Central Role of Glycogen Synthase Kinase-3β in Endoplasmic Reticulum Stress-induced Caspase-3 Activation*

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The endoplasmic reticulum (ER), which is associated with many neurodegenerative conditions, can lead to the elimination of affected cells by apoptosis through only partially understood mechanisms. Thapsigargin, which causes ER stress by inhibiting the ER Ca2+-ATPase, was found to not only activate the apoptosis effector caspase-3 but also to cause a large and prolonged increase in the activity of glycogen synthase kinase-3β (GSK3β). Activation of GSK3β was obligatory for thapsigargin-induced activation of caspase-3, because inhibition of GSK3β by expression of dominant-negative GSK3β or by the GSK3β inhibitor lithium blocked caspase-3 activation. Thapsigargin treatment activated GSK3β by inducing dephosphorylation of phospho-Ser-9 of GSK3β, a phosphorylation that normally maintains GSK3β inactivated. Caspase-3 activation induced by thapsigargin was blocked by increasing the phosphorylation of Ser-9-GSK3β with insulin-like growth factor-1 or with the phosphatase inhibitors okadaic acid and calyculin A, but the calcineurin inhibitors FK506 and cyclosporin A were ineffective. Insulin-like growth factor-1, okadaic acid, calyculin A, and lithium also protected cells from two other inducers of ER stress, tunicamycin and brefeldin A. Thus, ER stress activates GSK3β through dephosphorylation of phospho-Ser-9, a prerequisite for caspase-3 activation, and this process is amenable to pharmacological intervention.

Impaired function of the endoplasmic reticulum (ER), commonly referred to as ER stress, is an important factor in the neuropathology of a wide variety of neurological disorders (reviewed in Refs. 1–4). Exemplary among these is Alzheimer’s disease (AD). Studies related to AD have shown that the neurotoxic effects of the amyloid β-peptide are at least partially targeted to the ER (e.g. Refs 5 and 6). Also, AD-associated mutations of presenilin-1 disrupt calcium homeostasis and increase susceptibility to ER stress and apoptosis (7–12), whereas wild-type presenilin-1 is necessary for cellular responses to ER stress (13). Furthermore, an AD-associated splice variant of presenilin-2 increases vulnerability to ER stress (14). Substantial evidence links ER stress to several other neurological disorders as well as to the decline in neuronal function associated with aging (4). Because the ER is a central site of protein folding, ER stress can lead to increased intracellular levels of misfolded proteins, and eventual cell death by apoptosis, processes that may contribute to neurodegenerative disorders.

Several agents can be used to induce ER stress experimentally, and likely the most widely applied is thapsigargin. Thapsigargin inhibits the Ca2+-ATPase in the ER (15), which blocks sequestration of calcium by the ER, causing increases in the intracellular concentration of calcium, accumulation of unfolded or misfolded proteins, and activation of caspase-3-mediated apoptosis (16–18). The mechanisms mediating ER stress-induced activation of the apoptosis program remain incompletely elucidated, although both caspase-7 and caspase-12 have been implicated in addition to the crucial effector caspase-3 (6, 19, 20). We considered the possibility that glycogen synthase kinase-3β (GSK3β) may be involved in this apoptotic program, because it recently has been shown to be a key intermediate in several apoptotic signaling pathways that lead to activation of caspase-3 (reviewed in Ref. 21). This was first shown by the findings that GSK3β antisense oligonucleotides blocked apoptosis induced by the Alzheimer’s disease amyloid-β-peptide (22) and that transient overexpression of GSK3β caused PC12 and Rat-1 cells to undergo apoptotic programmed cell death (23). Further studies have extended the known links between GSK3β and apoptosis. Moderate overexpression of GSK3β (3.5-fold), which was insufficient to induce apoptosis alone, facilitated apoptosis induced by stressors (24, 25). The human immunodeficiency virus type 1 regulatory protein, Tat, induced neuronal apoptosis in a GSK3β-dependent manner, a signal mediated by platelet-activating factor receptor-induced activation of GSK3β (26, 27). GSK3β also has been implicated as contributing to neuronal cell death induced by ischemia (28, 29), excitotoxicity induced by glutamate receptor activation (30, 31), and models of Huntington’s disease (32, 33). Additionally, many studies of apoptotic conditions involving growth factor withdrawal or inhibition of the phosphatidylinositol 3-kinase/Akt signaling system, pathways that normally maintain GSK3β in an inhibited state through phosphorylation of Ser-9 (34), have shown that GSK3β promotes the subsequent apoptotic process (35–39). More that just neuronal apoptosis is promoted by GSK3β activity, because this relationship has been demonstrated in a wide variety of cell types, for example in vascular smooth muscle cells (40), fibroblasts (41), human erythroid progenitors (42), and cardiac cells (43). A number of these studies advantageously used lithium, along with other approaches, to identify the contributory effects of GSK3β to apoptosis. Lithium is useful in this regard, because it is a selective inhibitor of GSK3β (44, 45), a finding substantiated by...
an examination of 24 kinases, which showed that GSK3β, and the closely related GSK3α, to be the only kinases substantially inhibited by lithium (46). Extensive studies have clearly documented that several of lithium's prominent effects, such as inhibition of the phosphorylation of the microtubule-associated protein tau, increased levels of β-catenin, and protection from GSK3β-facilitated apoptosis, are directly dependent on lithium’s inhibition of GSK3β (24, 47–49; reviewed in Refs. 21 and 50). Based on the extensive evidence that GSK3β promotes apoptosis and that ER stress is induced in a variety of neurodegenerative disorders in which apoptosis may contribute to neuronal loss, we investigated whether there is an association between GSK3β activity and apoptotic signaling induced by ER stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments—** SH-SY5Y human neuroblastoma cells were grown in RPMI 1640 medium (Celgro, Herndon, VA) supplemented with 5% fetal clone II (HyClone, Logan, UT), 10% horse serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY). SH-SY5Y cell lines were previously described that stably express dominant-negative GSK3β (51) or stably overexpress active GSK3β at three to four times the normal level (24). Immortalized hippocampal cells (52) (generously provided by Dr. M. F. Mehler, Albert Einstein College of Medicine) were differentiated by incubation for 6 days at 39 °C in Neurobasal media containing B-27 supplement (53) prior to experimental manipulations. Cells were washed and preincubated in serum-free or B-27-free media overnight before experimental treatments. Where indicated, cells were treated with LiCl (Sigma), insulin-like growth factor-1 (IGF-1, Intergen, Purchase, NY), cyclosporin A, FK506 (Calbiochem, San Diego, CA), thapsigargin, tunicamycin, brefeldin A, okadaic acid, or calyculin A (Alexis, San Diego, CA).

