-gaaatcgccaatgccaactc-3′	62,
		Mouse	NM_009078	5′-ttccttggtcttagacctgcg-3′	30, 413

containing 10% FBS, and were allowed to adhere overnight. The following day, the medium was removed and replaced with phenol red-free DMEM containing 1% charcoal/dextran-treated FBS supplemented with 10^{-4} M formestane; 4 h before treatment of the cells with different concentrations of H_2O_2 or paraquat (PQ), cells were incubated with oestrogen, nebivolol, the ER antagonist ICI 182780 and L-NMMA, a non-selective NOS inhibitor, at indicated concentrations. After a 12 h incubation period, cell survival was measured using CytoTox-ONE Homogeneous Membrane Integrity assay according to the manufacturer's recommendations, and viable cells were reflected in relative fluorescence units.

Quantification of α, β and γ secretase activity, and sAPPα and Aβswe amounts

For quantification, 150 000 $N2A_{swe}$ cells per well were seeded on a 48-well plate. The next day, the cell culture medium was replaced with phenol red-free DMEM containing 1% charcoal/dextran-treated FBS supplemented with 10^{-4} M

formestane; after 4 h, cells were treated with several concentrations of E2 and nebivolol in the presence or absence of ICI 182780 for 36 h. All samples were normalized by determining protein concentration by the BCA method. To determine the secretase activity, cells were harvested and assays were performed, according to the manufacturer's instructions, using an α-secretase activity kit and a β-secretase activity assay kit; γ-secretase activity was measured by quantification of a fluorogenic γ-secretase substrate (Farmery *et al.*, 2003). For the determination of the amounts of sAPPα and $Aβ_{swe}$, 100 μL of cell culture supernatants was used in specific and sensitive sAPPα and Aβswe ELISAs (IBL, Hamburg, Germany).

Data analysis and statistical procedures

All experiments were performed independently, and a minimum of three times. Standard deviations and significance were calculated by using GraphPad Prism 4.2 software (GraphPad Software Inc., San Diego, CA, USA). Statistically significant differences between groups were determined by a one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

Materials

DMEM and FBS were purchased from Invitrogen Inc. Charcoal/dextran-treated FBS (Perbio, Bonn, Germany); formestane (Sigma, St Louis, MO, USA); [2,4,6,7,16,17-^{3}H]-E2 and scintillation fluid were from PerkinElmer LAS (Rodgau, Germany); the BCA-based assay was from Thermo Fisher Scientific (Bonn, Germany); the GRE was from Clontech Laboratories, Inc., (Mountain View, CA, USA); pRL, the Dual Luciferase Assay System and the CytoTox-ONE Homogeneous Membrane Integrity assay were from Promega (Mannheim, Germany); FuGene HD was from Roche (Mannheim, Germany); Agilent Total RNA Isolation Mini Kit was from Agilent Technologies; and Verso cDNA Kit and Absolute SYBRGreen Mix were from Thermo Fischer (Hamburg, Germany). The α-secretase activity kit was from R&D Systems (Minneapolis, MN, USA), the β-secretase activity assay kit was from BioVision (Mountain View, CA, USA) and the fluorogenic γ-secretase substrate was from Merck (Darmstadt, Germany).

17-β Oestradiol (E2) was purchased from Sigma Chemical Co., the ER antagonist ICI 182780 was from Tocris/Biotrend (Cologne, Germany), non-selective NOS inhibitor L-NMMA was purchased from Calbiochem/Merck (Darmstadt, Germany) and D-/L-nebivolol was provided by Berlin Chemie/Menarini AG (Berlin, Germany). Compounds were diluted in ethanol just before treatment of the cells. The drug/molecular target nomenclature conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2008).

Results

Nebivolol binds to ERs and induces a classical genomic ER response

To show the direct interaction of nebivolol with ER, we measured displacement by nebivolol of [^{3}H]-E2 bound to ER in

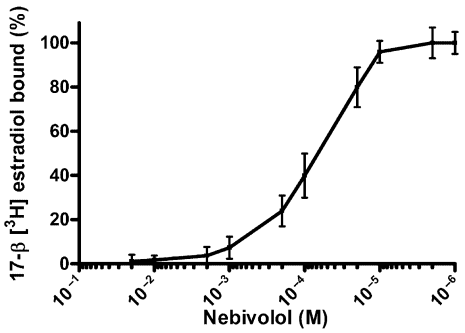

Figure 1 ER binding competition for nebivolol in N2A cells. In this assay, 2×10^{-9} M [^{3}H]-E2 was competing for binding to the ER with increasing concentrations of nebivolol from 1×10^{-6} M to 2×10^{-2} M. A 100-fold molar excess of unlabelled E2 was used to determine the specific displaceable binding. Specific binding was calculated based on the percentage of remaining isotopic activity. Results are expressed as the mean of ±SEM of three independent measurements. The nebivolol concentration at 50% inhibition of [^{3}H]-E2 binding (IC_{50}) = 0.8×10^{-5} M.

