Thrombin-Induced Oxidative Stress Contributes to the Death of Hippocampal Neurons: Role of Neuronal NADPH Oxidase

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The present study investigated whether thrombin can induce the production of reactive oxygen species (ROS) through activation of neuronal NADPH oxidase and whether this contributes to oxidative damage and consequentially to neurodegeneration. Immunocytochemical and biochemical evidence demonstrated that, in neuron-enriched hippocampal cultures, thrombin induces neurodegeneration in a dose-dependent manner. In parallel, ROS production was evident as assessed by analyzing DCF and hydroethidine. Real-time PCR analysis, at various time points after thrombin treatment, also demonstrated that expression of NADPH oxidase subunits (p47phox and p67phox) occurs. In addition, Western blot analysis and double-label immunocytochemistry showed an up-regulation in the expression of cytosolic components (Rac 1 and p67phox), the translocation of cytosolic proteins (p47phox and p67phox) to the membrane, and the localization of gp91phox or p47phox expression in hippocampal neurons of cultures and CA1 layer. The thrombin-induced ROS production, protein oxidation, and loss of cultured hippocampal neurons were partially attenuated by an NADPH oxidase inhibitor and/or by several antioxidants. Collectively, the present study is the first to demonstrate that, in cultured hippocampal neurons, thrombin-induced neurotoxicity is, at least in part, caused by neuronal NADPH oxidase-mediated oxidative stress. This strongly suggests that thrombin can act as an endogenous neurotoxin, and inhibitors of thrombin and/or antioxidants can be useful agents for treating oxidative stress-mediated hippocampal neurodegenerative diseases, such as Alzheimer's disease. © 2008 Wiley-Liss, Inc.

Key words: thrombin; hippocampus; NADPH oxidase; oxidative stress; neurodegeneration

Thrombin is generated from the precursor prothrombin, which is endogenously expressed in the rat, mouse, and human brain, including the hippocampus (Weinstein et al., 1995; Beilin et al., 2001). Prothrombin also exists and circulates in blood at micromolar levels (Fenton, 1986). Cerebrovascular injury triggers the rapid conversion of prothrombin to thrombin, resulting in extravasation into the central nervous system (CNS; Gingrich and Traynelis, 2000). There is accumulating evidence that thrombin induces various biological responses in the CNS, and, depending on the thrombin concentration, its effect on neurons and astrocytes is either protective or toxic. For example, increased thrombin in the brain has been shown to lead to the degeneration of spinal motoneurons (Turgeon et al., 1998) and astrocytes (Donovan et al., 1997). Moreover, we have recently reported that thrombin is toxic to cortical (Lee et al., 2006) and dopaminergic (Choi et al., 2003) neurons in neuron-enriched cultures.

There is growing evidence suggesting that microvascular damage is involved in the neuropathogenesis of Alzheimer's disease (AD; Grammas, 2000; Borroni et al., 2002). For instance, AD patients may have increased blood–brain barrier (BBB) permeability, resulting in increased serum protein accumulation within the brain extracellular space (Wardlaw et al., 2003). This suggests that certain blood-derived factors are capable of inducing various pathophysiological responses (Wardlaw et al., 2003). Among blood-derived factors, thrombin is elevated, whereas the activity of the thrombin inhibitor protease nexin I is significantly reduced in AD brains (Choi et al., 1995). A correlation between the incidence of head trauma and the occurrence of AD has also indicated that physical injury to the BBB may exacerbate...
the onset of the disease through thrombin activation and accumulation in the brain (Uryu et al., 2002). In addition, thrombin induces cell death in hippocampal neurons (Donovan et al., 1997; Striggow et al., 2000; Choi et al., 2005b). Collectively, these observations indicate that thrombin may act as an endogenous neurotoxin, leading to hippocampal neurodegeneration in neurodegenerative diseases such as AD. This hypothesis is supported by recent results showing that thrombin-induced neurotoxicity in the rat hippocampus is associated with cognitive deficits (Mhatre et al., 2004).