**Immunoblot Analysis—** For immunoblotting, cells were washed twice with phosphate-buffered saline and lysed with 100 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 mM sodium acid, and 0.2% Nonident P-40). The lysates were collected in centrifuge tubes, sonicated, and centrifuged at 16,000 × g for 10 min at 4 °C. Protein concentrations were determined using the BCA method (Pierce). Where indicated, cells were fractionated as described previously (54). For subcellular fractionation, lysed cells were collected in microcentrifuge tubes, and centrifuged at 2,700 × g for 10 min at 4 °C. The supernatant containing the cytosol was further centrifuged at 25,000 × g for 15 min at 4 °C to obtain the cytosolic fraction. The nuclei in the pellet were washed three times by gently resuspending the nuclei in 200 μl of wash buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 25 mM NaCl, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 μg/ml pepstatin A) and centrifuging at 2,700 × g for 5 min at 4 °C. For a final wash, the nuclei were resuspended in 100 μl of wash buffer, layered over a cushion of 1 ml of sucrose buffer (1% sucrose, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 μg/ml pepstatin A), and centrifuged at 2,700 × g for 5 min at 4 °C to remove residual sucrose buffer. Extracts were mixed with Laemmli sample buffer (2% SDS) and placed in a boiling water bath for 5 min. Proteins were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-GSK3β, anti-poly(ADP-ribose) polymerase (PARP), anti-proteolyzed PARP 85-kDa fragment (Pharmin
tgenTransduction Laboratories, San Diego, CA), anti-phospho-Ser-9-GSK3β, anti-phospho-Ser-473-Akt, anti-total Akt, anti-β-catenin, and anti-active caspase-3 (Cell Signaling, Beverly, MA) antibodies. Immuno
blobs were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence, and the protein bands were quantitated with a densitometer.

**Enzyme Activity Measurements—** Fluorometric assays of caspase-3 activity using the substrate Ac-DEVD-AMC (Alexis) were carried out as described previously (24). For this, fluorometric assays were conducted in 96-well clear bottom plates, and all measurements were carried out in triplicate wells. To each well 200 μl of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol) was added. Peptide substrates for caspase-3 (Ac-DEVD-AMC (Alexis Biochemicals, San Diego, CA) were added to each well to a final concentration of 25 ng/μl. When the caspase-3 inhibitor (Ac-DEVD-CHO) was used, it was added at a concentration of 2.5 ng/μl immediately before addition of the caspase-3 substrate. Cell lysates (20 μg of protein) were added to start the reaction. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer without the cell lysate. Assay plates were incubated at 37 °C for 1 h for measurement of caspase-3, and fluorescence was measured on a fluorescence plate reader (Bio-Tek, Winooski, VT) set at 360-nm excitation and 460-nm emission. Caspase activity was calculated as [mean AMC fluorescence from triplicate wells]/(background fluorescence) μg of protein.