N2A cells. Cells were incubated with 2×10^{-9} M [^{3}H]-E2 in the presence or absence of varying concentrations of nebivolol. A 100-fold molar excess of unlabelled E2 was used to determine the absolute displacement of radiolabelled E2 and as reference for the nebivolol-mediated competition of E2 from ER. The kinetics of [^{3}H]-E2 dissociation by nebivolol showed an IC_{50} to inhibit the binding of [^{3}H]-E2 at approximately 0.8×10^{-5} M nebivolol (Figure 1), indicating a direct ligand–receptor interaction of nebivolol with ERs.

Activation of ER-dependent downstream transcriptional activity was investigated in N2A, $N2A_{swe}$ and SK-N-MC cells stably transfected with ER. For quantification and normalization of transfection efficacy, cells were transiently cotransfected with luciferase reporter plasmids containing ERE, GRE or ARE, and a plasmid constitutively expressing the *Renilla* luciferase. Stimulation with corticosterone or testosterone induced a positive luciferase response of GRE and ARE luciferase-transfected N2A cells, but no activation by nebivolol in the concentration range 10^{-6} to 10^{-9} M (data not shown). A strong response of the ERE induced by nebivolol could be observed in N2A and $N2A_{swe}$ cells, which endogenously express both ERα and ERβ, and in SK-N-MC cells ectopically expressing either ERα or ERβ. In N2A and N2Aswe cells, nebivolol induced the same response of the ERE, at concentrations of 10^{-6} to 10^{-8} M, as the genuine ER agonist E2 (Figure 2A). SK transfectants showed a higher induction of ERE after treatment with E2 (Figure 2B), but nebivolol also induced a significant activation of the ERE. In detail, nebivolol induced the highest response in the SK-ERα expressing cells at a concentration of 10^{-7} M (4.2-fold), and in SK-ERβ transfectants at 10^{-8} M (4.3-fold). After co-incubation with the ER antagonist ICI 182780, neither E2 nor nebivolol activated an ERE-dependent response. From these results, we conclude that nebivolol, just like E2, can induce a classical genomic response via the ER subtypes ERα and ERβ.

Nebivolol protected N2Aswe cells against reactive oxygen species (ROS)-induced stress

Oestrogens and oestrogenic compounds have been shown to have different neuroprotective abilities under oxidative