NADPH oxidase is a multisubunit enzyme, composed of cytosolic subunits (gp40phox, p47phox, p67phox, and GTP-binding protein P21-Rac1), and membrane subunits (gp91phox and gp22phox), that catalyzes the generation of superoxide radicals from oxygen (Cross and Segal, 2004). When NADPH oxidase is activated, the entire cytosolic complex translocates to the membrane, where it assembles with other membrane-associated proteins and becomes activated. It has been demonstrated that neuronal reactive oxygen species (ROS) such as superoxide play a role in cell death (Noh and Koh, 2000; Tamamriello et al., 2000; Zekry et al., 2003; Jana and Pahan, 2004). These studies also showed that neuronal NADPH oxidase is an important potential source of ROS production. In addition, several studies have demonstrated that the brains of AD patients show evidence of oxidative stress, such as oxidative protein (Hensley et al., 1997) and lipid (Palmer and Burns, 1994) modification. Collectively, these observations suggest that neuronal NADPH oxidase-mediated oxidative stress, possibly originating from the neuron, contributes to AD neurodegeneration (Zekry et al., 2003). This hypothesis is supported by the finding that, in the brains of AD patients, neuronal NADPH oxidase induces ROS generation, leading to neuronal death (Shimohama et al., 2000). The present study examined whether neuronal NADPH oxidase can be activated by thrombin in neuron-enriched rat hippocampal cultures, in vitro, and whether NADPH oxidase-derived ROS participate in thrombin-induced degeneration of hippocampal neuron cultures.

MATERIALS AND METHODS

Thrombin, trolox, superoxide dismutase (SOD), and N-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Apocynin and diphenylene iodonium (DPI) were purchased from Calbiochem (San Diego, CA). 3-(4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) was purchased from USB (Cleveland, OH). Solutions of N-acetyl-L-cysteine (NAC) were adjusted with pH 7.4 before use.

Neuron-Enriched Hippocampal Culture

Rat hippocampal cultures were prepared from the 18-day-old rat fetal brain (Sprague-Dawley), and the hippocampi were mechanically triturated as described previously (Donovan et al., 1997). Dissociated cells were plated on 12-mm round acrylic plastic coverslips in 24-well plates at a density of 2 × 10^3 cells/coverlip. Plating media consisted of Neurobasal medium (NB; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin-streptomycin. For neuron-rich culture (>95% pure), at 2–4 days in vitro (DIV), the medium was replaced with growth media consisted of NBM supplemented with 2% B-27, 2 mM glutamine, penicillin-streptomycin, and 2.5 μM arabinoside C (ara C) as described previously (Gray and Patel, 1995). At DIV 6, cells were treated with thrombin or PBS in NBM containing 2 mM glutamine and penicillin-streptomycin deprived of serum. The purity of the cultures was confirmed by immunostaining with MAP-2 for neurons, glial fibrillary acidic protein (GFAP) for astrocytes, and OX-42 for microglia; composed of >95% neurons, 4–5% astrocytes, and <1% microglia.

Stereotaxic Surgery and Drug Injection

All experiments were in accordance with approved animal protocols and guidelines established by Ajou University. Female Sprague Dawley rats (260–280 g) were anesthetized with injection of chloral hydrate (360 mg/kg, i.p.) and positioned in a stereotaxic apparatus (Kopf Instrument, Tujunga, CA), and they received a unilateral administration of thrombin into the right hippocampus CA1 layer [anteroposterior (AP), −3.8; mediolateral (ML), −2.0; and dorsoventral (DV), −2.6 mm from bregma] according to the atlas of Paxinos and Watson (1998). Thrombin was injected at a rate of 0.2 μl/min using a 30-gauge Hamilton (Reno, NV) syringe attached to an automated microinjector (Buwon, Seoul, Korea). After injection, the needle was left in place for an additional 5 min before slow retraction. Intact (nontreated) or PBS-treated animals were used as controls. Animals were killed by an overdose of chloral hydrate for further studies.

MTT Reduction Assay

As previously described (Munoz et al., 2002), MTT stock solution was added to each culture after thrombin treatment so that the final concentration of MTT in the medium was 0.25 mg/ml (600 μM), and the cells were incubated for 3 hr at 37°C. After incubation, medium was removed, and DMSO was added to each well. The absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm.

Immunocytochemistry

As previously described (Lee et al., 2006), cultured cells were paraformaldehyde (4%) fixed, permeabilized with 0.2% Triton X-100 for 5 min, and then incubated overnight with the anti-MAP-2 (1:500; Sigma) for general neurons, anti-mouse GFAP (1:500; Sigma) for astrocytes, and mouse OX-42 (1:400; Serotec, Bicester, United Kingdom) for microglia. On the following day, cultured cells were rinsed with PBS + 0.5% BSA and incubated with appropriate biotinylated secondary antibody, followed by avidin-biotin complex (Elite Kit purchased from Vector, Burlingame, CA). The bound antiserum was visualized by incubating with 0.05% diaminobenzidine–HCl (DAB) and 0.003% hydrogen peroxide in 0.1 M
acid), 1% NP-40, and protease inhibitor mixture (Sigma).