The activity of GSK3β was measured as described previously (54). For this, immunoprecipitate caspase-3, 100 μg of protein was incubated with 0.75 μg of monoclonal GSK3β antibody overnight at 4 °C with gentle agitation. Extracts were incubated with 30 μl of protein G-Sepharose for 1 h at 4 °C. The immobilized immune complexes were washed twice with immunoprecipitation lysis buffer and twice with kinase buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, and 1 mM dithiothreitol). Kinase activity was measured by mixing immunoprecipitated GSK3β with 25 μl of kinase buffer containing 20 mM Tris, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, 250 μM ATP, 1.4 μCl of [γ-32P]ATP (Amer
sham Biosciences, Arlington Heights, IL), and 0.1 μg/ml recombinant tau protein (Panvera, Madison, WI). The GSK3β inhibitor lithium (20 mM (44)) was added in vitro to confirm that phosphorylation was medi
dated by GSK3β. The samples were incubated at 30 °C for 15 min, and 25 μl of Laemmli sample buffer (2% SDS) was added to each sample to stop the reaction. Samples were placed in a boiling water bath for 5 min, and proteins were separated in 7.5% SDS-polyacrylamide gels. The gels were vacuum-dried, exposed to a phosphor screen overnight, and quan
titated using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The efficiency of GSK3β immunoprecipitation was determined by immu
noblotting for GSK3β.

**RESULTS**

**Thapsigargin Treatment Induces Apoptosis and Activates GSK3β—** Characteristics associated with apoptosis were assessed in human neuroblastoma SH-SY5Y cells after the induction of ER stress with thapsigargin. These parameters included measurements of the activity of the effector caspase, caspase-3, proteolysis of PARP (a classical substrate cleaved by caspase-
3), immuno blot detection of the cleavage of pro-caspase-3 to active caspase-3 fragments, and changes in morphology. Caspase-3 activity, measured by fluorogenic substrate cleavage (24), increased between 2 and 4 h after treatment with 2 μM thapsigargin (Fig. 1A). Similar time courses after thapsigargin treatment were observed in Western blot analyses of the pro
teolysis of PARP from an intact 116-kDa protein to a stable 85-kDa breakdown product, and the production of 17- and 19-kDa activated caspase-3 (Fig. 1A). Examination of cells treated with thapsigargin and stained with Hoechst 33342 (24) revealed the characteristic morphology associated with apop
tosis, including nuclear condensation and cell shrinkage (data not shown). These results confirm previous reports that thapsigargin causes SH-SY5Y cells to undergo caspase-3-mediated apoptosis (55–57).

To test if GSK3β is involved in the apoptotic response to ER stress, the activity of GSK3β was assessed in SH-SY5Y cells after thapsigargin treatment. As described previously (54), GSK3β activity was measured by immunoprecipitatin GSK3β, measuring its catalysis of the phosphorylation of re
combinant tau protein, a well-characterized substrate of GSK3β (reviewed in Ref. 58), and confirming that phosphoryl
tion was mediated by immunoprecipitated GSK3β by inclu
dion of the GSK3β inhibitor lithium (20 mM (44)) in the kinase assay. These measurements revealed that there was a large and prolonged increase in GSK3β activity after thapsigargin treatment (Fig. 1B), GSK3β activity increased within 2 h after exposure of cells to 2 μM thapsigargin and was 277 ± 27% (n = 3) of control levels after 4 h of thapsigargin treatment (Fig. 1B).
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**Fig. 1.** Thapsigargin treatment activates caspase-3 and GSK3β. SH-SY5Y cells were treated with 2 μM thapsigargin. A, caspase-3 activity was determined by measuring cleavage of a fluorogenic substrate, Ac-DEVD-AMC, as described previously (24), and values are expressed as a percent of caspase activity in untreated cells. Means ± S.E., n = 3 experiments. PARP proteolysis (intact PARP is indicated by the band at 116 kDa, and proteolyzed PARP is indicated by the band at 85 kDa) and cleavage of procaspase-3 to active caspase-3 (∼17- and 19-kDa bands) were measured by immunoblot analyses. B, GSK3β activity was measured by immunoprecipitating GSK3β from cells 1–6 h after administration of 2 μM thapsigargin, and measuring the phosphorylation of recombinant tau using [32P]ATP as described previously (54). Quantitative values are expressed as a percentage of GSK3β activity in untreated cells. Means ± S.E., n = 3. C, β-catenin levels were measured in the indicated fractions following incubation with 2 μM thapsigargin (Tph) for 3 h, 20 mM lithium for 4 h, or pretreatment with lithium for 1 h followed by treatment with thapsigargin for 3 h.