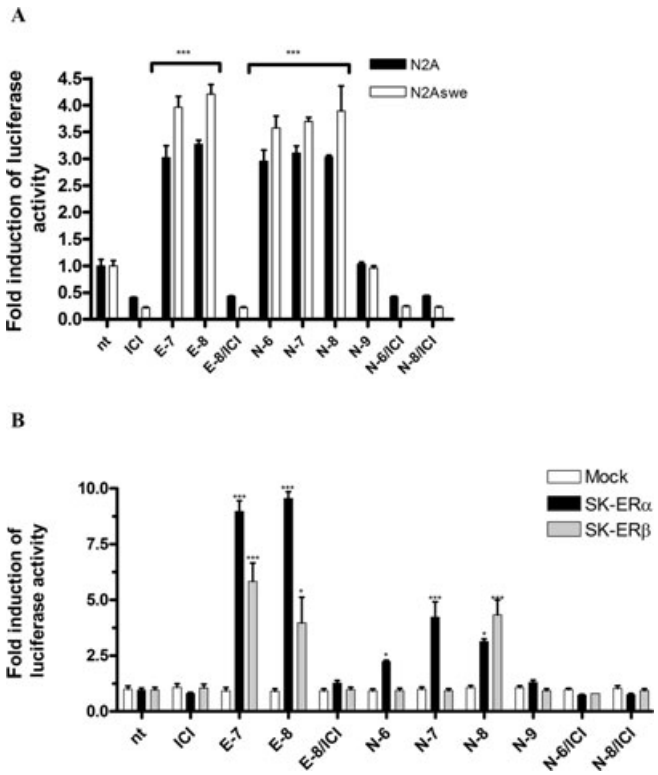

Figure 2 ER reporter assay with N2A and N2Aswe (A) and ER stable transfected SK-N-MC cells (B). Activation of the ER response element was plotted as fold induction of luciferase activity normalized to untreated cells (n.t.). Treatment: n.t., untreated; ICI, ICI 182780 at final concentration (f.c) of 10^{-6} M; E-7, E2 f.c. 10^{-7} M; E-8, oestrogen f.c. 10^{-8} M, N-6, nebivolol f.c. 10^{-6} M. The results shown represent more than three independent studies. Significance is defined as follows: $^{*}P < 0.05$; $^{***}P < 0.001$ compared with untreated cells (ANOVA).

Table 2 Nebivolol-induced gene expression in neuronal cells

Function	*Gene*	*Cell*		
		$N2A_{swe}$	*SK-ERα*	*SK-ERβ*
ER responsive	*PS2 (TFF1)*	→	↑	→
	BIRC3	↑	↑	→
	HSP 1B	↑	↑	→
	PR	↑	↑	↑
	AR	↓	→	→
APP processing				
α-Secretase				
	ADAM 9	↑	→	↑
	ADAM 10	→	→	→
	ADAM 17	→	→	→
β-Secretase		→	→	→
	BACE1	→	→	→
γ-Secretase				
	Presenillin 1	→	→	→

Comparison of gene expression between N2Aswe and SK-N-MC ERα- and ERβ-transfected cells after nebivolol treatment. Cells were treated with nebivolol (f.c. 10^{-8} M) for 18 h. Genes were considered differentially expressed when the difference in expression level between untreated and treated cells was more than 1.5-fold and a *P* value <0.05. (↑, significantly up-regulated; ↓, significantly down-regulated, →, genes were not significantly affected).

stress conditions (Behl, 2002). At pharmacological concentrations (10^{-6} M and higher) they have been demonstrated to be based on the antioxidative radical scavenging phenolic structure of 17β-oestradiol (Behl *et al.*, 1995; 1997). At physiological concentrations, oestrogens also acquire ER-mediated genomic activities (Manthey and Behl, 2006). Interestingly, antioxidative properties have been also described for nebivolol (Wagenfeld *et al.*, 2008). To investigate the protective properties of nebivolol in neuronal cells against oxidative stress induced by ROS, we exposed N2Aswe cells to H_2O_2 (0–1000 μM) and the herbicide PQ (0–1000 μM). While H_2O_2 causes a short acute burst of oxygen radicals, PQ induces a delayed accumulation of radicals (Yang and Sun, 1998). After both treatments, we observed a protective effect of E2 and nebivolol (Figure 3). At a nebivolol concentration of 10^{-8} M, a significantly increased cell survival could be detected: approximately 30% protection at 100 μM H_2O_2, 16% at 75 μM and 18% at 125 μM PQ compared to untreated controls (Figure 3). Interestingly, we found that nebivolol at a concentration range of 10^{-6} to 10^{-8} M promoted cell survival comparable to protection mediated by E2 at 10^{-8} M. The increased survival in this standard cell-based protection assays strongly indicates a direct neuroprotective effect of nebivolol. This effect was inhibited by the ER antagonist, ICI 182780, clearly suggesting that the mechanism of neuroprotection induced by nebivolol is ER-dependent genomic and not structurally based antioxidant. Furthermore, cotreatment with the non-selective NOS inhibitor L-NMMA did not affect the survival rates; this would seem to exclude the involvement of an NOS-dependent action in the neuroprotection provided by nebivolol.