Membrane factions were prepared from cultures after thrombin treatment. Cultures were lysed in ice-cold buffer consisting of the following (in mM): 20 HEPES, 250 sucrose, 150 NaCl, 10 mM Tris-HCl (pH 7.2), 0.5% sodium deoxycholate (deoxycholic acid), 1% NP-40, and protease inhibitor mixture (Sigma). The samples were centrifuged at 4°C for 20 min at 14,000 g, and the supernatant was transferred to a fresh tube. The pellets were further centrifuged for 1 hr at 100,000 g at 4°C, and the resulting supernatants and pellets were designated as the cytosolic and membrane fractions, respectively. Equal amounts of protein (30 μg) were mixed with loading buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% mercaptoethanol, and 0.002% bromophenol blue), boiled for 5 min, and separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using an electrophoretic transfer system (Bio-Rad, Hercules, CA). The membranes were washed with Tris-buffered saline solution (TBS) and then blocked for 1 hr in TBS containing 5% skim milk. The membranes were then incubated overnight at 4°C with one of the following specific primary antibodies: rabbit anti-p47phox (1:1,000; Santa Cruz Biotechnology), mouse anti-Calbindin (1:400; Chemicon, Temecula, CA) and rabbit anti-p47phox antibody (1:200; Santa Cruz Biotechnology), mouse MAP-2 (1:500; Sigma), and rabbit anti-gp91phox antibody (1:200; BD Biosciences, San Jose, CA). On the next day, cultures were rinsed and incubated with fluorescein isothiocyanate (FITC)-conjugated anti–mouse IgG (1:200; Kirkegaard & Perry, Gaithersburg, MD) and Texas red-conjugated anti-rabbit IgG (1:200; Vector) for 1 hr. For in vivo study, animals were transcardially perfused with a saline solution containing 0.5% sodium nitrate and heparin (10 U/ml) followed by 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). Brains were removed from the cranium and postfixed for 1 hr, washed in 0.1 M PB, and then immersed in 30% sucrose solution until they sank. Tissues were sectioned on a sliding microtome at a thickness of 40 μm, and every sixth serial section was selected and processed for immunostaining as described previously (Choi et al., 2005b). Free-floating sections were mounted on gelatin-coated slides and dried at room temperature. Brain sections were incubated in 0.2% Triton X-100 for 30 min, rinsed twice in PBS + 0.5% BSA, and then incubated overnight at 4°C with mouse NeuN (1:200; Cemicon, Temecula, CA) and rabbit anti-p47phox antibody (1:200; Santa Cruz Biotechnology), mouse MAP-2 (1:500; Sigma), and rabbit anti-gp91phox antibody (1:200; BD Biosciences). On the next day, slides were rinsed and incubated with FITC-conjugated anti–mouse IgG (1:200; Kirkegaard & Perry) and Texas red-conjugated anti-rabbit IgG (1:200; Vector) for 1 hr. Slides were then rinsed three times with PBS, cover-slipped with Vectashield medium (Vector), and analyzed with an Olympus IX71 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Cultures treated with thrombin (40 U/ml) were washed with RNase-free, ice-cold PBS, and then total RNA (1 μg) was extracted using RNAzol B (Invitrogen, San Diego, CA). RT was carried out using Superscript II reverse transcriptase (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. The primer sequences used in this study were as follows: 5′-GCTCACCAGGATCTTCAAACA-3′ (forward) and 5′-GCCCTTCTGCAGATATGGA-3′ (reverse) for p47phox; 5′-CAGGAGTCTCAAGATGCTG-3′ (forward) and 5′-GATCTGCTGAGATTATCC-3′ (reverse) for gp91phox; 5′-GAAAGCATGAAGGATGCCTGG-3′ (forward) and 5′-TCCTCAAGATGTTGCGACA-3′ (reverse) for p67phox; and 5′-ATAGCACAAGATCACTCCT-3′ (reverse) for p67phox. Real-time PCRs were performed in a reaction volume of 20 μl, including 1 μl RT product as a template, 10 μl of SYBR Green PCR master mix (Applied Biosystems, Warrington, United Kingdom), and 20 pmol of each primer described above. The PCR amplifications were performed with 40 cycles of 95°C for 30 sec and 60°C for 60 sec using ABI 7500 (Applied Biosystems, Foster City, CA). Average C_{S} values of p47phox, gp91phox, and p67phox from triplicate PCRs were normalized from average C_{S} values of glyceraldehyde-3-phosphate dehydrogenase. The ratios of expression levels of p47phox, gp91phox, and p67phox between control and control at each time point were calculated as 2^{ΔΔC_{T}}.

