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β -adrenoceptor blockers protect against staurosporine-induced apoptosis in SH-SY5Y neuroblastoma cells

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Abstract

The β -adrenoceptor blockers exhibit a well-characterized anti-apoptotic property in the heart and kidney while less is known about the effect of this class of drugs on neuronal apoptosis. We studied the effects of three β -adrenoceptor blockers propranolol (1-(isopropylamino)-3-(naphthalene-1-yl)oxypropan-2-ol), atenolol (2-[4-[2-hydroxy-3-(1-methylethylamino)propoxy]phenyl]ethanamide), and ICI 118551 (1-[2,3-(dihydro-7-methyl-1H-imidazo[4,5-f]indol-4-yl)oxy]-3-[(1-methylethylamino)-2-butanol], against staurosporine-induced apoptosis in SH-SY5Y human neuroblastoma cells. Staurosporine increased caspase 3-like activity, DNA fragmentation, PARP cleavage, and the number of TUNEL positive cells consistent with the induction of apoptosis. Propranolol and ICI 118551, but not atenolol, demonstrated a concentration-dependent inhibition of caspase 3-like activity. Propranolol and ICI 118551 directly inhibited the enzymatic activity of recombinant caspase 9 while atenolol did not; however, none of the β -adrenoceptor blockers that were examined directly blocked caspase 2 or 3 activity. In isolated mitochondria, propranolol and ICI 118551 inhibited staurosporine-induced cytochrome *c* release while atenolol did not. We conclude that propranolol and ICI 118551 protect SH-SY5Y cells against staurosporine-induced apoptosis through a dual action on the mitochondria and on caspase 9 in a cell type and an apoptotic paradigm where the conventional inhibitors of mitochondrial permeability transition such as cyclosporin A and bongkrekic acid demonstrate no protection.

Keywords

β -adrenoceptor blockers; apoptosis; staurosporine; SH-SY5Y; caspases

1. Introduction

Brain neurons undergo apoptotic cell death after acute injury and in chronic neurodegenerative disorders (Dickson, 2004; Dirnagl et al., 1999; Verma, 2000). Therefore, much effort has been put into the development of clinically acceptable therapeutics targeting the apoptotic cascade. Voltage gated calcium channel blockers, free radical scavengers, inhibitors of mitochondrial

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permeability pore, and caspase inhibitors are some examples of molecules explored as anti-apoptotic agents in the brain (reviewed in Merenda and Bullock, 2006). Although helpful in experimental investigations, none of the anti-apoptotic therapeutics has made much progress into the clinical arena. Furthermore, the conventional mitochondrial permeability transition inhibitor cyclosporin A demonstrates little anti-apoptotic activity in neurons (reviewed in Zoratti et al., 2005 but see Hansson et al., 2004) highlighting the importance of identifying a new class of anti-apoptotic drugs active in the brain.

β -adrenoceptor blockers are a clinically well-accepted and widely used class of drugs (Lopez-Sendon et al., 2004) with a well documented anti-apoptotic effect on the heart both *in vivo* and *in vitro* (Burniston et al., 2005; Communal et al., 1999; Zaugg et al., 2000). In contrast to the well-studied effects of β - adrenoceptor blockers on the heart and kidney, little is known about the action of these drugs on neurons. β -adrenoceptors are widely expressed in the brain and thought to mediate physiological responses to catecholamines. In the brain, β 1-adrenoceptors are expressed mainly in neurons whereas β 2-adrenoceptors are largely restricted to the glial cells (Nicholas et al., 1996). In the intact brain, both non-selective and β 1- adrenoceptor selective blockers have been shown to decrease infarct volume and enhance functional recovery in a transient focal ischemia model (Goyagi et al., 2006; and references cited therein). However, the cellular mechanism responsible for such apparent neuro-protection by β -adrenoceptor blockers is not known. A recent study demonstrated that propranolol, a non-receptor subtype selective β -adrenoceptor antagonist, inhibits Bax- and Bcl-2 homology 3 (BH3) peptide-induced cytochrome *c* release from isolated adult rat brain mitochondria (Polster et al., 2003). If β - adrenoceptor blockers have a similar effect on the mitochondrial function in a cellular context, this could explain the observed neuroprotective effect in the intact brain.

In this study, we investigated the potential anti-apoptotic activity of β -adrenoceptor blockers in a model system using the human SH-SY5Y cells. These cells are derived from a human catecholaminergic neuroblastoma but have been proposed as a useful *in vitro* model of normal neurons and often used to study neuronal death (Abramova et al., 2002; McGinnis et al., 1999; Moriya et al., 2000; Tang et al., 2005; Tieu et al., 1999). In addition, caspase 8 is not expressed in this cell line (Hopkins-Donaldson et al., 2000), therefore the extrinsic apoptotic pathway contributes little to cell death (Lopez and Ferrer, 2000), enabling us to focus only on the effects of β -adrenoceptor blockers on the intrinsic apoptotic pathway. We specifically asked whether all β -adrenoceptor blockers exhibit an anti-apoptotic property and whether the drugs possess a direct caspase-inhibitor-like property in addition to the previously described mitochondrial stabilization effect.

We report that propranolol (1-(isopropylamino)-3-(naphthalene-1-yloxy)propan-2-ol) as well as ICI 118551 (1-[2,3-(dihydro-7-methyl-1H-iden-4-yl)oxy]-3-[(1-methylethylamino)-2-butanol], an experimental β 2- adrenoceptor selective antagonist, was protective against staurosporine-induced apoptosis while atenolol (2-[4-[2-hydroxy-3-(1-methylethylamino)propoxy]phenyl]ethanamide), a relatively β 1-adrenoceptor selective antagonist, was not. Propranolol and ICI 118551 demonstrated direct inhibition of caspase 9 activity but at higher concentrations than necessary for its anti-apoptotic effect on intact cells. Propranolol and ICI 118551 blocked opening of the cyclosporin-A-insensitive mitochondrial permeability transition pore (mPTP) and the release of cytochrome *c* while atenolol did not. The blockade of cytochrome *c* release and inhibition of caspase 9 may be two mechanisms by which some β -adrenoceptor antagonists protect neurons from apoptosis.

2. Materials & Methods

2.1. Cell culture

The SH-SY5Y human neuroblastoma cells were grown in RPMI supplemented with 10% fetal calf serum and antibiotics. Cells were passaged weekly at about 1:20 split after trypsin digestion to allow growth to near-confluence over 1 week. All experiments were done on cells between passages 10 – 20 after purchasing the original cells from ATCC (Manassas, VA, USA).

For induction of apoptosis, SH-SY5Y cells plated on a poly-d-lysine-coated 6-well cell culture plate were serum-starved overnight and replaced with a serum-free medium supplemented in the morning with 10 μ M staurosporine. Preliminary studies demonstrated maximum caspase 3-like activity at 5–7 hours after initiation of staurosporine treatment with a decline in the detectable caspase 3-like activity at longer times (Supplemental data Fig. 1S). Therefore, all subsequent experiments were assayed after 6 hours of staurosporine treatment. The test β -adrenoceptor blockers were applied at the same time with the staurosporine-containing solution without pre-incubation. 2 μ M cyclosporin A and 10 μ M bongkreikic acid were applied 30 min before staurosporine or 5 μ M p-trifluoromethoxy-carbonyl-cyanide-phenyl-hydrazone (FCCP) treatments. Cells treated with vehicle (dimethyl sulfoxide (DMSO) 0.1%) were prepared as control.

