Norepinephrine-induced oxidative stress causes PC-12 cell apoptosis by both endoplasmic reticulum stress and mitochondrial intrinsic pathway: inhibition of phosphatidylinositol 3-kinase survival pathway

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Mao, Weike, Chikao Iwai, Peter C. Keng, Raju Vulapalli, and Chang-seng Liang. Norepinephrine-induced oxidative stress causes PC-12 cell apoptosis by both endoplasmic reticulum stress and mitochondrial intrinsic pathway: inhibition of phosphatidylinositol 3-kinase survival pathway. Am J Physiol Cell Physiol 290:C1373–C1384, 2006. First published December 7, 2005; doi:10.1152/ajpcell.00369.2005.—Norepinephrine (NE) induces endoplasmic reticulum (ER) unfolded protein response and reduces maturation and translocation of NE transporter to cell membrane via enhanced formation of reactive oxygen species in PC-12 cells. In the present study, we investigated whether ER stress is also implicated in the proapoptotic effect of NE. We found that the apoptotic effect of NE was associated with increased processing of ER-resident pro-caspase-12, cleavage of caspase-9 and -3, and mitochondrial release of cytochrome c. ER stress was evidenced by upregulation of ER chaperone GRP78 and transcription factor CHOP and the translocation of XBP-1 from the ER to the nucleus by NE. NE also induced phospho-Akt (Ser473), indicating suppression of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt survival pathway. Similar results were produced by thapsigargin. NGF, which promotes the PI3-kinase/Akt activity, reduced the effects of NE and thapsigargin on apoptosis and activation of caspase-12 and -3. However, the effects of NE, but not of thapsigargin, were abolished by pretreatment with SOD and catalase. In contrast, the PI3-kinase inhibitors LY-294002 and wortmannin abolished the protective effects of both SOD/catalase and NGF on NE-induced apoptosis. The functional importance of caspase-12 activation was supported by the use of Z-ATAD-FMK, which reduced the NE-induced processing of caspase-12 and cell apoptosis, but the caspase-12, -9, and -3 inhibitors had no effects on the increase in cytosolic cytochrome c produced by NE. In contrast, the release of mitochondrial cytochrome c was abolished by SOD/catalase and NGF. These results indicate that NE induced cell apoptosis by both ER stress and a mitochondrial death pathway and that the effects of NE were mediated via oxidative stress and inhibition of the PI3-kinase/Akt survival pathway.

caspase-12; superoxide dismutase; nerve growth factor; Z-Ala-Thr-Ala-Asp-fluoromethyl ketone; cytochrome c

norepinephrine (NE) is an important adrenergic neurotransmitter. At large doses, NE has been shown to induce cell apoptosis in a variety of cell types, including neurons, cardiomyocytes, and pheochromocytoma (PC)-12 cells (2, 5, 9). Recent studies have shown that the proapoptotic effect of NE involves activation of caspase-3 and -9, but the exact cellular targets and related gene expression induced by NE are still unknown (9). In addition, NE may cause damage to various cellular components by both enzymatic and nonenzymatic production of reactive oxygen species (12). Oxidative stress also may lead to modifications and alterations of endoplasmic reticulum (ER) chaperone proteins (45), causing the accumulation of unfolded or misfolded proteins, decreases in protein synthesis, and ultimately cell death (13, 39, 40). We recently reported that NE induced ER stress and sensor proteins via enhanced production of reactive oxygen species in PC-12 cells and that this mechanism is functionally important because it reduces glycosylation and translocation of NE transporter from cytosol to cell membrane (25).

Like the mitochondria, the ER is a repository for both pro- and antiapoptotic molecules. It is generally recognized that low-level stress to the ER activates antiapoptotic ER chaperone proteins such as the 78- and 94-kDa glucose-regulated proteins (GRP78 and GRP94, respectively), calreticulin, and protein disulfide isomerase, whereas severe ER stress causes cell death by activation of the proapoptotic transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), p28ap31, and caspase-12 (19, 21, 23, 30, 31). The evidence published to date indicates that caspase-12 is one of the initiator caspases and is produced by either calpain- or caspase-7-induced cleavage of pro-caspase-12 for ER stress-mediated cell apoptosis (30, 31, 38). The apoptotic effect of caspase-12 probably involves the activation of caspase-9 and -3 (27) or JNK induced by inositol-requiring enzyme 1 (IRE1) (48) and apoptosis signal-regulating kinase 1 (32), independently of apoptotic protease-activating factor 1 (Apaf-1), cytochrome c release, and mitochondria (27). However, it remains unknown what activates caspase-12 in ER stress. Recently, Shimoke et al. (44) showed that NGF attenuates ER stress-mediated apoptosis via suppression of caspase-12 activity and that this effect is mediated via the stimulation of phosphatidylinositol 3-kinase (PI3-kinase) and its effector, the serine/threonine protein kinase Akt. In this study, we hypothesize that NE produces oxidative stress in PC-12 cells and induces neuron apoptosis through activation of ER stress and caspase-12, and inhibition of the PI3-kinase/Akt survival pathway. This study was designed to investigate whether ER stress and activation of caspase-12 and -3 are implicated in the proapoptotic effects of NE in PC-12 cells. The effects of NE were compared with thapsigargin, a classic ER stressor that induces ER stress by inhibiting ER Ca2+-ATPase (8). We also sought to study whether enhanced oxidative stress by NE is an upstream event in ER dysfunction. Antioxidant enzymes SOD...
after ethanol pretreatment may include dead necrotic cells, we performed additional experiments using FITC-labeled annexin V to further classify caspase-3-associated apoptotic cell populations. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for membrane phosphatidylserine, which is translocated from the inner leaflet of the plasma membrane to the outer leaflet during the early phases of apoptosis (49). Propidium iodide was also included in this study to determine the necrotic cell population (annexin positive and propidium iodide positive). Intact cells exclude propidium iodide and are not stained by annexin V. Annexin V–FITC staining of PC-12 cells was performed according to the manufacturer’s instructions (BD Biosciences Pharmingen). For these studies, PC-12 cells were treated with 500 μM NE for 36 h, washed twice with cold PBS, and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Control PC-12 cells were treated identically, but without NE exposure. Cells (1×10^5) were then added to 5 μL of annexin V–FITC and 5 μL of propidium iodide, gently vortexed, and incubated for 15 min at room temperature in the dark. After adding 400 μL of 1× binding buffer to each tube, we analyzed the cells using flow cytometry as described above.