RT-PCR

Cultures treated with thrombin (40 U/ml) were washed with RNase-free, ice-cold PBS, and then total RNA (1 μg) was extracted using RNAzol B (Invitrogen, San Diego, CA). RT was carried out using Superscript II reverse transcriptase (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. The primer sequences used in this study were as follows: 5′-GCTCACCAGGATCTTCAAACA-3′ (forward) and 5′-GCCCTTCTGCAGATATGGA-3′ (reverse) for p47phox; 5′-CAGGAGTCTCAAGATGCTG-3′ (forward) and 5′-GATCTGCTGAGATTATCC-3′ (reverse) for gp91phox; 5′-GAAAGCATGAAGGATGCCTGG-3′ (forward) and 5′-TCCTCAAGATGTTGCGACA-3′ (reverse) for p67phox; and 5′-ATAGCACAAGATCACTCCT-3′ (reverse) for p67phox. Real-time PCRs were performed in a reaction volume of 20 μl, including 1 μl RT product as a template, 10 μl of SYBR Green PCR master mix (Applied Biosystems, Warrington, United Kingdom), and 20 pmol of each primer described above. The PCR amplifications were performed with 40 cycles of 95°C for 30 sec and 60°C for 60 sec using ABI 7500 (Applied Biosystems, Foster City, CA). Average C_{S} values of p47phox, gp91phox, and p67phox from triplicate PCRs were normalized from average C_{S} values of glyceraldehyde-3-phosphate dehydrogenase. The ratios of expression levels of p47phox, gp91phox, and p67phox between treatment with thrombin and control at each time point were calculated as 2^{ΔΔC_{T}}.
Measurement of ROS Generation

For measurement of intracellular ROS levels, cells were incubated with 10 μM 5- (and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate [CM-H2DCF-DA (here referred to as DCF)] (Invitrogen, San Diego, CA) for 10 min. The cells were then washed with D-PBS (in mM: 2.68 KCl, 1.47 KH2PO4, 136.89 NaCl, and 8.1 Na2HPO4), and the fluorescence images were taken with a 1X71 confocal laser microscope (Olympus). Fluorescence images of cells were analyzed using the Fluoview system (Olympus). DCF sample values were expressed as the -fold increase vs. control values.

Superoxide (O2- and O2- derived oxidants) generation was determined after incubation with 5 μM hydroethidine for 20 min at 37°C, as previously described (Bindokas et al., 1996; Rego et al., 2003). Hydroethidine was made in distilled water and was prepared freshly. HEPES-buffered saline (HBS; 1996; Rego et al., 2003). Hydroethidine was made in distilled water and was prepared freshly. HEPES-buffered saline (HBS: 144 mM NaCl, 10 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, 5 mM KCl, 10 mM D-glucose; 320 mM, pH 7.4) was used as the extracellular solution. The ethidium, obtained after oxidation of hydroethidine (dihydroethidium) by superoxide, was measured with excitation at 548 nm and emission at 620 nm using LS55 Luminescence Spectrometer (Perkin-Elmer, Waltham, MA). Levels of superoxide generation were expressed as the -fold increase vs. control values. In addition, for in situ visualization of oxidized hydroethidine, the images were taken using a laser confocal microscope (Olympus).

Detection of Protein Oxidation

The extent of protein oxidation was assessed by measuring protein carbonyl levels with an OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA) according to the protocol of the manufacturer, with some modifications (Singhal et al., 2002). Protein samples were prepared from cultures at 4 hr after thrombin treatment in the absence or presence of apocynin (1 mM). Subsequently, protein samples (15 μg) were mixed in a microcentrifuge tube with 5 μl of 12% SDS and 10 μl of 1× 2,4-dinitrophenylhydrazine (DNPH) solution. Ten microliters of 1× DNPH solution (a kit component) was added instead of the DNPH solution as the negative control. Tubes were incubated at RT for 15 min and then mixed with 7.5 μl of neutralization solution. Next, the samples were mixed in equal volumes of SDS sample buffer and separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked for 1 hr at RT in Tris-buffered saline containing 0.1% Tween 20 and 1% BSA. Membranes were incubated overnight at RT with the anti-DNPH antibody (1:150), then incubated at RT for 1 hr with secondary antibodies (1:300). Blots were developed using enhanced chemiluminescence reagents (Amersham Biosciences). Proteins that underwent oxidative modification (i.e., carbonyl group formation) were identified as a band in the samples derivatized with DNPH. The optical density of the bands was measured using the Computer Imaging Device and accompanying software (Fujifilm). Levels of protein carbonyls were quantified and expressed as the -fold increase vs. untreated controls.