2.2. Caspase-like activity assay

Cells treated with staurosporine and incubated for variable times as dictated by the experiment were washed in phosphate buffered saline, lysed in ice-cold caspase lysis buffer (10 mM Tris-HCl, 10 mM NaH₂PO₄, 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi, pH 7.5) for 15 min, and clarified by centrifugation. Protein concentration was standardized to 1 mg/ml in caspase lysis buffer and 50 μ g of protein was used for the assays. The caspase enzymatic reactions were carried out at 37°C in 20 mM PIPES, 100 mM KCl, 0.5 mM EDTA, 0.05% CHAPS, 5 mM dithio-threitol (pH 7.2) containing either 0.1 mM acyl-Asp-Glu-Val-Asp (Ac-DEVD) -7-amino-4-methylcoumarin (AMC) (caspase 3-like) or acyl-Leu-Glu-His-Asp (Ac-LEHD) -AMC (caspase 2- or 9-like) substrates. The rate of fluorescence increase was measured kinetically over 1 hour (ex: 360 nm, em: 460 nm) using a Synergy II microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and converted to AMC released/ min/ μ g protein using the calibration standard correlating fluorescence to AMC. Purified recombinant caspases, 2 units (caspase 2), 5 units (caspase 3) or 40 units (caspase 9) in ice-cold caspase lysis buffer described above, were used for the *in vitro* assays. Different units of caspases were used to compensate for the different efficacy of the reporter substrate. β -adrenoceptor blockers were added and the caspase enzymatic reactions carried out as described above with the exception that the read out was in fluorescent units/ min. Ac-LEHD-AMC was used to measure caspase 2 and 9, and Ac-DEVD-AMC for caspase 3 activity, respectively.

2.3. Apoptosis Assay

The TdT-mediated dUTP nick-end labeling (TUNEL) assay was carried out as per manufacturer's instructions (Dead End Fluorometric TUNEL Assay, Promega, Madison, WI, USA). At least 6 random fields from 3 independent experiments (> 600 cells) were photographed and counted.

To detect DNA fragmentation, cells were grown in a 6 cm culture plate and treated with vehicle, staurosporine or 100 μ M propranolol for 7 hours. Cells were collected by scraping and centrifuged, then the pellet was washed with phosphate buffered saline. The lysis buffer (1% Nonidet P40, 20 mM EDTA, 50 mM Tris-HCl, 1% sodium dodecyl sulfate (SDS), pH7.5) containing 5 μ g/ μ l RNase A was added and incubated for 2 hours at 56 °C. After subsequent incubation with 2.5 μ g/ μ l Proteinase K for 2 hours at 37 °C, fragmented DNA was precipitated

by adding ½ volume of 10 M ammonium acetate and 2.5 volume of absolute ethanol and then isolated by centrifugation at $10,000 \times g$ for 10 min at 4 °C. The DNA pellet was dissolved in 20 µl of nuclease-free water. The DNA samples were subjected to electrophoresis on 2 % agarose gel, and visualized under ultraviolet light after staining with ethidium bromide.

Cells with membrane externalization of phosphatidylserine was detected via binding of annexin-V-fluorescein isothiocyanate (FITC) using the ApoAlert Annexin-V kit (Clontech, Mountain View, CA, USA) and a Quanta SC flow cytometer (Beckman Coulter, Miami, FL, USA) as described previously (Lee et al., 2007). Non-viable (necrotic or late apoptotic) cells were detected with propidium iodide staining.

2.4. Western Blotting

Western blotting was carried out by standard methods. Samples were lysed in an ice-cold lysis buffer (1% Nonidet P40, 10 mM Tris pH 7.6, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF) containing freshly added protease inhibitor (Complete, Roche Biochemicals, Indianapolis, IN, USA) for 30 min on ice and sonicated (50% power for 2 second \times 10 pulses, Cole Palmer Model 130 Ultrasonic Processor). Lysed samples were centrifuged at $5000 \times g$ for 15 min at 4 °C and the supernatant was combined with SDS sample buffer and incubated at 100 °C for 5 min before loading on a 10% SDS-polyacrylamide gel. Proteins were separated by electrophoresis, and transferred to a nitrocellulose membrane (BioRad, Hercules, CA, USA). The membrane was blocked in 3% milk-Tris buffered saline (50 mM Tris-HCl, pH8.0, 100 mM NaCl) supplemented with 0.1% Tween 20 (TBST), probed with the following primary antibodies (all 1:1000 – 1:5000 dilution): poly-ADP-ribose polymerase (PARP) (#556494, BD Pharmingen, San Diego, CA, USA), cytochrome *c* (S2050, Clontech, Mountain View, CA, USA), actin (Sigma-Aldrich, St. Louis, MO, USA), all in 1% milk-TBST, and reacted with the horseradish peroxidase-conjugated secondary antibody (1:2000) in 1% milk-TBST. After reaction with the Western Lightning chemiluminescence reagent (NEN Life Science Products, Boston, MA, USA), the images were captured on the EpiChemi Darkroom System (UVP Inc, Upland, CA, USA). Densitometric quantitation was done using the LabWorks 4.0 software (UVP Inc).

2.5. Isolation of mitochondrial fraction and determination of cytochrome *c* release

Mitochondrial pellets were prepared as described by Kim et al. (2007). Pellets were resuspended in a mannitol-sucrose buffer consisting of 225 mM mannitol, 25 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4, protease inhibitors supplemented with 5 mM succinate, 3 mM ATP and 3 mM ADP. Staurosporine and β -adrenoceptor blockers were added and incubated at 30 °C. At the end of the stated incubation duration, the mitochondria were pelleted by centrifugation at $16,000 \times g$ for 20 min, and the supernatant assayed for the presence of released cytochrome *c* by western blotting.

2.6. Detection of β - adrenoceptor in SH-SY5Y cells

For detection of β -adrenoceptor mRNA, 1 µg of total RNA isolated (RNAeasy, Invitrogen, Carlsbad, CA, USA) from SH-SY5Y cells was subjected to a single tube reverse transcription polymerase chain reaction (rt-PCR) (Superscript III, Invitrogen) using the primer pairs (β 1: agcatcgagaccctgtgtgcaat and agttgaagaagacgaagggcgg (658 bp); β 2: ggatcatcacagccattgccaagt and agtaaggcctgacacaatccaca (335 bp); β 3: tctctgctggtgccctctttct and ctattctgaacagagccagaggt (621 bp)) specific for the human β - adrenoceptor subtypes. Negative controls were tubes subjected to the identical condition except for omitting the reverse transcription step. Positive controls were PCR reactions carried out with the same primer pairs using a human transcriptome cDNA library (#790000, Stratagene, La Jolla, CA, USA) as the template. Immunocytochemistry was performed with standard methods on 4% paraformaldehyde fixed cells using anti- β 1 adrenoceptor (sc568, Santa Cruz Biotech, Santa

Cruz, CA, USA) or anti- β 2 adrenoceptor (sc569, Santa Cruz Biotech) polyclonal antibody both at 1:200 dilution in phosphate buffered saline with 0.2% Tween 20 and 2% normal goat serum. Images were captured on an epifluorescent microscope with a 100 \times oil immersion objective lens after reacting with an Alexa 594 -conjugated goat anti-rabbit secondary antibody.