Nebivolol displayed an oestrogenic gene expression profile

To determine further the properties of nebivolol as an ER agonist, we compared nebivolol-induced gene expression with oestrogen-induced gene expression in $N2A_{swe}$ and SK-ERα and SK-ERβ cells, focusing on a battery of well-known oestrogen-regulated genes. It is well known that ER-driven gene expression is largely modulated by and dependent on the activity of several cofactors. The presence of these cofactors is tissue and cell type specific, suggesting that common or general ER-marker genes do not exist (O'Lone *et al.*, 2004). We tested a group of known neuronal ER-responsive genes, and found that few of them were controlled by nebivolol and E2 (Table 2 and Figure 4). The mRNA expression of the trefoilfactor 1 (PS2), a commonly known ERα-responsive gene (Brown *et al.*, 1984), was increased by E2 and nebivolol in SK-ERα cells (Figure 4A), as well as the mRNA of Birc3 (Figure 4B) and Hsp 1B (Figure 4C). In $N2A_{swe}$ cells, pS2 mRNA or expression changes through nebivolol or oestrogen were not detectable. Interestingly, the increased expression of BIRC3 and Hsp 1B mRNA by nebivolol was only seen at 10^{-8} M. Expression analysis of the progesterone receptor (Figure 4D) showed a general up-regulation in all cells after treatment with E2 and nebivolol. But exclusively in the controls (mock-transfected SK cells and cotreatment with ICI 182780), an up-regulation was not found. In addition, in $N2A_{swe}$ cells, androgen