Measurement of the Densities of the Immunoblot Bands

For semiquantitative analysis of immunoblot bands, the density of each band was measured with a computer imaging device and accompanying software (Bio-Rad, Hercules, CA). The background value was subtracted from all other readings.

Statistical Analysis

All values are expressed as mean ± SEM. Statistical significance (P < 0.05 for all analyses) was assessed by ANOVA using Instat 3.05 (GraphPad, San Diego, CA), followed by Student-Newman-Keuls analyses.

RESULTS

Thrombin Induces Neurodegeneration in Neuron-Enriched Hippocampal Cultures

In nontreated, neuron-enriched control hippocampal cultures, immunocytochemistry showed that MAP-2 immunopositive (MAP-2-ip) neurons have large and healthy cell bodies with long, branched processes (Fig. 1A). By contrast, cultures treated with 100 U/ml thrombin for 24 hr displayed a profound loss of MAP-2-ip neurons, and most of the remaining MAP-2-ip neurons appeared injured and were characterized by shrunken cell bodies and short processes (Fig. 1B). In addition, thrombin-induced neurotoxicity occurred in a dose-dependent manner, as determined by MAP-2 immunocytochemistry and MTT reduction assays. Treatment of neurons with 5 U/ml thrombin did not affect the MAP-2-ip cell number or the MTT reduction assay value (data not shown). In contrast, when quantified and expressed as a percentage of control values, treatment of neurons with 20–100 U/ml thrombin attenuated the number of MAP-2-ip neurons by 29–80% and reduced MTT conversion by 20–77% (Fig. 1C).

Thrombin Induces ROS Production and NADPH Oxidase Expression in Neuron-Enriched Hippocampal Cultures

Thrombin was found to activate the NADPH oxidase complex in cultured human vascular smooth muscle cells, which, in turn, may generate ROS and oxidative stress (Patterson et al., 1999). These ROS can lead to degeneration of hippocampal (Abramov et al., 2007), cortical (Noh and Koh, 2000; Kim et al., 2002), and mesencephalic (Choi et al., 2005a) neurons, in vivo and/or in vitro. Thus, we investigated whether thrombin mediates the production of ROS in neuron-enriched hippocampal cultures. To examine this possibility, 2,7'-dichlorodihydrofluorescein diacetate (DCF) was used to visualize intracellular ROS (Greenlund et al., 1995). At 4 hr after thrombin (40 U/ml) treatment, ROS level was significantly increased (Fig. 2B,C; *P < 0.001) compared with untreated control cells (Fig. 2A,C).

Recent studies have demonstrated that NADPH oxidase is a significant source of ROS (Noh and Koh, 2000; Kim et al., 2002; Choi et al., 2005b). We therefore investigated whether thrombin-induced ROS...
production is mediated by NADPH oxidase in neuron-enriched hippocampal cultures. Real-time PCR analysis showed that thrombin induces a significant up-regulation of p47phox and p67phox mRNA expression as early as 30 min posttreatment compared with control cells (Fig. 2D). To determine the protein expression of p47phox and gp91phox in cultured hippocampal neurons, double-immunofluorescence staining was performed with a combination of antibodies against p47phox and MAP-2 or gp91phox and MAP-2. At 4 hr after thrombin treatment, immunoreactivity to p47phox (Fig. 2E, red) and gp91phox (Fig. 2F, red) was increased in MAP-2-ip neurons (Fig. 2E,F, green). Moreover, in CA1 layer of the rat hippocampus, the expression of p47phox (Fig. 2G, green) or gp91phox (Fig. 2H, green) protein was localized to NeuN-ip (Fig. 2G, red) or MAP-2-ip (Fig. 2H, red) neurons, respectively, at 8 hr after intrahippocampal thrombin injection.

Western blotting showed that, compared with non-treated (0 hr) controls, thrombin caused a significant up-regulation in the total levels of Rac1 and p67phox proteins at the indicated times (Fig. 2I,J). Expression of the p67phox protein was maintained up to 6 hr after thrombin treatment, and Rac1 protein expression peaked at 4 hr after thrombin treatment.