**Effects of NE and thapsigargin on caspase-3, caspase-9, caspase-12, cytochrome c, and ER stress response signals.** To study the association of cell apoptosis with caspase-3 activation, we treated PC-12 cells with either 500 μM NE or 1 μM thapsigargin and harvested the cells after 36 h of treatment to measure the amount of active caspase-3 using a FITC-conjugated MAb active caspase-3 apoptosis kit (BD Biosciences Pharmingen) according to the manufacturer’s instructions. Briefly, the cells were washed twice with cold PBS, resuspended in a Cytofix/Cytosperm solution, and incubated for 20 min on ice. Cells were then washed twice with PermWash buffer and resuspended in the buffer plus anti-active caspase-3 MAb for 30 min at room temperature to measure FITC-conjugated active caspase-3 analysis using flow cytometry. We also measured the protein expression levels of caspase-3, caspase-9, cytochrome c, pro-caspase-12, and ER stress response proteins GRP78, CHOP, and XBP-1 using Western blot analysis. For these studies, PC-12 cells were exposed to NE and thapsigargin for various durations as specified in Results. At the end of NE or thapsigargin treatment, cells were rinsed once with PBS and solubilized in 1× Cell Signaling Technology lysis buffer (in mM: 20 Tris·HCl, 150 NaCl, 1 Na2EDTA, 1 EDTA, 1% Triton X-100, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, and 1 Na3VO4; pH 7.5) supplemented with 1 μg/ml leupeptin and 1 mM PMSF for 30 min on ice. The whole cell lysate was obtained after centrifugation at 12,000 g and 4°C for 15 min. Protein (30–50 μg) was loaded onto ~8% to ~12% SDS-PAGE gels and transferred electrochemically (100 V, 60 min) onto polyvinylidene difluoride membranes (PerkinElmer, Boston, MA). Blots were then probed with the following antibodies: anti-caspase-12 (1:500 dilution), anti-caspase-3 (1:1,000 dilution), anti-GRP78 (1:500 dilution), and anti-CHOP (1:200 dilution). Equal protein loading was confirmed using β-actin Western blot analysis. For the XBP-1 assay, protein was prepared from both nuclear and ER-enriched fractions and subjected to Western blot analysis using anti-XBP-1 MAb (1:250 dilution). Cytosolic and mitochondrial fractions were collected for cytochrome c protein assay using anti-cytochrome c antibody (1:500 dilution). To prepare the subcellular fractions, cell pellets were resuspended in sucrose-supplemented cell extraction buffer (in mM: 250 sucrose, 20 HEPES, 10 KCl, 1.5 MgCl2, 1 EDTA, 1 EGTA, 1 DTT, and 0.1 PMSF; pH 7.5) supplemented with protease inhibitors (in μg/ml: 5 pepstatin A, 10 leupeptin, and 2 aprotinin). After being placed on ice for 15 min, the cells were homogenized using a Polytron homogenizer (setting 6 for 20 s). The homogenate was centrifuged at 1,000 g for 10 min and collected as a nuclear fraction. The supernatant was then centrifuged at 15,000 g for 20 min to get rid of mitochondria and resuspend at 100,000 g for 60 min to isolate an ER-enriched membrane fraction. Both nuclear and mitochondrial fractions were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL and stained for 15 min with FITC–annexin V and 5 μL of propidium iodide. After washing twice with cold PBS, the samples were filtered through a 37-μm nylon filter and analyzed using flow cytometry to further identify caspase-3-associated apoptotic cell populations.
ER membrane fractions were dissolved in 1× Cell Signaling Technology lysis buffer and used for XBP-1 Western blot analysis. After being incubated with the specified antibodies, the immunoblots were exposed to horseradish peroxidase-conjugated secondary antibody for 1 h so that the unique protein bands could be visualized using an ECL detection kit (Amersham Biosciences, Piscataway, NJ) on a Microtek 6800 ScanMaker (Microtek USA, Carson, CA). The optical density of the bands was determined using NIH 1.6 gel image software, and the readings were normalized to a control sample in arbitrary densitometric units.

We also measured the mRNA expression of GRP78 and CHOP normalized by the GAPDH housekeeping gene using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA was isolated from control and NE-treated PC-12 cells using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations as detailed previously (51). cDNA was synthesized using RT, and then the first-stranded cDNA were used for RT-PCR. The primers used were as follows: GRP78 sense primer, 5′-TTTCCTCGTTGAGCCGCTCG-3′; GRP78 antisense primer, 5′-GTTCTGCTTGATGTGTGTCC-3′; GAPDH sense primer, 5′-TCAGTGAATTTGGGCGGAC-3′ and CHOP antisense primer, 5′-T TTCCTCGTTGACCGCTCG-3′; and GAPDH antisense primer, 5′-GGCAAAAGGTCCATCATCTC-3′ and CHOP antisense primer, 5′-GGCCATCCACAGTCTTCT-3′. PCR consisted of an initial denaturation cycle at 94°C for 2 min, followed by 30 cycles for CHOP and GAPDH or by 25 cycles for GRP78 containing a denaturation step at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 68°C for 1 min. An additional cycle at 72°C for 7 min ended the PCR process. The PCR products were separated using 5% polyacrylamide gel, dried by vacuuming, and exposed to Kodak Biomax MR film at −70°C overnight. The autoradiographs were quantified and normalized to a control sample in arbitrary units. The GRP78 and CHOP mRNA readings were then divided by the GAPDH mRNA reading of the same sample to indicate gene expression.