In situ activation of GSK3β by thapsigargin treatment was further confirmed by measuring the level of β-catenin. Phosphorylation of cytosolic β-catenin by GSK3β promotes its degradation, whereas inhibition of GSK3β allows the stabilization, accumulation, and nuclear translocation of β-catenin (59). An additional pool of β-catenin is sequestered at the plasma membrane, and its stability is unaffected by GSK3β. Treatment of SH-SY5Y cells with thapsigargin caused depletion of cytosolic β-catenin (Fig. 1C), and a modest reduction of nuclear β-catenin, whereas membrane-bound β-catenin was unaltered. Treatment with lithium to inhibit GSK3β attenuated thapsigargin-induced depletions of cytosolic and nuclear β-catenin. These results are indicative of GSK3β activation by thapsigargin and inhibition by lithium. Thus, thapsigargin treatment caused the activation of GSK3β, a previously unknown response to ER stress, concomitantly with the initiation of apoptotic signaling, which raised the possibility that GSK3β may be involved in the signaling pathway linking ER stress to caspase-3 activation.

**GSK3β Is an Obligate Intermediate in Thapsigargin-induced Apoptosis**—Considering recent findings that GSK3β can promote apoptosis (reviewed in Ref. 21), the thapsigargin-induced activation of GSK3β raised the question of whether this is an essential component of the apoptosis signaling cascade that is induced by ER stress leading to activation of caspase-3. To test this, the effects of thapsigargin on caspase-3 activation were examined under conditions where the activity of GSK3β was modified. To test if increased GSK3β activity is stimulatory, thapsigargin-induced PARP proteolysis was compared in control SH-SY5Y cells, vector-transfected cells, and two different stable lines of SH-SY5Y cells that overexpress active GSK3β 3- to 4-fold above the endogenous level of GSK3β, which have been described previously (24). Thapsigargin-induced PARP proteolysis was similar in wild-type and vector-transfected SH-SY5Y cells but was much greater in cells overexpressing GSK3β (Fig. 2A), indicating that increased active GSK3β promotes thapsigargin-induced caspase activation. In opposition to overexpression of GSK3β, thapsigargin-induced PARP proteolysis was examined in SH-SY5Y cells stably expressing a dominant-negative mutant of GSK3β (60). Although incubation with 2 μM thapsigargin caused a time-dependent increase in PARP proteolysis in control cells, there was little PARP proteolysis in cells expressing dominant-negative GSK3β (Fig. 2B).

To test further if GSK3β is a necessary intermediate in thapsigargin-induced caspase-3 activation, cells were pretreated with lithium, a selective inhibitor of GSK3β (44–46). Thapsigargin-induced PARP proteolysis was concentration-dependently inhibited in cells pretreated with 1–20 mM lithium (Fig. 2C). Pretreatment with 20 mM lithium, which we have shown inhibits GSK3β in vitro by ~80% (49), reduced thapsigargin-induced PARP proteolysis by 60–80%, indicating a close correspondence between inhibition of GSK3β activity and caspase-3 activation. Furthermore, examination of the lithium concentration-dependent attenuation of thapsigargin-induced PARP proteolysis revealed less protection in cells overexpressing GSK3β than in wild-type or vector-transfected SH-SY5Y cells due to the greater activity of GSK3β (Fig. 2C), substantiating the conclusion that lithium’s protective action is due to inhibition of GSK3β. Taken together, these results indicate that GSK3β is a necessary and regulatory component of thapsigargin-induced signaling leading to activation of caspase-3.

**Mechanism of Thapsigargin-induced GSK3β Activation**—Although GSK3β is a constitutively active enzyme, the activity of GSK3β is modulated by phosphorylation, with phosphorylation of Ser-9 decreasing activity and phosphorylation of Tyr-216 increasing activity (reviewed in Ref. 21). To examine if either of these post-translational modifications of GSK3β was altered by thapsigargin treatment to account for the thapsigargin-induced activation of GSK3β, the phosphorylation state of GSK3β in SH-SY5Y cells was examined by immunoblot analyses. These measurements revealed a time-dependent decrease in phospho-Ser-9-GSK3β (Fig. 3A), whereas phospho-Tyr-216-GSK3β immunoreactivity was unaltered (data not shown). Hence, thapsigargin treatment activated GSK3β by reducing the inhibitory Ser-9 phosphorylation of GSK3β. Because Akt (also known as protein kinase B) is a primary kinase responsible for phosphorylating Ser-9 of GSK3β (34), we tested if thapsigargin affected the activation-associated phosphorylation of Ser-473 of Akt. These measurements demonstrated that treatment with thapsigargin greatly decreased phospho-Ser-473-Akt immunoreactivity (Fig. 3B). Thus, thapsigargin treatment reduced both the inhibitory phosphorylation of Ser-9 on GSK3β and the activating phosphorylation of Ser-473 on Akt, leading to increased GSK3β activity.