It has previously been shown that translocation of NADPH oxidase subunits from the cytosol to the membrane leads to activation of NADPH oxidase and therefore ROS production (Infanger et al., 2006). For this reason, cell cultures treated with 40 U/ml thrombin for 4 hr were separated into membrane and cytosolic components and examined by Western blotting. After thrombin treatment, the cytosolic NADPH oxidase subunit (p47phox and p67phox) levels significantly increased in the membrane components (Fig. 2K,L) compared with controls. This indicates that translocation and activation of the NADPH oxidase complexes occur.

**Thrombin Induces the NADPH Oxidase-Mediated Production of ROS in Neuron-Enriched Hippocampal Cultures**

We additionally performed in situ analysis of ROS production by using hydroethidine (Choi et al., 2005b). Consistent with DCF results (Fig. 2A–C), the fluorescent products of oxidized hydroethidine significantly increased 4 hr after 40 U/ml thrombin treatment of hippocampal cultures (Fig. 3B,C; P < 0.01) compared with controls (Fig. 3A,C). In contrast, pretreatment with an NADPH oxidase inhibitor, apocynin (1 mM), significantly reduced thrombin-induced ethidium accumulation by 29% (Fig. 3C; P < 0.05).

To examine further the extent of oxidative damage mediated by thrombin-induced NADPH oxidase, we analyzed carbonylated protein levels in hippocampal cultures. The carbonyl levels were assessed by Western blotting and the band intensities compared. Our results indicate that, compared with the controls, the level of carbonylated proteins significantly increases in cultures treated with thrombin for 4 hr (Fig. 3D,E; P < 0.01). Next, we assessed NADPH oxidase-mediated oxidative damage by pretreating hippocampal cultures with apocynin (1 mM) for 30 min. This pretreatment significantly reduced the level of thrombin-induced carbonylated protein in the hippocampal culture by 52% (Fig. 3D,E; P < 0.05). Pretreatment of cultures with 0.5 mM apocynin had no effect (data not shown).

**NADPH Oxidase Contributes to Thrombin-Induced Neurodegeneration in Hippocampal Cultures In Vitro**

We hypothesized that the ROS generated by thrombin-induced activation of neuronal NADPH oxidase contributes to neuronal cell death in neuron-enriched hippocampal cultures. To test this hypothesis, we investigated whether the NADPH oxidase inhibitor apocynin alters the effects of thrombin on cultured hippocampal neurons. MAP-2 immunocytochemical analysis showed that pretreatment of hippocampal neurons with
Figure 2.
Role of NADPH Oxidase in Thrombin-Induced Neurotoxicity 1059

1 mM apocynin partially protected these cells against thrombin-induced neurotoxicity by 25% (Fig. 4; P < 0.001); however, a lower level of apocynin (0.5 mM) had no effect. These results were further corroborated by MTT reduction assays showing that apocynin had protective effects not only at the 1 mM pretreatment concentration but also at the lower concentration of 0.5 mM. Reductions of 22% (P < 0.001) and 14% (P < 0.001) were observed for 1 mM and 0.5 mM apocynin pretreatment, respectively, after neuron-enriched hippocampal cultures were treated with thrombin (Fig. 4). The discrepancy between this finding and the MAP-2 immunocytochemical data, which showed no effect of 0.5 mM apocynin on cells, may be due to differences in the sensitivities of the methods used. In addition, another NAPDH oxidase inhibitor, diphenyleneiodonium (DPI), had no effects at 10 nM but was neurotoxic at 1 μM (data not shown).

In separate experiments, we examined whether antioxidants alter the effects of thrombin on cultured hippocampal neurons. The MTT reduction assay demonstrated that pretreatment with antioxidants such as trolox (100, 200, 400 M), SOD (100 U/ml), or NAC (20 mM) for 30 min partially protected cultured hippocampal neurons against thrombin-induced neurotoxicity by 12–27% (Fig. 5; P < 0.01 ~ 0.001), whereas treatment with trolox, SOD, or NAC alone did not influence neuronal survival.

**DISCUSSION**

In the present study, we found that thrombin leads to the up-regulation and activation of neuronal NADPH oxidase in cultured hippocampal neurons, resulting in ROS production and subsequent oxidative modification of proteins. This oxidative stress was partially prevented by NADPH oxidase inhibitor, apocynin, and other antioxidants, leading to an increase in neuronal survival. Collectively, our data demonstrate that oxidative stress, originating from neuronal NADPH oxidase, is associated with thrombin-induced neurodegeneration in neuron-enriched hippocampal cultures.