As described below, NE increased caspase-12 processing and decreased pro-caspase-12 protein in this study. To study whether this decrease in pro-caspase-12 protein was associated with changes in caspase-12 mRNA, real-time quantitative PCR was performed to measure the gene expression of caspase-12, RNA from control and NE-treated PC-12 cells was analyzed using TRIzol reagent (Invitrogen) as detailed previously (51). Genomic DNA contamination from RNA was removed by on-column DNase treatment using RNaseasy Mini Mix (Qiagen, Valencia, CA) according to the manufacturer's recommendations. One micromolar of total RNA was reverse transcribed using the iScript DNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer’s instructions. Gene expression was examined using SYBR Green fluorescence with primers designed with DS Gene version 1.5 software and default selection parameters optimized by us. For caspase-12 mRNA quantification, we used primers 5′-GAGTGCGCCATGGCATACACATCTAA-3′ and 5′-TTCCGGCTTCTCTTCCATCTAC-3′. GAPDH primers 5′-TCCACCCACCTGGTCTGTA-3′ and 5′-ACCAGTCCATTGCCATAC-3′ were used to normalize caspase-12 gene expression. A 25-µl PCR was run containing (in µl) 12.5 of 2× SYBR Green Supermix (Bio-Rad Laboratories), 1.25 each of forward and reverse primers, and 2 each of cDNA template and RNase/DNase-free water. All reactions were performed in triplicate, and the experiment was replicated three times. Reactions were run in a MyIQ single-color, real-time PCR detection system (Bio-Rad Laboratories) with the following cycle parameters: 1 cycle of 3 min at 95°C, 40 cycles of 10 s at 95°C, 10 s at 58°C, and 1 min at 68°C and finally a melt curve to assess uniform product formation. A standard curve using 10-fold serial dilutions of a nontemplate control was used to determine amplification efficiency and relative expression levels.

**Influence of SOD and catalase on the effects of NE and thapsigargin.** To study whether the effects of NE were mediated via formation of reactive oxygen species, we pretreated the PC-12 cells with 20 μg/ml SOD plus 20 μg/ml catalase for 1 h before the addition of 500 μM NE for 36 h. For comparison, PC-12 cells were similarly treated with 1 μg of thapsigargin. We measured the effects of antioxidant enzymes on cell apoptosis and protein expressions of cytochrome c, caspase-3, pro-caspase-12, GRP78, CHOP, and XBP-1 as described previously. To determine whether the doses of SOD and catalase chosen for the study were sufficient to reduce the oxidative stress induced by 500 μM NE, we measured the generation of reactive oxygen species produced by NE with and without SOD and catalase using the fluorescent dye CM-H2DCFDA (24, 37). CM-H2DCFDA is a nonpolar compound that, after entering cells, is converted by cellular esterases to dichlorodihydrofluorescein (H2DCF), a nonfluorescent polar derivative. The latter compound is membrane impermeable and is widely used to measure intracellular reactive oxygen species because it is readily oxidized by reactive radicals to yield the highly fluorescent 2′,7′-dichlorofluorescein (DCF). H2DCF is sensitive not only to nonenzymatic oxidation by H2O2 in the presence of peroxidase but also to a wide range of other oxidizing species such as O2·-, OH, peroxynitrite, nitric oxide, and FeSO4 (14, 29). For this study, 500 μM NE was added to cell medium in the presence and absence of 20 μg/ml SOD plus 20 μg/ml catalase for 6 h. During the last hour of incubation, the cells were loaded with 10 μM CM-H2DCFDA. Cells were then treated with trypsin, centrifuged, and resuspended at 1×10⁶/ml. The integrated fluorescence intensity of DCF was measured using a Coulter EPICS Elite flow cytometer with excitation at 488 nm and emission at 530 nm.

**Effects of caspase inhibitors on the proapoptotic effects of NE.** To identify the relative contributions of different caspase activation levels on NE-induced apoptosis, PC-12 cells were preincubated with 2 μM Z-ATAD-FMK, 2 μM Z-LEHD-FMK, or 2 μM Z-DEVD-FMK for 30 min and then incubated with 500 μM NE for 36 h. At these doses, the caspase inhibitors produce maximum inhibition of the enzyme activity in cell culture. Cell death, procaspase-12, caspase-9, and caspase-3, and cytochrome c were measured.

**Effects of NE and thapsigargin on the PI3-kinase/Akt signaling pathway.** The amount of phospho-Akt protein was measured using Western blot analysis as a measure of activation of the PI3-kinase/Akt pathway. Western blot analysis was performed as described above using anti-Akt and anti-phospho-Akt (Ser473) antibodies (both at 1:1,000 dilution). The studies were performed in PC-12 cells treated with NE or thapsigargin. Antioxidant enzymes (SOD plus catalase) were also used to determine whether oxidative stress played a role in the PI3-kinase/Akt survival pathway.

In addition, we performed experiments to determine whether the apoptotic effects of NE and thapsigargin could be affected by LY-294002 (10 μM) or Wortmannin (5 μM), two well-known inhibitors of the PI3-kinase/Akt pathway, or by NGF (100 ng/ml), which activates the PI3-kinase/Akt pathway. We measured the effects of the PI3-kinase activator and inhibitors on cell apoptosis, caspase-3, caspase-12, cytochrome c, and phospho-Akt (Ser473) as described above. We also determined whether the addition of LY-294002 or Wortmannin reversed the protective effects of SOD plus catalase or NGF on the NE- and thapsigargin-induced cell apoptosis.