To further examine the relationship between the activities of Akt, GSK3β, and caspase-3, we tested if receptor-mediated activation of Akt affected the changes in phosphorylation of Akt and GSK3β and caspase-3 activity induced by thapsigargin in SH-SY5Y cells. Administration of insulin-like growth factor-1 (IGF-1), a growth factor, which activates receptors endogenously expressed in SH-SY5Y cells known to activate Akt (61), counteracted the inhibitory effect of thapsigargin on Akt, causing an increase in phospho-Ser-473-Akt (Fig. 3C), blocked the
and two different stable lines of SH-SY5Y cells that overexpress GSK3\(^{WT}\) compared in control SH-SY5Y cells (\(\text{WT}\)), vector-transfected cells (\(\text{V}\)), a dominant-negative mutant of GSK3\(^{\text{thapsigargin}}\) (data not shown).

PARP was equivalent in all cell lines in the absence of treatment with thapsigargin-induced PARP proteolysis (Fig. 2C). This is in accordance with previous reports that PP2A, but not PP1, dephosphorylates both GSK3\(^{\beta}\) and Akt (66–68). Thus, activation of GSK3\(^{\beta}\) via dephosphorylation of phospho-Ser-9 is mediated by a PP2A-dependent mechanism and activation of GSK3\(^{\beta}\) promotes caspase-3 activation following thapsigargin treatment.

In addition to directly inhibiting GSK3\(^{\beta}\) (44), lithium also has been reported to increase the inhibitory serine phosphorylation of GSK3\(^{\beta}\) (29, 69), indicating that lithium has dual mechanisms for inhibiting GSK3\(^{\beta}\). Examination of phospho-Ser-9-GSK\(^{\beta}\) levels revealed that, although thapsigargin treatment caused a reduction, the level of phospho-Ser-9-GSK\(^{\beta}\) was increased in SH-SY5Y cells pretreated with lithium (Fig. 3F). This finding is in accordance with emerging evidence that lithium counteracts the effects of PP2A (70, 71), to increase levels of phospho-Ser-9-GSK\(^{\beta}\), and suggests that lithium may protect cells from ER stress-induced caspase-3 activation both by direct inhibition of GSK3\(^{\beta}\) and by increasing the inhibitory phosphorylation of Ser-9 of GSK3\(^{\beta}\).

To determine if similar signaling activities are generated by ER stress in another neuronal model system, and particularly in non-proliferating cells, the responses to treatment with thapsigargin were examined in immortalized hippocampal cells that had been differentiated for 6 days. Treatment with 2 \(\mu\text{M}\) thapsigargin resulted in activation of caspase-3 and proteolysis of PARP, although with a somewhat delayed time course compared with SH-SY5Y cells, and pretreatment with 20 mM lithium effectively blocked these effects, consistent with a facilitating effect of GSK3\(^{\beta}\) on apoptotic signaling (Fig. 3G). A slightly higher concentration of thapsigargin (4 \(\mu\text{M}\)) activated caspase-3 and caused proteolysis of PARP in differentiated hippocampal cells (Fig. 3H) in a similar time frame as was obtained with 2 \(\mu\text{M}\) thapsigargin in SH-SY5Y cells. Thapsigargin treatment also caused a profound dephosphorylation of phospho-Ser-9-GSK and of phospho-Ser-473-Akt (Fig. 3H). Changes in SH-SY5Y cells caused by increasing the inhibitory phosphorylation of Ser-9 of GSK3\(^{\beta}\).

Considering previous reports that the calcium-activated protein phosphatase 2B (calcineurin; PP2B) can cause apoptosis (62) and is activated following thapsigargin treatment (63, 64), we tested whether inhibition of PP2B altered thapsigargin-induced PARP proteolysis. SH-SY5Y cells were pretreated for 30 min with either 1 \(\mu\text{M}\) cyclosporin A or 1 \(\mu\text{M}\) FK506, two selective inhibitors of PP2B, and then incubated with 2 \(\mu\text{M}\) thapsigargin for 3 h. Neither PP2B inhibitor altered thapsigargin-induced PARP proteolysis or changed the levels of phospho-Ser-9-GSK\(^{\beta}\) or phospho-Ser-473-Akt (Fig. 3D).