2.7. Chemicals

ICI 118551 was purchased from Tocris Bioscience (Ellisville, MO, USA). Staurosporine, Ac-DEVD-AMC, Ac-LEHD-AMC, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), and human recombinant caspases were from Biomol International (Plymouth Meeting, PA, USA). All other chemicals were purchased from Sigma-Aldrich.

2.8. Data analysis

Data are presented as mean \pm S.E.M. and the statistical significance between the control and a treatment group determined by the Mann-Whitney nonparametric test (SigmaStat v3.5, Systat Software Inc., Point Richmond, CA, USA) at the indicated P-level.

3. Results

Staurosporine induced apoptosis in SH-SY5Y neuroblastoma cells as assessed by caspase 3-like activity in a concentration and time-dependent manner, detection of PARP cleavage product by Western blot, and by externalization of phosphatidylserine as determined by flow cytometer (Supplemental Fig. 1–Supplemental Fig. 2). All subsequent experiments were performed at a fixed staurosporine concentration of 10 μ M and assays performed at 6–7 hours at the peak of caspase 3-like activity. We examined the effect of propranolol, atenolol, and ICI 118551, on staurosporine-induced apoptosis. The anti-apoptotic property of propranolol at the 7 hour time point was confirmed by its ability to reduce the number of TUNEL positive cells (Fig. 1 and Table I). At the 24 hour time point, there was no statistically significant difference in the number of TUNEL positive cells between the staurosporine and the staurosporine + propranolol groups suggesting that propranolol did not inhibit but delayed the onset of apoptosis (Table I). The anti-apoptotic effect of propranolol was confirmed by the decrease in staurosporine-induced DNA fragmentation and PARP cleavage (Fig. 2A, B) while propranolol by itself had no effect on any of the indicators of apoptosis examined. Examination of the PARP cleavage over an extended time after staurosporine stimulation also suggested that propranolol delayed the onset of apoptosis (Fig. 2C), however, this interpretation must be viewed with caution since prolonged exposure of the cells to the serum starved experimental condition in itself induces apoptosis. Propranolol and ICI 118551 inhibited the staurosporine induction of caspase 3-like activity with an IC_{50} of $30 \pm 2.8 \mu$ M and $2.5 \pm 1.0 \mu$ M, respectively, while atenolol did not (Fig. 3).

Staurosporine, a protein kinase C inhibitor, induces apoptosis in a wide variety of cells. Its major site of action is the intrinsic pathway, although components of the extrinsic pathway also have been implicated (Stepczynska et al., 2001; Zhang et al., 2004). In SH-SY5Y cells devoid of caspase 8, staurosporine induces apoptosis mostly via activation of the upstream initiator caspases 2 and 9, with a consequent activation of the downstream executor caspase 3 (Lopez and Ferrer, 2000). The β -adrenoceptor blockers could inhibit staurosporine-induced apoptosis by interfering with caspase activation. In fact, the broad-spectrum caspase inhibitor zVAD-fmk inhibited staurosporine-induced caspase 3-like and caspase 9-like activity in a concentration-dependent manner much like the effect of propranolol as assessed by the substrates DEVD-AMC and LEHD-AMC (Fig. 4). However, it is not possible to convincingly examine the effect of propranolol on the individual caspases within a cellular context since no currently available caspase inhibitors (e.g. Ac-DEVD-aldehyde, Ac-LEHD-aldehyde, Ac-VDVAD-aldehyde) are strictly selective (Talanian et al., 1997). Therefore, we examined the

effect of propranolol on the purified recombinant caspases activity in an *in vitro* experiment (Fig. 5). Caspase 2, 3, and 9 were chosen because these three caspases largely mediate staurosporine-induced apoptosis in SH-SY5Y cells (Lopez and Ferrer, 2000). Propranolol, atenolol, or ICI 118551 up to 100 μ M did not have any significant direct effect on either caspase 2 or caspase 3 activities. In contrast, caspase 9 was directly inhibited by ICI 118551 and propranolol albeit at concentrations approximately one order of magnitude greater than the apoptosis-inhibitory activity of these β -adrenoceptor blockers observed within the *in vivo* cellular context. zVAD-fmk inhibited all *in vitro* caspase activities in a concentration-dependent manner as expected.

Polster et al. (2003) demonstrated that propranolol inhibited BH3 peptide and Bax -induced cytochrome *c* release from isolated brain mitochondria. In SH-SY5Y cells, staurosporine similarly induced cytochrome *c* release from isolated mitochondria in a time dependent manner with a reciprocal decrease in the mitochondrial cytochrome *c* consistent with an earlier report (Kim et al., 2007), and propranolol significantly reduced this release (Fig. 6A, B). ICI 118551 also reduced the staurosporine-induced cytochrome *c* release from the mitochondria but to a lesser degree than propranolol, suggesting that the two β -adrenoceptor blockers may utilize different mechanisms to manifest the anti-apoptotic effect. FCCP, a mitochondrial depolarizer and a pro-apoptotic agent (Gautier et al., 2000), induced caspase 3-like activation in SH-SY5Y cells. Staurosporine-induced cytochrome *c* release has been reported to depend on opening of the mPTP in some preparations but not in others (Scarlett et al., 2000; Wigdal et al., 2002). We examined whether the staurosporine-induced caspase 3 activation in SH-SY5Y was inhibited by the classical mPTP blockers cyclosporin A and bongkreikic acid. Staurosporine-induced caspase 3 activation in SH-SY5Y cells was not blocked by cyclosporin A or bongkreikic acid (Fig. 6C, left) but rather paradoxically increased. The caspase-3 activation by FCCP was not blocked by cyclosporin A or bongkreikic acid either (Fig. 6C, right). However, propranolol inhibited the caspase 3 activity induced by both staurosporine and FCCP indicating that this β -adrenoceptor blocker demonstrated anti-apoptotic activity in a cell type where the conventional mPTP blockers were unable to provide protection.

Lastly, we sought to demonstrate the presence of β -adrenoceptor in SH-SY5Y cells. Rt-PCR demonstrated the presence of both β 1- and β 2- adrenoceptor mRNA (Fig. 7A) and immunocytochemistry confirmed the presence of immunoreactivity to both anti- β 1- adrenoceptor and anti- β 2- adrenoceptor antibodies consistent with both β -adrenoceptor proteins being expressed in these cells (Fig. 7B). However, the immunoreactivity was mostly peri-nuclear to diffusely cytoplasmic and not strictly plasma membrane limited. Direct β -adrenoceptor stimulation with norepinephrine, epinephrine, or isoproterenol did not increase the number of TUNEL positive cells or caspase 3-like activation, indicating that these receptors do not serve an acute pro-apoptotic role in the SH-SY5Y cells (Fig. 8). Thus the observed anti-apoptotic effect of the β -adrenoceptor antagonists was most likely not mediated by inhibition of an occult stimulation of these receptors.