**Effects of NGF on ER stress response proteins and intracellular reactive oxygen species.** We studied the effects of NGF on the PI3-kinase/Akt pathway. Also, because NGF has been reported to have an antioxidant effect (43), we examined whether NGF reduced the induction of GRP78, CHOP, and nuclear XBP-1 produced by 36 h of 500 μM NE using the methods described above. For these studies, PC-12 cells were incubated in 2% serum RPMI 1640 medium and pretreated with 100 ng/ml NGF for 12 h before the introduction of NE. In addition, experiments were performed to determine whether NGF reduces the generation of reactive oxygen species produced by NE. In the latter study, 500 μM NE was added to the cell medium in the presence or absence of NGF for 4 h. During the last hour of incubation, the cells were loaded with 10 μM CM-H2DCFDA and the
between two groups.

**Statistical Analysis**

Experimental data were analyzed using the RS/I research system (BBN Technologies, Cambridge, MA), and the results are presented as means ± SE. The statistical significance of differences among the different experimental groups was analyzed using either one- or two-way ANOVA. Bonferroni simultaneous intervals for all comparisons were used to determine the statistical significance of differences between two groups. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**NE-Induced Cell Apoptosis and Caspase-3 Activation**

**NE-induced apoptosis in PC-12 cells.** Using microscopy, we observed shrinkage of the cells and vacuolization in the cytoplasm as well as gradual detachment from culture dishes after NE exposure. Cell death was identified using propidium iodide. Figure 1 shows that the number of dead cells identified using propidium iodide increased as the duration of exposure to 500 \( \mu \)M NE increased. The percentage of propidium iodide-positive cells also increased with the concentration of NE in the incubation medium. At 500 \( \mu \)M NE, \( \sim 55-65\% \) of the cells were propidium iodide positive after 36 h of treatment; a similar effect was observed after 36 h of treatment with 1 \( \mu \)M thapsigargin.

Because propidium iodide after ethanol pretreatment stained both apoptotic and necrotic cells, we also measured the effect of NE on cell apoptosis and necrosis using annexin V-FITC and propidium iodide staining without ethanol pretreatment. We found that the percentage of the cell population showing the cells necrosis (annexin V positive and propidium iodide positive) was consistently low under both the untreated control condition (2.0 ± 0.7\%, \( n = 6 \)) and after 36 h of 500 \( \mu \)M NE treatment (3.8 ± 0.4\%, \( n = 8 \)). On the other hand, the percentage of apoptotic cells (annexin V positive and propidium iodide negative), which was low in the untreated control group (3.5 ± 0.3\%, \( n = 6 \)), was increased markedly in PC-12 cells (48.3 ± 1.7\%, \( n = 8 \); \( P < 0.0001 \)) after 36-h treatment with NE. Because there was no striking increase in necrotic cells treated with NE, the propidium iodide-positive cells represented predominantly apoptotic cells. Thus, in subsequent studies, the percentage of propidium iodide-positive cells was used as an index of apoptosis.

The effects of NE on cell apoptosis were also studied by measuring active caspase-3 activity and caspase-3 protein content. Flow cytometric measurements showed that active caspase-3 activity (integrated fluorescence intensity) increased 8- to 10-fold, from 0.64 ± 0.07 (control) to 6.84 ± 0.78 and 4.26 ± 0.28 (both \( P < 0.001 \)) after 36-h treatments with 500 \( \mu \)M NE and 1 \( \mu \)M thapsigargin, respectively. Simultaneously, we found that both treatments reduced the uncleaved 32-kDa caspase-3 protein and induced a threefold increase in the cleaved form of caspase-3 (17 kDa) (Fig. 2).

**NE-Induced Activation of Caspase-12 and ER Stress Signaling Proteins**

Figure 3 shows the effects of NE on the processing of pro-caspase-12 protein (55 kDa) in whole cell lysate. The top panel shows that 500 \( \mu \)M NE produced a progressive decrease in the pro-caspase-12 protein level in PC-12 cells during a 36-h period. The figure also shows that the effect of NE on the reduction of pro-caspase-12 was dose dependent; the lower doses of NE (<100 \( \mu \)M) had no effect on pro-caspase-12 content. The effect of 500 \( \mu \)M NE was similar to that induced by 1 \( \mu \)M thapsigargin. Thus, in subsequent studies, 500 \( \mu \)M NE for 36 h was chosen and compared with 1 \( \mu \)M thapsigargin unless indicated otherwise.

However, despite the obvious reduction of pro-caspase-12, Western blot analysis of whole cell lysates showed no cleaved caspase-12. Thus, we repeated the measurement of caspase-12 in the ER membrane fraction of PC-12 cells. Figure 4 shows the presence of both pro-caspase-12 (55 kDa) and cleaved caspase-12 (42 kDa) in the ER-enriched fraction. It also shows that as in whole cell lysates, pro-caspase-12 decreased in the ER membrane fraction after 500 \( \mu \)M NE and 1 \( \mu \)M thapsigargin treatment. Concomitantly, cleaved caspase-12 (42 kDa) increased 3.5-fold, confirming the increased processing of pro-caspase-12 by NE and thapsigargin.
Caspase-12 mRNA measured using real-time quantitative PCR. The expression level of caspase-12 mRNA was calculated as relative copy numbers normalized to GAPDH mRNA. We found no significant change in caspase-12 mRNA levels in PC-12 cells after 30 h of NE treatment (21.0 ± 5.2, n = 5, t = 0.79; P = 0.46) compared with control (15.1 ± 5.3, n = 4).