In contrast to the lack of effects of inhibitors of PP2B, treatment with the phosphatase inhibitors okadaic acid or calyculin A provided protection from the effects of thapsigargin at concentrations reported to be selective (65) for protein phosphatase 2A (although additional inhibition of protein phosphatase 1 cannot entirely be ruled out). Pretreatment with 1 \(\mu\text{M}\) okadaic acid or 1 \(\mu\text{M}\) calyculin A blocked thapsigargin-induced PARP proteolysis and increased the levels of phospho-Ser-9-GSK and of phospho-Ser-473-Akt (Fig. 3D). This is in accordance with previous reports that PP2A, but not PP1, dephosphorylates both GSK3\(^{\beta}\) and Akt (66–68). Thus, activation of GSK3\(^{\beta}\) via dephosphorylation of phospho-Ser-9 is mediated by a PP2A-dependent mechanism and activation of GSK3\(^{\beta}\) promotes caspase-3 activation following thapsigargin treatment.

ER Stress Induced by Tunicamycin and Brefeldin-A—Similar, but not identical, results were obtained in SH-SY5Y cells using two other agents that cause ER stress, tunicamycin, which causes ER stress by inhibiting N-linked glycosylation and protein folding in the ER, and brefeldin-A, which perturbs ER-Golgi protein trafficking. Changes in SH-SY5Y cells caused by tunicamycin, probably the second most widely used agent to...
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Fig. 3. Phosphorylation of GSK3β and caspase-3 activation. A, in SH-SY5Y cells, phospho-Ser-9-GSK3β immunoreactivity decreased 1–4 h after treatment with 2 μM thapsigargin, whereas total GSK3β levels were unaltered. Quantitative values are expressed as the percentage of phospho-Ser-9-GSK3β in untreated cells. Means ± S.E., n = 3. B, in SH-SY5Y cells, activation-associated phospho-Ser-473-Akt immunoreactivity decreased 1–4 h after treatment with 2 μM thapsigargin, whereas total Akt levels were unaltered. C, IGF-1 attenuated proapoptotic actions of thapsigargin. SH-SY5Y cells were treated with thapsigargin (2 μM, 3 or 4 h), with or without a 30-min pretreatment with 50 ng/ml IGF-1, followed by measurements of phospho-Ser-473-Akt, total Akt, phospho-Ser-9-GSK3β, total GSK3β, and PARP proteolysis. D, SH-SY5Y cells were pretreated for 30 min with 1 μM cyclosporin A (CSA) or 1 μM FK506 followed by incubation with 2 μM thapsigargin for 3 h, and samples were immunoblotted for PARP, phospho-Ser-9-GSK3β, and phospho-Ser-473-Akt. E, pretreatment of SH-SY5Y cells for 30 min with 1 μM okadaic acid (OA) or 1 μM calyculin A (Cal A) blocked thapsigargin-induced PARP proteolysis, dephosphorylation of phospho-Ser-9-GSK3β, and the dephosphorylation of phospho-Ser-473-Akt. F, phospho-Ser-9-GSK3β levels were immunoblotted in SH-SY5Y cells treated with 2 μM thapsigargin for 2, 3, or 4 h, with or without a 1-h preincubation with 20 mM lithium. G, pretreatment with 20 mM lithium for 1 h blocked caspase-3 activation and PARP proteolysis induced by 2 μM thapsigargin (Tg) measured 4, 6, and 8 h after treatment of differentiated immortalized hippocampal cells. H, treatment of differentiated hippocampal cells with 4 μM thapsigargin for 3 h induce ER stress after thapsigargin, were similar to those caused by thapsigargin. Treatment with tunicamycin (1–6 μg/ml) caused concentration-dependent increases in PARP cleavage and in the appearance of active caspase-3, and it reduced the level of phospho-Ser-9-GSK3β (Fig. 4A). Pretreatment with lithium completely blocked tunicamycin-induced PARP cleavage, activation of caspase-3, and dephosphorylation of GSK3β (Fig. 4A). The protection from the deleterious effects of tunicamycin afforded by lithium was compared with the effects of IGF-1 and phosphatase inhibitors. Fig. 4B shows that, as was found with thapsigargin, in SH-SY5Y cells treated with tunicamycin (2 μg/ml), both lithium and IGF-1 attenuated PARP proteolysis, caspase-3 activation, and dephosphorylation of phospho-Ser-9-GSK3β. Furthermore, the PP2A/PPI inhibitors okadaic acid and calyculin A, but not the PP2B inhibitors FK506 and cyclosporin A, were similarly protective. Somewhat similar effects were obtained with brefeldin A. Treatment of SH-SY5Y cells with 10 μg/ml brefeldin A for 4 h also activated caspase-3 and increased the proteolysis of PARP (Fig. 4C). As with thapsigargin and tunicamycin, apoptotic signaling induced by brefeldin A was blocked by pretreatment with lithium, IGF-1, and calyculin A and was unaffected by cyclosporin A. Unlike thapsigargin and tunicamycin, there was not an evident dephosphorylation of phospho-Ser-9-GSK3β 4 h after brefeldin A treatment, perhaps indicating that endogenous GSK3β need not be further activated to contribute to brefeldin A-induced apoptotic signaling. However, examination of early times after treatment with brefeldin A revealed a rapid, but transient, decrease in phospho-Ser-9-GSK3β, indicating that all three treatments that induce ER stress cause dephosphorylation of phospho-Ser-9-GSK3β but with differences in the duration of the decrease.