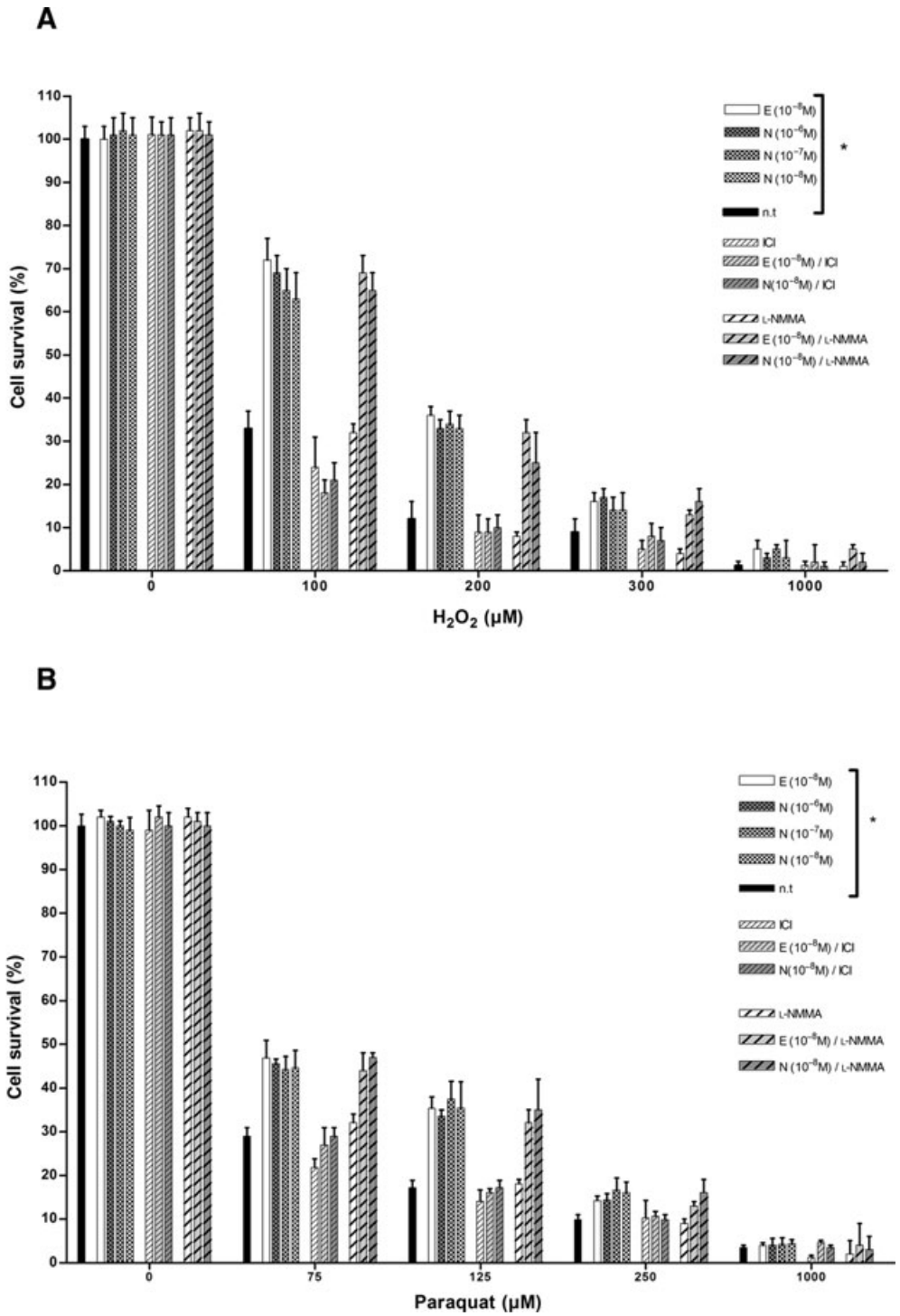

Figure 3 Nebivolol and E2 increased the survival of N2Aswe cells after H_2O_2 or PQ-induced oxidative stress. Cells were pretreated with oestrogen (f.c. 10^{-8} M), different nebivolol concentrations as indicated, ICI 182780 (ICI, f.c. 10^{-6} M), L-NMMA (f.c. 10^{-6} M) and oestrogen and nebivolol together with ICI 182780 and L-NMMA, followed by exposure to H_2O_2 (A) and PQ (0–1000 μM) (B). Data show mean cell survival from a representative experiment ($n = 9$). *$P < 0.05$, compared with untreated cells (n.t) (ANOVA).

receptor mRNA was down-regulated by E2 (–2.2-fold) and nebivolol (–3.3-fold at 10^{-6} M, –7-fold at 10^{-8} M), whereas SK transfectants showed no significant regulation of androgen receptor (AR) expression except the up-regulation in SK-ERβ cells after E2 (+1.8-fold) (Figure 4E).

Nebivolol and E2 lowered Aβ generation by activating α-secretase activity in N2A$_{swe}$ cells

Oestrogens modulate the biochemical processing of the AD-associated APP (Xu *et al.*, 1998; Manthey *et al.*, 2001). To investigate a potential effect of nebivolol on APP