4. Discussion

In this study, we demonstrated that the β -adrenoceptor blockers propranolol and ICI 118551 protect SH-SY5Y cells against staurosporine-induced apoptosis. Both β -adrenoceptor blockers directly inhibited recombinant caspase 9 activity *in vitro*; however, they did so at concentrations greater than necessary for the manifestation of their cytoprotective effect in intact cells. Therefore, it is unlikely that the direct inhibition of caspase 9 activity alone plays a significant role in the drugs' anti-apoptotic effect. Although β -adrenoceptors are present in these cells, the anti-apoptotic effect of β -adrenoceptor blockers is unlikely to be mediated by the actual blockade of the receptors because direct stimulation of the β -adrenoceptors by catecholamines does not induce apoptosis. Furthermore, concentrations of β -adrenoceptor blockers necessary

for the anti-apoptotic (1 – 100 μM) effect were orders of magnitude greater than the binding affinity of these ligands for the β - adrenoceptors (1 – 100 nM) (Hoffman et al., 2004).

Polster et al. (2003) suggested that amphiphilic molecules such as propranolol and dibucaine inhibit cytochrome *c* release by acting on events downstream of Bax incorporation into the outer mitochondrial membrane, perhaps by blocking Bax-induced changes in the lipid structure (Basanez et al., 2002). In their model of isolated brain mitochondria, the BH3 peptide and Bax-induced release of cytochrome *c* was not due to the opening of conventional mPTP since cyclosporin A did not block this release. Our experiments on intact SH-SY5Y cells demonstrated a similar non-cyclosporin A and non-bongkrekiic acid-blockable activation of the apoptotic cascade by staurosporine or FCCP which was blocked by two of the three β -adrenoceptor antagonists tested. Cyclosporin A and bongkrekiic acid are well known inhibitors of apoptosis in many cell types (reviewed in Bouchier-Hayes et al., 2005; Zoratti et al., 2005). Cyclosporin A is thought to acts through inhibition of association between the matrix cyclophilin D and the inner membrane adenine nucleotide translocator while bongkrekiic acid acts by binding to the adenine nucleotide translocator fixing its conformational state to the closed state. Both adenine nucleotide translocator and cyclophilin D are thought to be components of the putative mPTP. However, the ability of these classical mPTP inhibitors to prevent opening of the mPTP and the subsequent induction of apoptosis in SH-SY5Y cells appears to depend on the pro-apoptotic stimulus employed (Abramova et al., 2002; McGinnis et al., 1999; Moriya et al., 2000) and we confirmed the earlier report by McGinnis et al. (1999) that staurosporine-induced apoptosis in SH-SY5Y cells was not blocked by the classical mPTP inhibitors. It is likely that the β - adrenoceptor blockers are inhibiting the initiation of cyclosporin A and bongkrekiic acid insensitive mitochondria-mediated apoptotic cascade in the intact SH-SY5Y cells similar to that described in the isolated brain mitochondria (Polster et al., 2003). The site of action of the β - adrenoceptor blockers remains undefined at this time but most likely not mediated by the β - adrenoceptors. The opening of the mPTP is a complex process with many different inducers, modulators and inhibitors, dependent on the cellular model (reviewed by Zoratti et al., 2005). Therefore our finding may not be generalizable, however, β -adrenoceptor blockers may prove to be better anti-apoptotic drugs than the conventional mPTP inhibitors when targeting neurons.

The role of reactive oxygen species in the opening of mPTP is well-known (He and Lemasters, 2002; Kowaltowski et al., 1996), and some β -adrenoceptor blockers have been reported to exhibit anti-oxidant properties (Jaboureck-Bouttier et al., 1999; Nishina et al., 2001). Carvedilol, an α - and β - adrenoceptor antagonist, demonstrated neuroprotection in an *in vitro* model of free radical-mediated neuronal injury (Yue et al., 1994), *in vivo* global ischemic model (Lysco et al., 1992), and *in vivo* focal cerebral ischemia model (Savitz et al., 2000) through its anti-oxidant effect. However, the IC_{50} required to inhibit lipid peroxidation in the swine brain homogenate for propranolol and atenolol were >3000 and $1000 \mu\text{M}$, respectively (Yue et al., 1994), thus much higher than the concentrations used in our experiment. Therefore it is likely that the anti-apoptotic effect of propranolol was not due to its antioxidant property.

Although we focused on the β - adrenoceptor blockers' effect on caspases and mitochondrial integrity, staurosporine induces cell death in many ways. Decrease in the expression of the anti-apoptotic Bcl 2 and increase in the expression of the pro-apoptotic p53 (Tieu et al., 1999), Bax translocation to the mitochondria secondary to intracellular alkalization (Tafani et al., 2002), and increase in the phosphorylation of glycogen synthase kinase-3 β (Bhat et al., 2000) are some of the other reported staurosporine-induced cell death mechanisms. Further studies will be required to rule out the possibility that the β - adrenoceptor blockers' anti-apoptotic effect may be mediated by effects on any of the above mechanisms.

As a class of drugs, β -adrenoceptor blockers share common molecular characteristics but also individually possess unique structural signatures. Regardless of the precise mechanism responsible for the anti-apoptotic action, further structure-activity analysis common to propranolol and ICI 118551 exhibiting an anti-apoptotic property, but not shared with atenolol devoid of the protective effect, may lead to identification of critical molecular signatures essential to the development of a more potent clinically acceptable novel anti-apoptotic small molecule derivative of the β -adrenoceptor blockers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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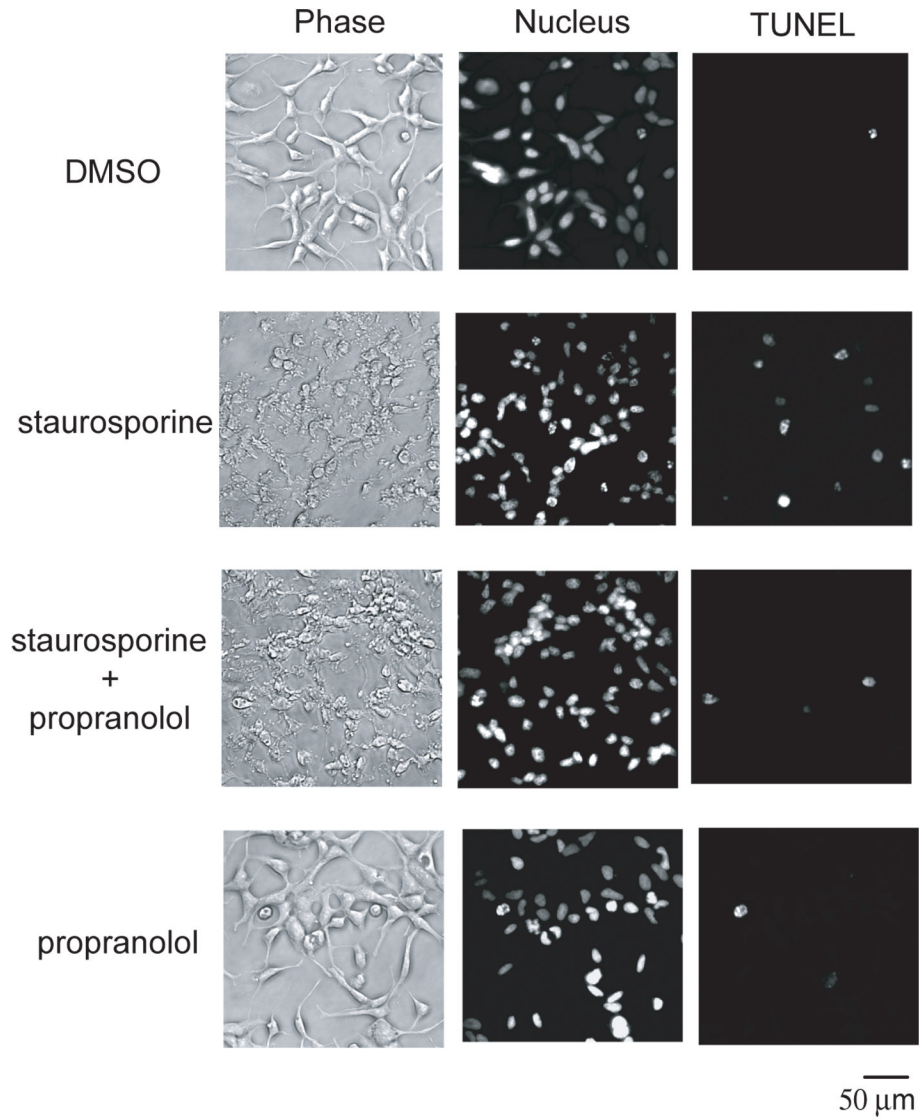