DISCUSSION

Impaired ER function can cause accumulation of unfolded and misfolded proteins, actions that can initiate the apoptotic signaling cascade, and indications of neuronal ER stress have been identified in aging and a number of neurodegenerative conditions (1–4). Furthermore, the ER appears to have a key role in apoptosis initiated from other cellular sites, because trafficking of members of the bcl-2 family of apoptosis regulators to the ER is a critical action modulating many types of apoptosis (72–74). Thapsigargin is one of the most useful agents available to identify cellular responses to ER stress because of its specific and potent action of inhibiting the Ca2+-ATPase in the ER (15), which leads to caspase-3-mediated apoptosis (18). The present investigation revealed a previously unrecognized and obligatory early step in thapsigargin-induced apoptosis, because thapsigargin treatment activated GSK3β. This response was found to be due to dephosphorylation of phospho-Ser-9-GSK3β, and blockade of caspase-3 activation by inhibition of GSK3β directly or by phosphatase inhibition demonstrated that this is a critical intermediate step coupling ER stress to caspase-3 activation.

A number of recent findings have linked GSK3β to apoptosis, but the present results represent the first to find that GSK3β is involved in the response to ER stress. For example, overexpression GSK3β was shown to be sufficient to induce apoptosis (23), moderately overexpressed GSK3β facilitated apoptosis induced by staurosporine or heat shock (24) or mitochondrial complex 1 inhibitors (25), and inhibition of the phosphatidylinositol 3-ki-
tunicamycin and brefeldin A.

A.

| Tunicamycin | Lithium |
|-------------|---------|
| 0 | - |
| 1 | - |
| 2 | - |
| 4 | + |
| 6 | + |

B.

| Tunicamycin | Lithium |
|-------------|---------|
| 0 | - |
| 1 | - |
| 2 | - |
| 4 | + |
| 6 | + |

C.

| Tunicamycin | Lithium |
|-------------|---------|
| 0 | - |
| 1 | - |
| 2 | - |
| 4 | + |
| 6 | + |

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nase pathway, which normally maintains phosphorylation of Ser-9-GSK3β, led to GSK3β-dependent apoptosis (35, 36). These and other recent findings (reviewed in Ref. 21) support the concept that disinhibition of GSK3β promotes apoptotic signaling. The present findings expand the conditions in which GSK3β promotes apoptosis to those involving ER stress as activation of GSK3β was found to be a critical process in thapsigargin-induced apoptosis based on measurements of the effects of both increasing and decreasing GSK3β activity. Increased GSK3β activity achieved by its moderate overexpression facilitated thapsigargin-induced caspase-3 activation, whereas inhibition of GSK3β by expression of dominant-negative GSK3β greatly attenuated thapsigargin-induced caspase-3 activation. Furthermore, inhibition of GSK3β with lithium reduced caspase-3 activation to a level comparable with its inhibition of GSK3β, suggesting that, although lithium has other targets, its inhibitory effect on GSK3β was likely to be the basis of its protective effect. This conclusion was further substantiated by the finding that higher concentrations of lithium were necessary for it to provide protection in cells overexpressing GSK3β. These findings demonstrate that GSK3β is an important step in the initiation of apoptosis caused by thapsigargin treatment.

ER stress induced by thapsigargin treatment activated a signaling pathway that included dephosphorylation of phospho-Ser-473-Akt and activation of GSK3β through dephosphorylation of phospho-Ser-9-GSK3β, and PP2A/PP1 inhibitors blocked these dephosphorylation actions and caspase-3 activation. PP2A is known to dephosphorylate phospho-Ser-9-GSK3β (66, 67), and PP2A but not PP1 was shown to dephosphorylate Akt (68, 75). With thapsigargin treatment, PP2A/PP1 inhibitors blocked dephosphorylation of both Akt and GSK3β, thus, it is not possible to distinguish between two possible PP2A-dependent mechanisms causing GSK3β dephosphorylation and activation: this could result from a direct effect of PP2A on phospho-Ser-9-GSK3β or as an indirect effect of PP2A resulting from the inactivation of Akt. Presently, it appears that both mechanisms may contribute to GSK3β activation following thapsigargin treatment. Protein phosphatases constitute a critical component of many signaling systems that initiate the apoptotic program. For example, both PP2B (64) and PP2A (76) dephosphorylate phospho-BAD, thereby converting it to its proapoptotic dephosphorylated form, as well as regulating other proteins involved in apoptosis. However, in SH-SY5Y cells treated with thapsigargin, PP2B activation occurs after caspase activation in a caspase-dependent manner, suggesting that PP2B contributes to late events in cell death (57). In contrast to the lack of effects of inhibitors of PP2B on caspase-3 activation induced by thapsigargin, PP2A inhibitors blocked