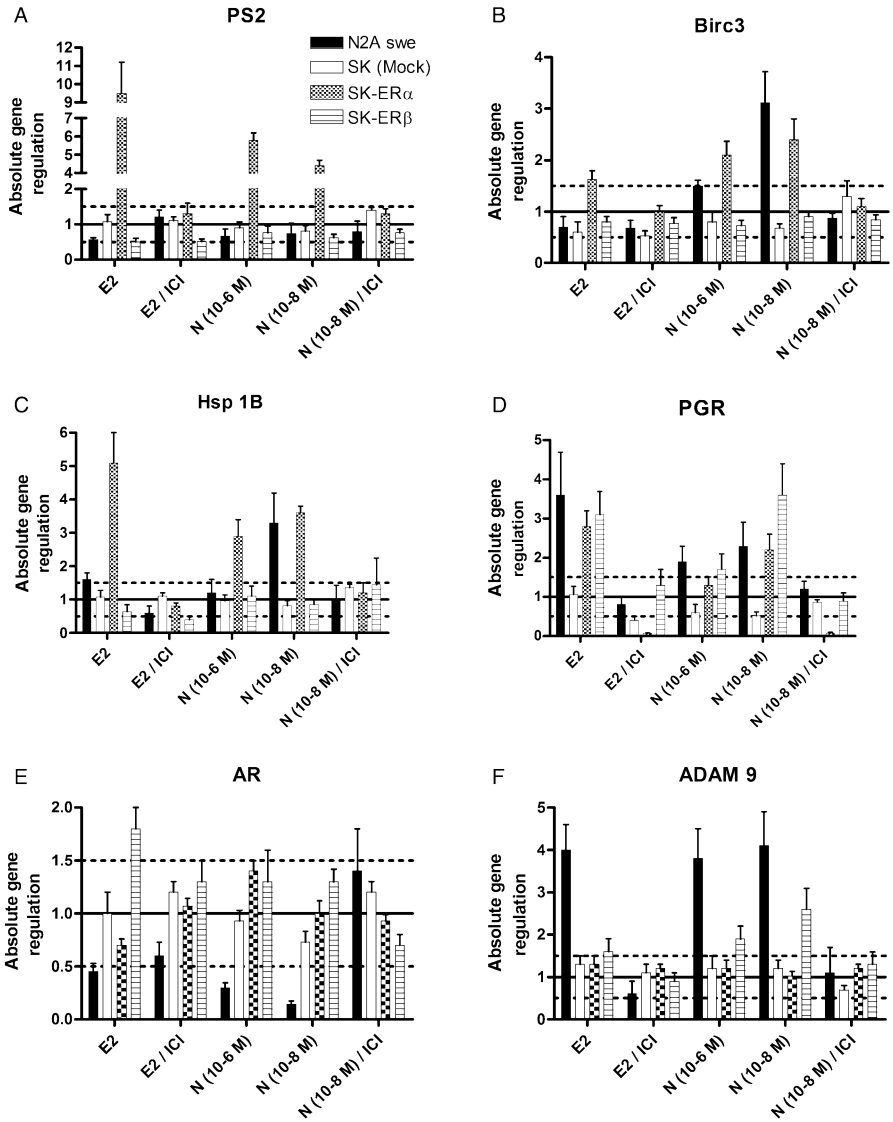

Figure 4 Relative mRNA expression ratios of E2 and nebivolol-regulated genes in N2Aswe and SK-N-MC ERα and ERβ over-expressing cells. Cells were treated with E2 (f.c. 10^{-8} M), nebivolol (10^{-6} M and 10^{-8}M) and oestrogen and nebivolol (10^{-8}M) together with ICI 182780 for 18 h. Genes were considered differentially expressed when the expression levels between untreated and treated cells (expressed as a ratio) were more than 1.5-fold difference (dotted lines) and a *P* value <0.05. Fold change of gene expression levels was based on the ΔΔCt method with normalization of the raw data to housekeeping genes by using the relative expression software tool (REST-MCS V2.0).

processing, the activities of APP cleavage by the three known secretases (α, β, γ) and selected cleavage products of APP were determined. Interestingly, we found treatment with nebivolol or E2 enhanced the activity of the α-secretase. The significant stimulation of α-secretase activity with E2 or nebivolol was approximately 1.5- to 1.8-fold at 10^{-7} and 10^{-8} M E2 compared to untreated controls, while E2 caused a slightly higher increase in α-secretase (Figure 5A). This effect could be completely suppressed by cotreatment with the ER antagonist ICI 182780 (Figure 5A). In addition, neither the activity of γ-secretase nor the activity of the Aβ-generating β-secretase was influenced by oestrogen or nebivolol (data not shown). As cleavage products of the APP processing, we found an accumulation of the potentially neurotrophic sAPPα peptide in $N2A_{swe}$ cells after treatment with E2 and nebivolol and, consistently, a decline of the neurotoxic $Aβ_{40/42}$ peptide amount. Cotreatment with the ER antagonist ICI 182780 repressed these processes, indicating an ER-mediated mechanism (Figure 5A–C). For further analysis of the enhanced α-secretase activity related to a possible ER-mediated genomic effect, we analysed mRNA expression of α-, β- and γ-secretases (Table 2). Indeed, we found that nebivolol and E2 induced an up-regulation of the potential α-secretase ADAM 9 in N2Aswe (fourfold) and SK-ERβ (2.6- and 1.6-fold, respectively) cells (Figure 4F). Expression levels of ADAM10 and 17, the other putative α-secretases, and presenillin1 (PSEN1) as part of the β-secretase complex and β-site APP-cleaving enzyme 1 (BACE1, γ-secretase) were not affected by nebivolol and E2 (data not shown).