Fig. 1. Propranolol, a clinically used β -adrenoceptor blocker, decreased the number of TUNEL positive cells

A phase contrast (left), nuclear stain (middle), and TUNEL stain (right) images of the same field of view for cells subjected to the noted conditions. Staurosporine increased the number of TUNEL positive cells while propranolol decreased this effect.

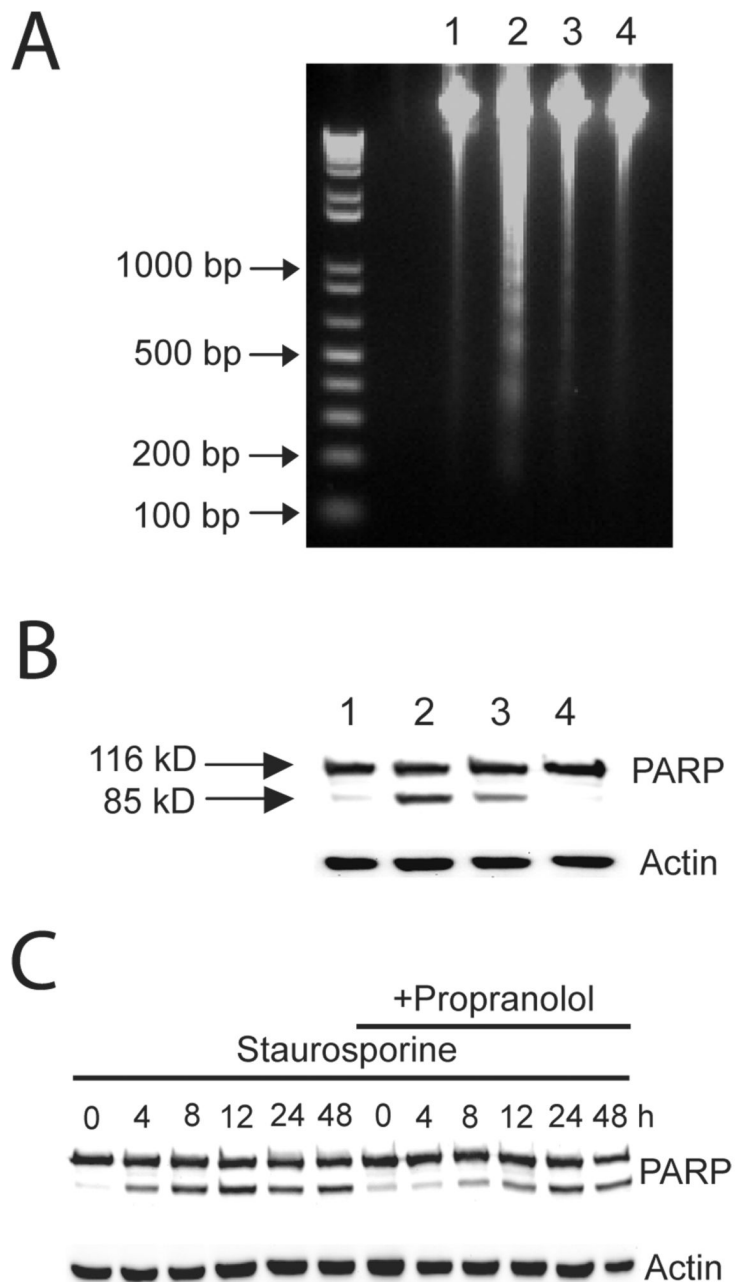


Fig. 2. Propranolol decreased staurosporine-induced DNA fragmentation and PARP cleavage
A. A 2% agarose gel demonstrating DNA fragmentation and reversal of this effect by propranolol (lanes 1: DMSO, 2: staurosporine (10 μ M), 3: staurosporine + propranolol (100 μ M), and 4: propranolol alone (100 μ M). **B.** A western blot of the cytosolic fraction probed with anti-PARP antibody demonstrating the cleavage of the PARP protein (85 kD). Anti-actin antibody probing of the same membrane confirmed equal loading of the lanes. The lanes correspond to the same conditions as above and assayed at 7 hours after initiation of staurosporine treatment. **C.** Same condition as above except assayed at later time points. Propranolol was unable to inhibit PARP cleavage at 24–48 hours indicating that the onset of staurosporine-induced apoptotic program was delayed but not inhibited.