To study the transcriptional activation of the ER stress response protein, we measured the effects of NE and thapsigargin on GRP78 and CHOP protein and mRNA levels. We found that NE produced a transient increase in the GRP78 protein, with mRNA peaking at 6 h (Table 1) and returning to baseline at 24 and 36 h of treatment. CHOP protein was also increased by NE; this effect was more prolonged and was present at 36 h of treatment. We also found that CHOP mRNA was increased as well. Similar changes were induced by thapsigargin (Table 1).

Translocation of XBP-1 protein from the ER to the nucleus occurs during ER stress. XBP-1 samples were obtained from both untreated control PC-12 cells and after 6- and 24-h treatment with 500 μM NE. We found that NE decreased XBP-1 in the ER membrane after 6 and 24 h of treatment, whereas XBP-1 increased concomitantly in the nuclear fraction (Fig. 5). The magnitude of changes in nuclear XBP-1 produced by 500 μM NE at 24 h of treatment was similar to that produced by 1 μM thapsigargin (Table 1).

Effects of SOD plus Catalase on NE-Induced ER Stress and Cell Apoptosis

To determine whether the effects of NE and thapsigargin were mediated via the formation of reactive oxygen species, we measured reactive oxygen species in PC-12 cells using DCF fluorescence and treated the PC-12 cells with antioxidant enzymes SOD and catalase before adding NE or thapsigargin. As expected, NE treatment produced a marked increase in DCF fluorescence in PC-12 cells (1.64 ± 0.14 integrated fluorescence intensity, n = 6; P < 0.0001) compared with untreated controls (0.38 ± 0.02 integrated fluorescence intensity, n = 6). At the doses administered, SOD and catalase exerted no effect on DCF fluorescence in control PC-12 cells (0.37 ± 0.04 integrated fluorescence intensity, n = 6; P = 0.80) but were sufficient to produce a marked inhibition of the increase in DCF fluorescence induced by 500 μM NE (0.87 ± 0.10 integrated fluorescence intensity, n = 6; P = 0.001).

Table 2 shows that SOD plus catalase treatment markedly reduced the proapoptotic effects of NE and the decrease in the processing of pro-caspase-12 produced by NE, but the antioxidant enzymes had relatively little effect in the thapsigargin experiments. We also found that the antioxidant enzymes prevented the cleavage of caspase-3 produced by NE, but not the effect of thapsigargin. In addition, antioxidant enzyme treatment prevented NE-induced increases in GRP78 and CHOP and in the nuclear translocation of XBP-1. In contrast, the antioxidant enzymes had no effect on the actions of thapsigargin on GRP78, CHOP, and XBP-1 nuclear translocation.

Activation of ER Stress and Mitochondrial Intrinsic Pathway in NE-Induced Cell Apoptosis

Figure 6 shows that in addition to activation of caspase-12 and caspase-3 in PC-12 cells, NE and thapsigargin also acti-
Induced caspase-9 was demonstrated by the increases in cleaved 37-kDa caspase-9 and 17-kDa caspase-3 produced by NE. However, the caspase-12 inhibitor had no effect on the NE-induced increase in cytosolic cytochrome c (Table 3).

To study whether the PI3-kinase/Akt survival pathway regulates the apoptotic effects of NE and thapsigargin, we measured the amount of phosphorylated Akt at the Ser473 protein in PC-12 cells. Figure 7 shows that NE treatment reduced phospho-Akt (Ser473) beginning at 12 h of incubation, which corresponded to the beginning of NE-induced apoptosis (Fig. 1). There was no change in the amount of total Akt protein. By 36 h of incubation, phospho-Akt (Ser473) had decreased to ~40% of control. The figure also shows that phospho-Akt (Ser473) was reduced to a similar extent by 1 μM thapsigargin. The addition of SOD plus catalase prevented the reduction of phospho-Akt (Ser473) induced by NE, but like the lack of effect of the antioxidant enzymes on thapsigargin-induced apoptosis, SOD plus catalase did not affect the reduction of phospho-Akt (Ser473) by thapsigargin.

The functional importance of the PI3-kinase/Akt pathway was further studied by treating PC-12 cells with either the PI3-kinase activator NGF or the PI3-kinase inhibitors LY-294002 and wortmannin. We also studied the antioxidant effect of NGF on PC-12 cells using DCF flow cytometric fluorescence. Table 4 shows that NGF itself had no effect on DCF.

Table 1. Effects of NE and thapsigargin on GRP78, CHOP, and nuclear XBP-1

<table>
<thead>
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<th>Parameter</th>
<th>Control</th>
<th>NE</th>
<th>Thapsigargin</th>
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<tr>
<td>Protein GRP78</td>
<td>1.00 ± 0.09</td>
<td>2.15 ± 0.14*</td>
<td>2.28 ± 0.24*</td>
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<td>mRNA GRP78</td>
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<td>2.01 ± 0.15*</td>
<td>2.39 ± 0.23*</td>
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<tr>
<td>Protein CHOP</td>
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<td>3.31 ± 0.64*</td>
<td>4.47 ± 0.33*</td>
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<td>mRNA CHOP</td>
<td>1.00 ± 0.18</td>
<td>4.96 ± 0.84*</td>
<td>10.51 ± 1.81*</td>
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<tr>
<td>XBP-1 (nuclear)</td>
<td>1.00 ± 0.18</td>
<td>3.55 ± 0.74*</td>
<td>2.69 ± 0.39*</td>
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</table>

Values are means ± SE; n = 6 for each group. NE, norepinephrine. Pheochromocytoma (PC)-12 cells were incubated for 36 h for 78-kDa glucose-regulated protein (GRP78) and CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) assays and for 24 h for nuclear X box binding protein 1 (XBP-1). All 3 endoplasmic reticulum (ER) stress signal proteins were increased by NE and thapsigargin (see text for further explanation). GRP78 and CHOP mRNA levels were normalized to GAPDH. The peak effects shown in this table occurred at 6 h of treatment for GRP78 at 36 h for CHOP, and at 24 h for nuclear XBP-1. *P < 0.05 vs. control without NE or thapsigargin treatment. There were no differences in the responses between the NE and thapsigargin experiments.