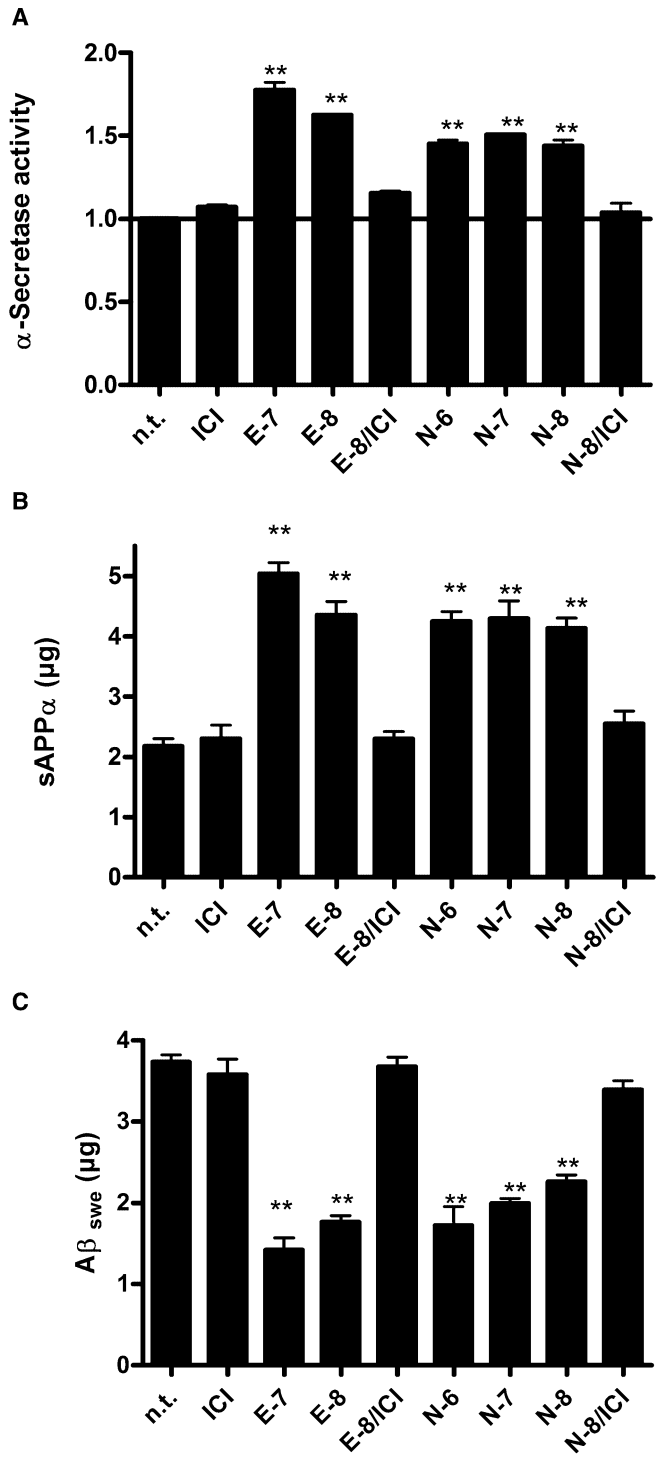

Figure 5 Modulation of the generation of sAPPα and Aβswe induced by the enhanced α-secretase activity observed after nebivolol and E2 treatment. (A) Quantification of sAPPα (ELISA). (B) Quantification of Aβ swe (ELISA). (C) α-Secretase activity assay; activity was plotted as fold induction normalized to untreated cells (n.t.). Treatments: n.t., untreated; ICI, ICI 182780 at final concentration (f.c) of 10^{-6} M; E-7, oestrogen f.c. 10^{-7} M; E-8, oestrogen f.c. 10^{-8} M, N-6, nebivolol f.c. 10^{-6} M, N-7, nebivolol f.c. 10^{-7} M, N-8, nebivolol f.c. 10^{-8} M. The histograms represent results from more than three independent studies. $^{**}P < 0.005$, compared with untreated cells of the same kind (ANOVA).

Discussion

Nebivolol is an established, highly selective β_1-adrenoceptor antagonist used for the treatment of hypertension with unique vasodilator effects based upon its ability to activate eNOS via ER-mediated pathways (Grundt *et al.*, 2007; Van Bortel *et al.*, 2008). Based on the initial finding that nebivolol can display oestrogenic properties in endothelial preparations and human MCF-7 cells (Garban *et al.*, 2004), together with the well-known activities of oestrogens in neuronal cells, we were interested in the possible effects of nebivolol in neuronal cells and any potential overlap of the actions of nebivolol and E2-mediated neuroprotective activities (Behl and Manthey, 2000; Maggi *et al.*, 2004; Brann *et al.*, 2007).

We found that nebivolol induces an ER-mediated response in established neuronal *in vitro* systems. Nebivolol activated both known ER subtypes even at low concentrations of 10^{-8} M, while an induction of other steroid receptors as androgen receptor or glucocorticoid receptor does not occur. In addition, the analysis of oestrogen-responsive gene expression revealed that nebivolol displayed similar effects to E2, but differences occurred when studying different cell lines. For instance, nebivolol but not E2 induced an up-regulation of the Birc3 mRNA, an anti-apoptotic protein, in N2Aswe cells. In SK-ERα cells, this up-regulation of Birc3 was only seen after treatment with E2, not with nebivolol. With regard to expression levels of the AR mRNA, in N2Aswe cells we only observed minor changes in the expression level with E2, but a pronounced down-regulation with nebivolol. Whereas in SK cells transfected with either ERα or ERβ, no changes were observed with either E2 or nebivolol. This could be due to a different cofactor composition, which triggers gene expression or requires the presence of both ERs (Chang *et al.*, 2006). The ability of nebivolol to act as an oestrogen agonist and in part as an antagonist means that nebivolol has been designated to be a selective ER modulator (SERM).