with 20 mM lithium for 1 h, followed by incubation with 1, 2, 4, or 6 ng/ml tunicamycin for 3 h, and proteolyzed PARP, active caspase-3, and phospho-Ser-9-GSK3β were measured in immunoblots. B, SH-SY5Y cells were pretreated with 20 mM lithium for 1 h, or for 30 min with 50 ng/ml IGF-1, 1 μM okadaic acid (OA), 1 μM calyculin A (Cal), 1 μM FK506 (FK), or 1 μM cyclosporin A (Cyc), followed by incubation with 2 μg/ml tunicamycin for 3 h, and proteolyzed PARP, active caspase-3, phospho-Ser-9-GSK3β, and phospho-Akt were measured in immunoblots. Quantitative values are expressed as a percentage of PARP proteolytic fragment or active caspase-3 in untreated cells. Means ± S.E., n = 3.
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caspase-3 activation, indicating that PP2A contributes to an early event in the apoptotic program initiated by thapsigargin. It is notable that another cell stress, hypersomotic stress, activated Akt via phosphorylation mediated by PP2A without changing the cellular activity of PP2A (75). Thus, the action of PP2A appears to be important in the apoptotic signaling cascades following both hypersomotic stress and ER stress induced by thapsigargin, perhaps through subcellular re-localization of the enzyme. PP2A and GSK3β also were implicated in the signaling mechanisms leading to caspase-3 activation following ER stress induced by tunicamycin and by brefeldin A. With both agents, caspase-3 activation was attenuated by lithium, IGF-1, and inhibition of PP2A, but not inhibition of PP2B. Although the mechanism whereby thapsigargin treatment leads to PP2A-mediated phosphorylation of Akt and GSK3β is unknown, it is evident that phosphorylation of the inhibitory site on GSK3β contributes to early events in apoptotic signaling. There are many potential targets whereby GSK3β could promote apoptosis. For example, GSK3β directly phosphorylates, and thereby regulates, at least eight transcription factors, consequently impacting the expression of many genes (reviewed in Ref. 21). Among these are several survival-promoting transcription factors, such as cAMP response element-binding protein, of which inhibition by GSK3β (49) may contribute to facilitation of apoptosis. Also, activation of GSK3β inhibits protein synthesis (77), a logical response to ER stress to reduce the further production of proteins, and this action has been linked to the promotion of apoptosis by GSK3β (78). However, the one or more precise proapoptotic targets of GSK3β during ER stress remain to be identified. Considering the well-known antiapoptotic actions of Akt (79–81) along with the proapoptotic actions of GSK3β (reviewed in Ref. 21), it is evident that inactivation of Akt and activation of GSK3β following thapsigargin-induced ER stress provides a strong stimulus for activation of the apoptotic program.

ER stress appears to be an early event contributing to neuronal dysfunction and death in AD and likely also in aging and in other neurodegenerative conditions (1–4). Many pathways involving multiple kinases have been identified that inhibit GSK3β activity, but fewer mechanisms are known that increase the activity of GSK3β. Activation of GSK3β primarily requires phosphorylation of phospho-Ser-9-GSK3β, an action previously shown to be mediated by PP2A and here shown to be caused by ER stress. This raises the possibility that GSK3β may be activated by ER stress in neurodegenerative conditions where this occurs, such as in affected neurons in AD. In this regard, it is relevant that GSK3β is a prime candidate kinase for causing the hyperphosphorylation of tau that is associated with neurofibrillar tangles in AD (reviewed in Ref. 21). In the few studies that have assessed GSK3β in AD, increased levels of GSK3β were found in AD, compared with non-diseased, human brain; immunohistochemical measurements found GSK3β associated with neurofibrillary tangles in AD brain (82–86); and active GSK3β was found to be sequestered in pre-tangle neurons (86). Furthermore, the AD-associated Aβ peptide is known to activate GSK3β (22), and the GSK3β inhibitor lithium provides protection from Aβ toxicity (87, 88). Taken together, these studies suggest that alterations in the control of GSK3β may result from ER stress, which could contribute to the neuropathology of AD as well as other neurodegenerative conditions in which ER stress occurs.

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