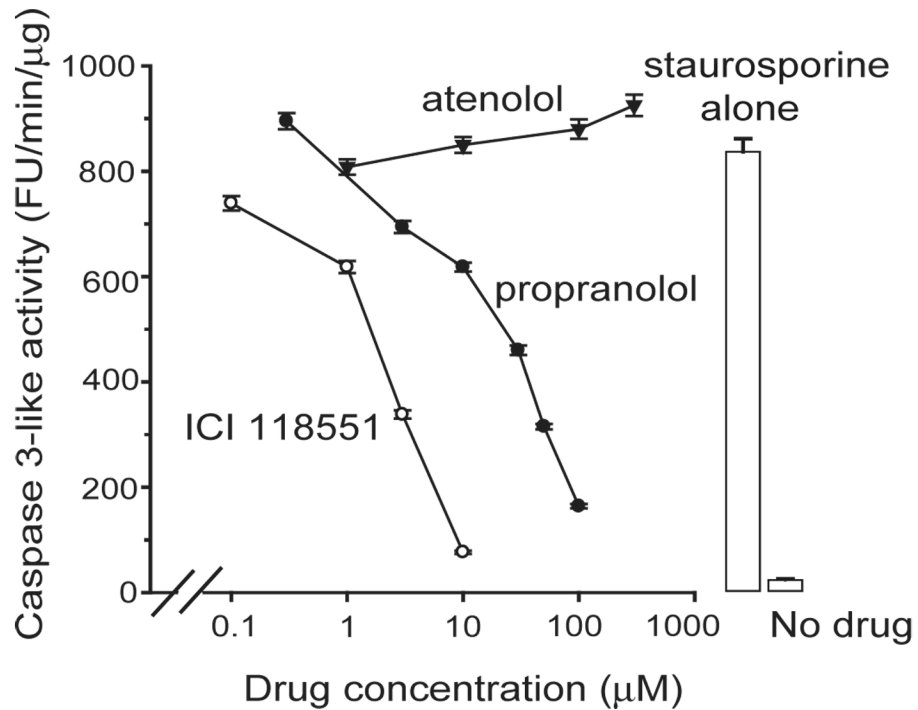


Fig. 3. Not all of the β -adrenoceptor blockers inhibited staurosporine-induced increase in caspase-3-like activity

Inhibition concentration-response for the various β -adrenoceptor blockers with caspase-3-like activity reported as FU (fluorescence unit)/ min/ μ g protein. Data fit with $y=y_0+A(IC_{50}^n/(IC_{50}^n+x^n))$ where IC_{50} = half-inhibition concentration, n = Hill slope, gave IC_{50} of 30 ± 2.8 and $2.5 \pm 1.0 \mu$ M, and n of 1.3 ± 0.4 and 1.4 ± 0.4 , for propranolol and ICI 118551, respectively. Results from 3 experiments each with triplicate measurements.

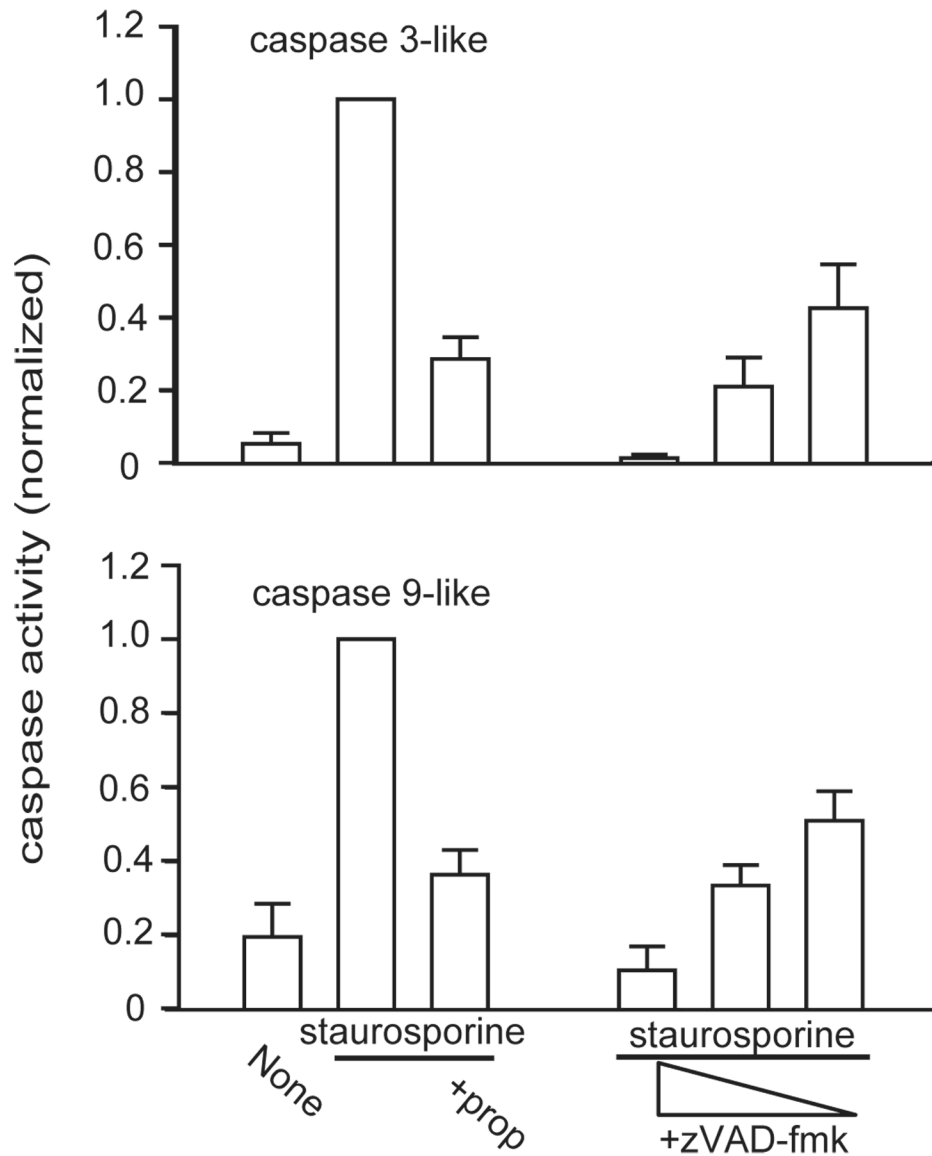


Fig. 4. Propranolol inhibited staurosporine-induced activation of caspase 3 and 9-like activities Propranolol (prop) (100 μ M) inhibits staurosporine-activation of both caspase 3 and its upstream initiator caspase 9-like activities much like the broad-spectrum caspase inhibitor zVAD-fmk (10, 1, 0.1 μ M). Results from two independent experiments each with triplicate measurements.

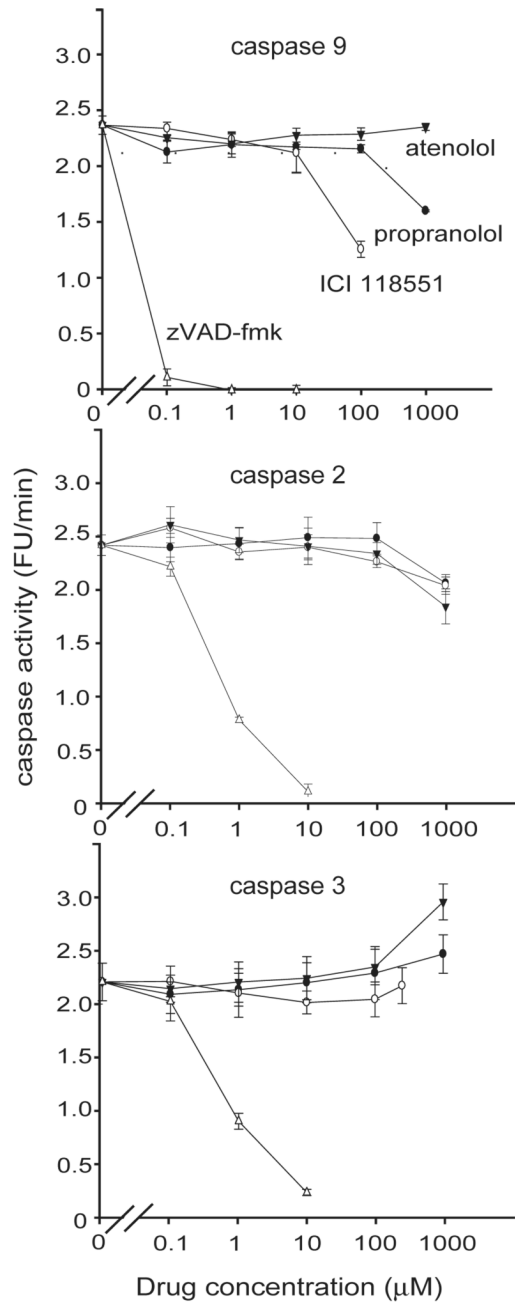


Fig. 5. Propranolol and ICI 118551 inhibited staurosporine activation of caspase 9 in vitro