Table 2. Effects of SOD and catalase on NE and thapsigargin

<table>
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<th>Parameter</th>
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<tr>
<td>Cell apoptosis, %</td>
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<td>54.6 ± 5.6*</td>
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<td>SOD + catalase</td>
<td>7.4 ± 1.1</td>
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<td>0.34 ± 0.02*</td>
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<td>3.35 ± 0.66*</td>
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<tr>
<td>GRP78, arbitrary units</td>
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<td>6.68 ± 1.67*</td>
<td>5.70 ± 0.95*</td>
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<td>Control</td>
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<td>1.47 ± 0.35*</td>
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<td>SOD + catalase</td>
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<td>1.47 ± 0.35*</td>
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<tr>
<td>CHOP, arbitrary units</td>
<td></td>
<td>3.55 ± 0.74*</td>
<td>2.69 ± 0.39*</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.18</td>
<td>3.55 ± 0.74*</td>
<td>2.69 ± 0.39*</td>
</tr>
<tr>
<td>Nuclear XBP-1, arbitrary units</td>
<td></td>
<td>3.55 ± 0.74*</td>
<td>2.69 ± 0.39*</td>
</tr>
<tr>
<td>SOD + catalase</td>
<td>1.33 ± 0.14</td>
<td>1.42 ± 0.24*</td>
<td>2.73 ± 0.31*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. Effects of NE and thapsigargin are shown for cell apoptosis, pro-caspase-12 protein, 17-kDa caspase, and CHOP proteins after 36 h of treatment; for GRP78 protein at 6 h of treatment; and for nuclear XBP-1 protein at 24 h of treatment. *P < 0.05 vs. control without NE or thapsigargin treatment. †P < 0.05 vs. respective control NE or thapsigargin treatment groups.
fluctuation but effectively abolished the increase in DCF fluorescence produced by NE.

Figure 8 shows that NGF reduced the increases in GRP78, CHOP, and nuclear XBP-1 produced by NE but had no effect on thapsigargin. NGF also reduced the decreases in phospho-Akt (Ser473) and pro-caspase-12 and the increases in cleaved caspase-9 produced by NE but had no effect on the ER unfolded protein response by expressing numerous chaperone proteins and, when the damage is severe, to cause cell apoptosis. In this study, we have found that excessive NE deprivation of glucose and oxygen, have been shown to induce apoptosis in PC-12 cells, but the PI3-kinase inhibitors eliminated the neuroprotective effects of SOD plus catalase in NE-treated PC-12 cells (Fig. 10, Table 2). Coincident with the antiapoptotic effect, LY-294002 and wortmannin increased cell apoptosis to 66 ± 4% and 60 ± 5% in thapsigargin- and NGF-treated PC-12 cells, respectively. Neither LY-294002 nor wortmannin alone affected cell apoptosis in PC-12 cells, but the PI3-kinase inhibitors eliminated the neuroprotective effects of SOD plus catalase in NE-treated PC-12 cells (Fig. 10, Table 2). Coincident with the antiapoptotic effect, LY-294002 and wortmannin increased cell apoptosis to 66 ± 4% and 60 ± 5% in thapsigargin- and NGF-treated PC-12 cells, respectively.

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demonstrates for the first time an intimate interplay of ER stress with oxidative stress and inhibition of the PI3-kinase/Akt pathway in the NE-induced apoptosis of PC-12 cells. Also, NE activated a mitochondrial apoptotic pathway, as evidenced by the increased cleaved 37-kDa caspase-9, as well as cytochrome c translocation from the mitochondria to the cytosol. These findings suggest the possible involvement of both ER- and mitochondria-dependent pathways in NE-induced apoptosis in PC-12 cells. Recent reports also have shown that the typical ER stress inducers thapsigargin and brefeldin A induced the degradation of caspase-12, -9, and -3 (11, 20). Cytochrome c release from the mitochondria also has been observed in ER stress-induced apoptosis in several cell lines (18). The relative importance and mechanisms of cross talk between the ER and the mitochondria in cell apoptosis induced by NE are largely unknown. It is thought that caspase-9 and -3 may be activated by a mitochondria-dependent cytochrome c/Apaf-1 pathway and by an Apaf-1-independent ER/caspase-12 pathway (39, 40). In this study, we found no effect of the caspase-12 inhibitor Z-ATAD-FMK on mitochondrial cytochrome c release, but the caspase-12-specific inhibitor was effective not only in protecting cells from apoptosis and caspase-3 cleavage but also in decreasing the degradation of both procaspase-12 and -9. These results are in agreement with those reported in other studies, showing the linkage of the activation of mitochondria-dependent pathways in NE-induced apoptosis in PC-12 cells. Recent reports also have shown that the typical ER stress inducers thapsigargin and brefeldin A induced the degradation of caspase-12, -9, and -3 (11, 20). Cytochrome c release from the mitochondria also has been observed in ER stress-induced apoptosis in several cell lines (18). The relative importance and mechanisms of cross talk between the ER and the mitochondria in cell apoptosis induced by NE are largely unknown. It is thought that caspase-9 and -3 may be activated by a mitochondria-dependent cytochrome c/Apaf-1 pathway and by an Apaf-1-independent ER/caspase-12 pathway (39, 40). In this study, we found no effect of the caspase-12 inhibitor Z-ATAD-FMK on mitochondrial cytochrome c release, but the caspase-12-specific inhibitor was effective not only in protecting cells from apoptosis and caspase-3 cleavage but also in decreasing the degradation of both procaspase-12 and -9. These results are in agreement with those reported in other studies, showing the linkage of the activation of mitochondria-dependent pathways in NE-induced apoptosis in PC-12 cells.