To investigate the potential neuroprotective activity of nebivolol further, we exposed $N2A_{swe}$ to oxidative stress, a condition frequently observed in acute brain injuries (e.g. cerebral ischaemia, stroke) or during chronic neurodegeneration (e.g. AD). It is well known that E2 can protect against oxidative stress *in vitro* and *in vivo* (Singer *et al.*, 1996; Harms *et al.*, 2001; Behl, 2002; Miller *et al.*, 2005), and several groups have described the antioxidant protective effects of nebivolol (Janssen *et al.*, 2001; De Groot *et al.*, 2004; Wagenfeld *et al.*, 2008). Here, we found that $N2A_{swe}$ cells pretreated with nebivolol or E2 showed an increased cell survival rate of more than 20% against H_2O_2- or PQ-induced ROS stress compared to untreated controls or cells co-incubated with the ER antagonist ICI 182780. Metoprolol, another β_1-selective antagonist neither led to an activation of the ER nor did it show any significant protective effect in the cell survival assays (data not shown). In addition, in both neuronal *in vitro* models, the expression of β_1-adrenoceptors was not detectable (data not shown). When taken together, these results clearly indicate that the protective activity of nebivolol is dependent on ERs as reported for E2's ER-dependent neuroprotection (Garcia-Segura *et al.*, 2001; Harms *et al.*, 2001; Gelinas *et al.*, 2004). Oestrogenic up-regulation and activation of eNOS via E2 or nebivolol, which mediates the beneficial vasorelaxing

effects in endothelial cells (Darblade *et al*., 2002; Grundt *et al*., 2007), appeared not to be involved in the neuronal cells. In fact, we did not detect any changes in the expression levels of eNOS, iNOS and nNOS in N2A and SK-N-MC cells following E2 and nebivolol treatment (10^{-6}–10^{-9} M) (data not shown). Furthermore, cotreatment of E2 or nebivolol with the non-selective NOS inhibitor L-NMMA had no effect on the cell survival after ROS treatment.

To study a pathogenic mechanism relevant for neurodegenerative conditions, we analysed the AD-associated processing of APP in N2Aswe cells after administration of E2 and nebivolol. The cerebral extracellular protein deposition, the 'senile plaques', which are a major neuropathological hallmark of AD, consists mainly of the extracellularly aggregated 40–42 residue peptide called Aβ. Amyloid β derived from APP after consecutive cleavage by β- and γ-secretases and is called amyloidogenic processing. An alternative proteolysis of APP with α- (instead of β-) secretase followed by γ-secretase cleavage leads to generation and release of other non-amyloidogenic fragments and the neurotrophic sAPPα peptide (non-amyloidogenic processing; De Strooper and Annaert, 2000). A beneficial effect of E2 in *in vitro* and *in vivo* models of AD has been frequently reported (Brann *et al*., 2007), and genomic (direct and indirect genomic) as well as non-genomic modes of action of E2 have been proposed (Behl, 2002). We examined the secretase activities and the amount of Aβ and sAPPα after treatment of N2Aswe cells with E2 and nebivolol. Indeed, we detected an enhanced generation of sAPPα and a decreased production of Aβ after E2 and nebivolol treatment, which could be inhibited by cotreatment with ICI 182780 indicating again an ER-mediated effect. Furthermore we found that α-secretase activity was increased, but β- and γ-secretase activities were unchanged. We demonstrated that E2 and nebivolol induced an increased expression of the putative α-secretase ADAM 9, and this could be a potential molecular mechanism for the promotion of the non-amyloidogenic APP processing. Interestingly, the expression of other putative α-secretases, such as ADAM 10 and 17 (Asai *et al*., 2003; Deuss *et al*., 2008), as well as β-secretase (presenilin 1) and γ-secretase (BACE1), was not affected by nebivolol. In conclusion, α-secretase cleavage of APP both precludes the deposition of the Aβ and releases the neuroprotective sAPPα; pharmacological up-regulation of α-secretase may provide an alternative therapeutic approach for AD (Pietrzik and Behl, 2005).

In summary, based on our findings we propose that nebivolol: (i) displays oestrogenic activity in neuronal cells; (ii) may potentially act as a SERM; and (iii) has antioxidative protective features and modulates APP processing in a beneficial neuroprotective manner. The potential of nebivolol to provide these neuroprotective effects should be studied further in *in vivo* models.

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Conflict of interest

Authors declare no conflict of interest.

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