Several studies have demonstrated that thrombin induces the degeneration of hippocampal (Striggow et al., 2000), spinal motor (Turgeon et al., 1998), and dopaminergic (Choi et al., 2003) neurons in culture. These results are consistent with our current data showing thrombin-induced neurotoxicity in neuron-enriched hippocampal cultures. The numerous in vitro findings that thrombin induces neurotoxicity are confirmed in vivo by results showing that intrahippocampal injection of thrombin produces a substantial loss of hippocampal CA1 neurons as revealed by NeuN immunostaining and Nissl staining (Choi et al., 2005b). Moreover, studies have shown that ischemic insults produce thrombin-induced neurodegeneration of the hippocampal CA1 layer in vivo (Striggow et al., 2000), and intranigral injection of thrombin causes a loss of dopaminergic neurons in the substantia nigra in vivo (Choi et al., 2003). Interestingly, thrombin was found to modulate the production of amyloid protein precursors and their cleavage into fragments, which are detected in the amyloid plaques of AD brains (Igarashi et al., 1992; Chong et al., 1994). In addition, an increase in thrombin immunoreactivity was observed in the neuritic plaques of AD brains (Akiyama et al., 1992), where a decrease in the level of the major protease inhibitor nexin I was detected (Vaughan et al., 1994; Choi et al., 1995). Collectively, these results indicate that thrombin may be involved in the neuropathological process in AD brains, including the hippocampus. This hypothesis is strongly supported by our present data.
supported by the recent findings that thrombin-induced neurotoxicity in the hippocampus is associated with cognitive impairment, including deficits in learning and memory (Mhatre et al., 2004).

ROS such as superoxide (O$_2^-$) and O$_2^-$-derived oxidants may induce or exacerbate neurotoxicity by causing oxidative stress to neurons (Zekry et al., 2003; Jana and Pahan, 2004). Several studies have demonstrated that the brains of AD patients show evidence of oxidative stress, including oxidative modifications to proteins (Hensley et al., 1995), lipids (Palmer and Burns, 1994), and DNA (Mecocci et al., 1993). Recent findings demonstrate that NADPH oxidase, expressed in neurons (Serrano et al., 2003), is a multicomponent enzyme, and its activation is responsible for generating ROS in sympathetic neurons (Tammariello et al., 2000) and cortical neurons (Noh and Koh, 2000). These ROS can cause neurodegeneration via oxidative damage, such as protein oxidation (Lyra et al., 1997; Markesbery and Carney, 1999). The results of the present study show that, in neuron-enriched hippocampal cultures, thrombin induces the up-regulation of p47$_{phox}$ and p67$_{phox}$ mRNA and Rac1 and p67$_{phox}$ proteins and causes the translocation of cytosolic NADPH oxidase subunits (p47$_{phox}$ and p67$_{phox}$) to the plasma membrane. In thrombin-treated hippocampal cultures, there was not only an activation of neuronal NADPH oxidase but also a significant enhancement of ROS production and protein oxidation. Our results are quite comparable with several reports showing that amyloid protein precursor (Nikura et al., 2004)- or zinc (Noh and Koh, 2000)-induced loss of cortical neurons in cultures is mediated by neuronal