Fig. 7. NE (500 μM) decreased phosphorylation of Akt at Ser473 [phospho-Akt (Ser473)] in a time-dependent manner during a 36-h period (top). Representative Western blot analysis is shown. Bottom, phospho-Akt (Ser473) was reduced to a similar extent by NE (500 μM for 36 h) and thapsigargin (1 μM for 36 h), and antioxidant treatment (SOD + catalase) abolished decrease in phospho-Akt produced by NE. Decrease in phospho-Akt produced by thapsigargin was unaffected by antioxidant enzymes. Bars, SE. n = 6 in each group. *P < 0.05 vs. control group without NE or thapsigargin treatment. †P < 0.05 vs. antioxidant enzyme treatment.

Fig. 8. NGF treatment reduced peak increases in 78-kDa glucose-regulated protein (GRP78), proapoptotic transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), and nuclear XBP-1 produced by 500 μM NE at 6, 24, and 24 h of treatment, respectively. NGF had no effect on changes produced by thapsigargin. Optical density readings were normalized against a control sample in arbitrary units. Bars, SE. n = 6–9 in each group. *P < 0.05 vs. control group without NE or thapsigargin treatment. †P < 0.05 vs. groups without NGF treatment.

Table 4. Effects of NE and NGF on intracellular reactive oxygen species in PC-12 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Integrated Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.51±0.04</td>
</tr>
<tr>
<td>NGF</td>
<td>0.54±0.05</td>
</tr>
<tr>
<td>NE</td>
<td>1.49±0.14</td>
</tr>
<tr>
<td>NE + NGF</td>
<td>0.41±0.03†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. Reactive oxygen species were measured using fluorescent probe 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA). One-way ANOVA revealed significant differences among groups (P = 40.72, df = 3,20; P < 0.0001). *P < 0.05 vs. control without NE or NGF treatment. †P < 0.05 vs. NE without NGF treatment.
Caspase-12 is an ER-resident member of the interleukin-1β-converting enzyme subfamily of caspases, which is activated by ER stress inducers such as tunicamycin, thapsigargin, and Ca^{2+} ionophores (21, 30, 40, 46). Caspase-12 is an important regulator of cell apoptosis, because caspase-12-deficient mice showed decreased sensitivity to apoptosis induced either by tunicamycin on renal tubular epithelial cells or by β-amyloid on neuronal cells (16). Oxidative stress is involved in the induction of ER stress and caspase-12 activation (13, 35). In this study, we found that ER stress was induced by NE. Furthermore, the decrease in pro-caspase-12 and the increase in cleaved caspase-12 produced by NE were not associated with significant changes in caspase-12 mRNA, suggesting that the increased processing of caspase-12 is a posttranscriptional event. This action of NE was mediated, at least in part, by increased reactive oxygen species as demonstrated by DCF fluorescence. Increased oxidative stress by NE also has been demonstrated by decreases in reduced glutathione and in the ratio of reduced to oxidized glutathione in PC-12 cells (24). However, because DCF fluorescence can be induced by a broad range of oxidizing radicals (14, 29), it is not known which reactive oxygen species are produced by NE. Reactive oxygen species comprise O_2^{-}, H_2O_2, and -OH. O_2^{-} is first converted into H_2O_2, which in turn, in the presence of metal ions such as Cu^{2+} or Fe^{2+}, is further oxidized to -OH and

caspase-12 in response to ER stress to cytochrome c-independent cleavage of pro-caspase-9 and other ER stress-specific caspase cascades (27). Furthermore, because the increase in cytosolic cytochrome c by NE also was not affected by either Z-LEHD-FMK or Z-DEVD-FMK, the release of cytochrome c from the mitochondria by NE is likely to occur upstream from activation of caspase-9 and -3.

XBP-1 is a b-Zip transcription factor that mediates DNA binding and an adjacent leucine zipper structure controlling protein dimerization. XBP-1 mRNA undergoes splicing in response to ER stress, and the expressed XBP-1 proteins translocate to the nucleus and induce the expression of themselves and other ER stress-specific genes (46). We found that XBP-1 protein increased in the nuclear fraction beginning as early as 6 h after NE and thapsigargin treatment. NE also increased another nuclear transcription factor, CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), which acts to amplify the proapoptotic genes by stimulating the downstream IRE1 during ER stress (48). CHOP also has been shown to sensitize cells to ER stress by downregulating Bcl-2 (26).

Fig. 10. NE and thapsigargin increased cell apoptosis in PC-12 cells. PI3-kinase inhibitors LY-294002 and wortmannin had no direct effect on apoptotic effects of NE (500 μM for 36 h) and thapsigargin (1 μM for 36 h) but prevented antiapoptotic effect of SOD plus catalase in NE experiment (see Table 2 for comparison). Bars, SE. n = 6 in each group; *P < 0.05 vs. control group without NE or thapsigargin treatment.
eventually to H₂O and O₂. Because Cu²⁺ potentiates the effect of NE on cell apoptosis and oxidative stress in PC-12 cells (24), oxidative stress induced by NE probably occurs at the level of increased O₂⁻ or H₂O₂, which is then converted to ·OH by the metal-catalyzed oxidation reaction. Increased interstitial ·OH has been demonstrated in the intact rat heart after NE administration and cardiac sympathetic nerve stimulation using a microdialysis technique (33).