The direct effect of β -adrenoceptor blockers on purified recombinant caspases 9, 2, and 3 activities were examined *in vitro*. ICI 118551 (10–100 μ M) and propranolol (100–1000 μ M) directly inhibited caspase 9 activity. All caspases were inhibited by zVAD-fmk (0.1–10 μ M). Representative of 3 independent experiments each with triplicate measurements. The ordinate is presented as fluorescence unit (FU) / min since the amount of recombinant caspase used for the assay was defined by the activity unit rather than μ g protein (see Methods).

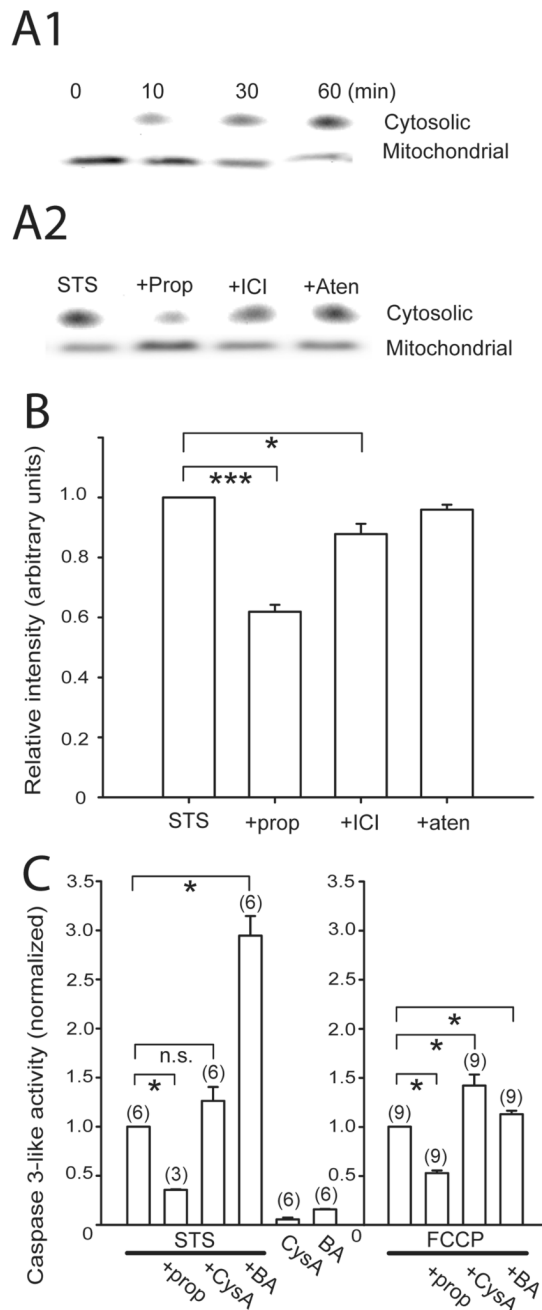


Fig. 6. Cytochrome c release from mitochondria induced by staurosporine was blocked by propranolol

A. Time-dependent increase in cytosolic- and a reciprocal decrease in the mitochondrial-cytochrome *c* induced by 10 μ M staurosporine (A1) and the effect of β -adrenoceptor blockers on this release at 60 min (A2). The concentration of the drugs were staurosporine (STS) 10 μ M, propranolol (prop) 100 μ M, ICI 118551 (ICI) 10 μ M, and atenolol (aten) 100 μ M. The cytosolic and mitochondrial cytochrome *c* appeared differently on the Western blot due to the different extraction buffers used for the two cellular fractions (see Methods). **B.** A bar plot of densitometric quantification of immunoblots of cytosolic cytochrome *c* normalized to the STS only condition from four separate experiments. The relative intensities (in arbitrary units) of

propranolol-treated (0.62 ± 0.02) and ICI 118551-treated (0.88 ± 0.03) samples were significantly reduced from staurosporine alone while atenolol had no effect (0.96 ± 0.02). **C.** Propranolol inhibited both STS- and FCCP-induced caspase 3-like activation that was not blocked by $2 \mu\text{M}$ cyclosporin A (CysA) or $10 \mu\text{M}$ bongkrelic acid (BA). The relative caspase 3-like activity was normalized to the STS-only (left) or p-trifluoromethoxy-carbonyl-cyanide-phenyl-hydrazone (FCCP)-only conditions (right). Statistical significance at * $P < 0.05$ and *** $P < 0.001$. n.s. = not significant. Number of independent measurements in parentheses.