Fig. 3. Effect of apocynin on thrombin-induced O$_2^-$ and O$_2^-$-derived oxidant production and protein oxidation in neuron-enriched hippocampal cultures. A, B: Thrombin (40 U/ml; B) or vehicle (control; A) was added to hippocampal cultures for 4 hr, and O$_2^-$ and O$_2^-$-derived oxidant production were assayed using hydroethidine (5 µM) as described in Materials and Methods. Scale bar = 20 µm. C: Effect of apocynin on thrombin-induced O$_2^-$ and O$_2^-$-derived oxidant production in cultures. Thrombin (40 U/ml) was added to neuron-enriched hippocampal cultures in the absence or presence of apocynin (1 mM) pretreatment for 30 min. At 4 hr later, O$_2^-$ and O$_2^-$-derived oxidant production were assayed with 5 µM hydroethidine as described in Materials and Methods. Graph represents the means ± SEM of five cultures in the three separated platings. *P < 0.01 compared with control, **P < 0.05 compared with thrombin treatment (ANOVA and Student-Newman-Keuls analyses). D: Effect of apocynin on thrombin-induced protein oxidation in cultures. Thrombin (40 U/ml) was added to neuron-enriched hippocampal cultures in the absence or presence of apocynin (1 mM) pretreatment for 30 min. At 4 hr later, cultures were lysed, and samples were analyzed by Western blotting for protein carbonyls as markers of oxidatively modified proteins. E: Error bars represent the means ± SEM of triplicate cultures for four separate platings. *P < 0.01 vs. untreated control cultures; **P < 0.05 vs. cultures treated with thrombin only (ANOVA and Student-Newman-Keuls analyses).
NADPH oxidase. These results collectively suggest that thrombin activates neuronal NADPH oxidase, which stimulates ROS production and thereby results in neurodegeneration in hippocampal cultures. This interpretation is further complemented by our present findings that both the NADPH oxidase inhibitor apocynin and various antioxidants (trolox, SOD, NAC) protect against thrombin-induced neurodegeneration in neuron-enriched hippocampal cultures. Additionally, it is likely that neuronal NADPH oxidase also participates in degeneration of hippocampal neurons in vivo, insofar as NADPH oxidase subunits (p47phox, gp91phox) are colocalized within hippocampal neurons of the CA1 layer following intrahippocampal thrombin injection.

Although our results point to a likely role for neuronal NADPH oxidase, recent findings demonstrate that seizure-induced hippocampal damage may be attributable to activation of microglial NADPH oxidase and consequently the production of ROS (Patel et al., 2005). It has been shown that microglial NADPH oxidase-deficient mice are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity because of the absence of ROS production, which results in oxidative damage (Wu et al., 2003). Recently, it has been shown that nanometer-sized diesel exhaust particles induce neurotoxicity through the activation of microglial NADPH oxidase and consequent oxidative stress (Block et al., 2004). In addition, β-amyloid-induced neurotoxicity in cultured hippocampal neurons is mediated by astrocyte NADPH oxidase (Abramov et al., 2007). Therefore, it is likely that NADPH oxidase, originating from microglia (Cross and Segal, 2004; Patel et al., 2005) or astrocytes (Noh and Koh, 2000), may also participate in thrombin-induced neurotoxicity. This is supported by our recent study demonstrating that thrombin induces microglial NADPH oxidase in the hippocampus in vivo (Choi et al., 2005b). Collectively, although thrombin-induced microglial NADPH oxidase has a major role in neurodegeneration, our current data allow us to suggest that thrombin-induced neuronal NADPH oxidase, at least in part, contributes to death of hippocampal neurons. This neuronal-mediated effect would be in addition to the microglial-mediated neurotoxicity of thrombin on hippocampal neurons.

The present study has demonstrated, for the first time, that the expression and activation of NAPDH oxidase in thrombin-treated hippocampal cultures, at least in part, leads to oxidative stress and eventual neurodegeneration. Combined with extensive clinical findings and in vivo and in vitro data indicating that oxidative stress is a likely common mechanism for cellular damage in various neurological diseases, this suggests that thrombin inhibition or enhancing antioxidants may be beneficial for the treatment of neurodegenerative diseases such as AD.

REFERENCES

Fig. 4. Effects of NADPH oxidase inhibitors on thrombin-induced neurodegeneration in neuron-enriched hippocampal cultures. Vehicle as a control or thrombin (40 U/ml) for 24 hr was added to neuron-enriched hippocampal cultures in the absence or presence of the NADPH oxidase inhibitor apocynin (0.5–1 mM) pretreatment for 30 min. Cell viability was determined by counting the number of MAP-2-ip cells and MTT reduction assay. Graph represents the mean ± SEM of triplicate cultures for five separate platings expressed as a percentage of the control. *P < 0.001 compared with untreated control cultures; **P < 0.001, ~P < 0.01 vs. thrombin-only treated cultures (ANOVA and Student-Newman-Keuls analyses).

Fig. 5. Antioxidants prevent thrombin-induced neuronal death in neuron-enriched hippocampal cultures. Thrombin (40 U/ml) was added to cultures in the absence or presence of trolox (100, 200, 400 μM) or SOD (100 U/ml) or NAC (20 mM) pretreatment for 30 min. Cell viability was measured by using the MTT reduction assay. Graph represents the mean ± SEM of five cultures for four separate platings. *P < 0.001 vs. untreated control cultures; [P < 0.001, ▲P < 0.01 vs. thrombin-only treated cultures (ANOVA and Student-Newman-Keuls analyses).


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