In mammalian cells, Cu-Zn-SOD is the most abundant and ubiquitous isoform of the antioxidant enzyme SOD and has great physiological significance and therapeutic potential. Perturbation of normal cellular antioxidant function by mutating human Cu-Zn-SOD has been shown to induce oxidative stress and early cleavage of caspase-12 in neuronal death and disease progression in amyotrophic lateral sclerosis (53). Edaravone, a potent and novel scavenger of free radicals, not only can reduce HO⁻ and related lipid peroxidative damage in mice exposed to hypoxia/ischemia but also can attenuate ER stress and apoptosis (35). Administration of antioxidants also has been shown to reduce oxidative stress and myocyte apoptosis in animals after prolonged NE infusion (36).

PI3-kinase is a heterodimer composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (4). When stimulated by growth hormones such as insulin and NGF, PI3-kinase activates the downstream serine/threonine kinase Akt (also known as PKB) by phosphorylation. Akt inhibits apoptosis by phosphorylating the phospho-Bcl-2-associated death promoter (BAD) component of the BAD/Bcl-xL complex on a serine residue (7). Phosphorylated BAD binds to 14-3-3 protein, causing dissociation of the BAD/Bcl-xL complex, which is then capable of suppressing the intrinsic death pathway. BAD phosphorylation plays an important role in the mitochondrial release of cytochrome c after PI3-kinase inhibition in neonatal rat brain slices during hypoxia (15). Activated Akt is also capable of phosphorylating a number of other downstream target proteins such as Bim, the Bcl-2 homology 3-domain-only protein (52), Forkhead transcription factor (1), caspase-9 (3), inhibitor of IKK (41), or GSK-3β (6), all of which act to promote its antiapoptotic effects. The importance of the PI3-kinase/Akt pathway in mediating ER stress-induced cell death has been established on the basis of the use of either LY-294002 or the overexpression of kinase-dead mutant Akt (17).

Our study has shown that suppression of PI3-kinase/Akt activity was associated with cell apoptosis induced by both NE and thapsigargin but that the addition of SOD plus catalase increased phospho-Akt and reduced caspase-12 and -3 activation and cell apoptosis in only NE-treated PC-12 cells. The functional importance of the PI3-kinase/Akt pathway in NE-induced cell apoptosis was further supported by our experiments using LY-294002 and wortmannin, which not only exaggerated the NE induced inhibition of phospho-Akt, caspase-12 activation, and apoptosis but also abolished the protective effect of SOD plus catalase on NE-induced ER stress and apoptosis. We also showed that NGF attenuated inhibition of phospho-Akt by NE and protected cells against caspase-12-dependent apoptosis. However, unlike SOD plus catalase, NGF also exerted a protective effect on thapsigargin-induced ER stress and cell apoptosis. Our findings are consistent with an earlier observation that NGF reduced ER stress-induced activation of caspase-12 by heightening the PI3-kinase/Akt signaling in PC-12 cells (44). Nevertheless, a significant difference exists regarding the effects of NGF between NE and thapsigargin experiments in that NGF attenuated the ER stress-induced activation of GRP78 and CHOP proteins only in the cells treated with NE. The effects of NGF on GRP78 and CHOP probably were caused by an antioxidant action; NGF has been shown to prevent the conversion of O₂⁻.
to \( \text{H}_2\text{O}_2 \) and to protect PC-12 cells against oxidative stress (43). The antioxidant effect of NGF on NE-induced oxidative stress was demonstrated using DCF fluorescence in our study (Table 4). NGF also has been shown to induce gene expression of catalase and glutathione peroxidase in PC-12 cells in a time- and dose-dependent manner (42). However, this effect of NGF on increased activity and transcriptional regulation of catalase, which is present only after 3 days of incubation, probably does not play an important role in acute experiments with NGF.

A close interaction between the ER stress-induced activation of caspase-12 and the inhibition of the PI3-kinase/Akt pathway was demonstrated in PC-12 cell apoptosis in our study. However, little is known of the molecular mechanisms that link the ER stress pathway to the inhibition of PI3-kinase. In addition, how the activated Akt decreases caspase-12 is not known. Because the amino acid sequence of caspase-12 does not include a potential phosphorylation site for Akt, Akt activation probably exerts its inhibitory effect on caspase-12 by an indirect mechanism. Shimoke et al. (44) showed that the antiapoptotic effect of NGF is not mediated via the activity of JNK. They speculated that caspase-12 could be reduced by the antiapoptotic effect of NGF is not mediated via the activity of the PI3-kinase/Akt pathway on caspase-12 activation and the inhibition of the PI3-kinase pathway. Further studies are needed to determine whether the protective effect of NGF and activation of the PI3-kinase/Akt pathway on caspase-12 activation and apoptosis are mediated via the reduction of translocation of Akt to the ER.

Kitamura et al. (20) showed that both the ER- and mitochondria-dependent pathways are involved in thapsigargin-induced apoptosis in human neuroblastoma cells. The results of our present study indicate that both of these pathways are also operative in NE-induced PC-12 cell apoptosis. Our results show that NGF prevented cytochrome \( c \) release from mitochondria by NE. This finding is consistent with a recent report that showed that NGF inhibits apoptosis induced by Bcl-xS, a truncated, proapoptotic member of the Bcl-2 protein family, and cytochrome \( c \) release in PC-12 cells via the PKC, PI3-kinase, and MEK signaling pathways (22). NGF also inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosphorylation, and cytochrome \( c \) release (47). However, like SOD plus catalase, NGF may exert a protective effect on cytochrome \( c \) release through its antioxidant property. Overexpression of Cu-Zn-SOD has been shown to prevent oxidative ER stress and the early release of mitochondrial cytochrome \( c \) in mice with ischemia in the brain (10). Thus we speculate that interventions that reduce oxidative stress and promote PI3-kinase/Akt activity may offer novel therapeutic advantages in human diseases in which NE or oxidative stress is increased.

**REFERENCES**