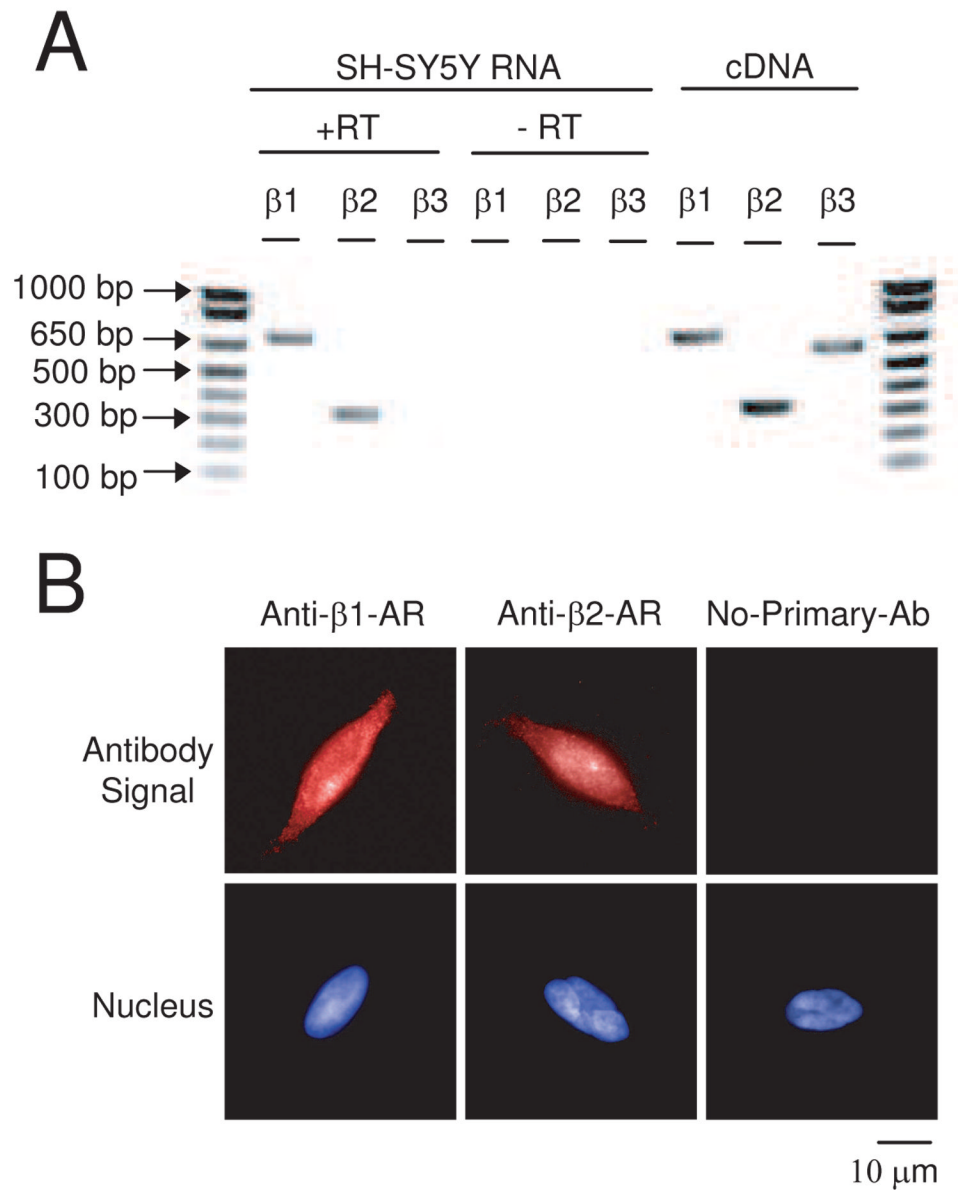


Fig. 7. β-adrenoceptors exist in SH-SY5Y cells

A. rt-PCR confirmation of the presence of β1- and β2- adrenoceptor mRNA. rt-PCR was performed on total RNA isolated from the cells demonstrating products of the expected size. The negative rt control (-RT) where the reverse transcription step was omitted resulted in no product. The same primer pairs using a cDNA brain library control template served as a positive control for the PCR reaction. **B.** Immunocytochemistry with β1- (left) or β2- (middle) adrenoceptor selective antibodies demonstrated immunoreactivity consistent with the expression of the receptor protein. Fluorescent antibody signal (upper panels) and nuclear staining (lower panels) views of the same field are shown. Omission of the primary antibody (right) gave no signal.

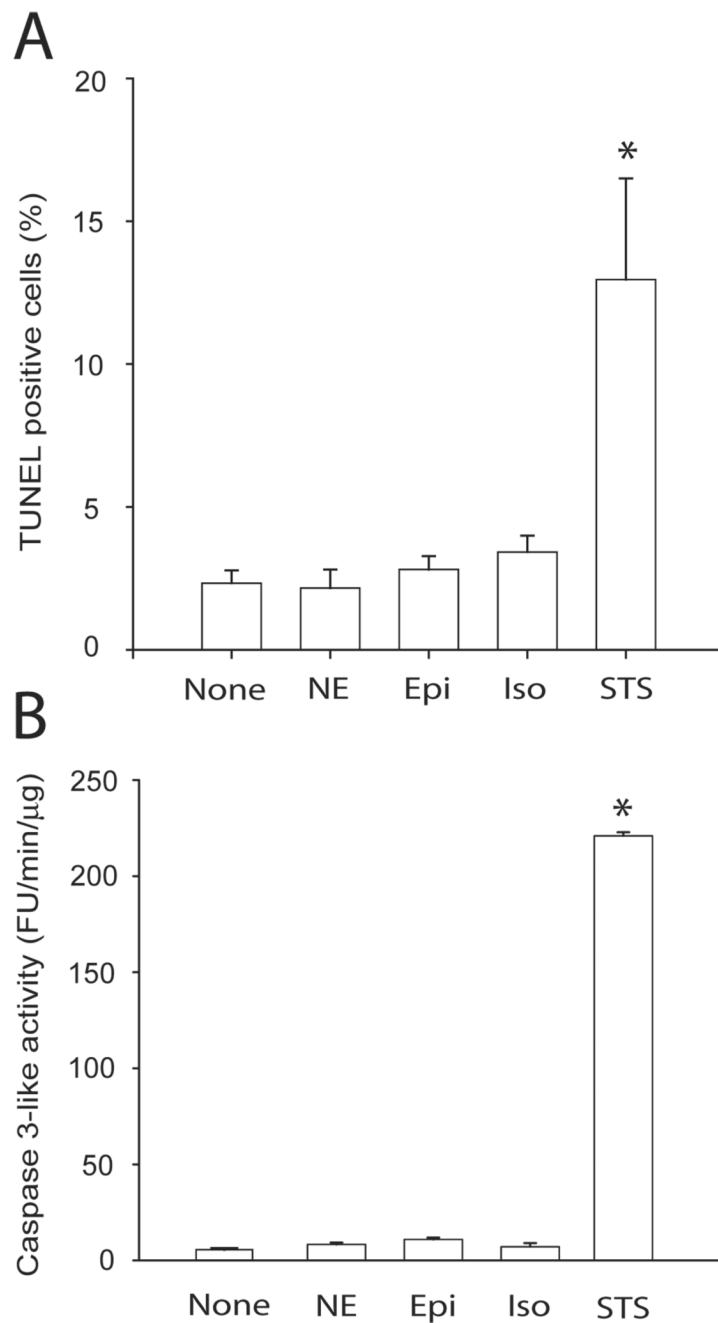


Fig. 8. Direct catecholamine stimulation does not increase the number of TUNEL positive cells or caspase 3-like activity in SH-SY5Y cells

A. A histogram summary of TUNEL positive cells (mean \pm S.E.M. from triplicate counts). TUNEL labeling was performed in cells with no treatment (None) or 8 hours after treatment with 100 μ M norepinephrine (NE), epinephrine (Epi), isoproterenol (Iso), or 10 μ M staurosporine (STS). **B.** Similar experiments but assayed for caspase 3-like activity. Results from N=3 independent experiments. * $P < 0.01$ and no statistically significant difference between the other 4 groups.

Table I**Propranolol decreases the number of TUNEL positive cells induced by staurosporine**

The numbers are percentage (mean \pm S.E.M.) of TUNEL positive cells at 7 or 24 hours after initiation of the staurosporine (10 μ M) treatment with or without propranolol (100 μ M). * $P < 0.05$, ns=not significant.

	7 hours		24 hours	
Control (DMSO)	1.8 \pm 0.6		2.6 \pm 0.5	
Staurosporine	12.1 \pm 1.1	}	21.9 \pm 3.8	}
Staurosporine + Propranolol	5.0 \pm 1.1		16.0 \pm 2.3	
Propranolol	2.9 \pm 0.7		1.9 \pm 0.4	
		*		*
		*		ns
		ns		ns