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Neurobiology of Disease

Tuberous Sclerosis Complex Activity Is Required to Control Neuronal Stress Responses in an mTOR-Dependent Manner

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Tuberous sclerosis complex (TSC) is a neurogenetic disorder caused by loss-of-function mutations in either the TSC1 or TSC2 genes and frequently results in prominent CNS manifestations, including epilepsy, mental retardation, and autism spectrum disorder. The TSC1/TSC2 protein complex plays a major role in controlling the Ser/Thr kinase mammalian target of rapamycin (mTOR), which is a master regulator of protein synthesis and cell growth. In this study, we show that endoplasmic reticulum (ER) stress regulates TSC1/TSC2 complex to limit mTOR activity. In addition, Tsc2-deficient rat hippocampal neurons and brain lysates from a Tsc1-deficient mouse model demonstrate both elevated ER and oxidative stress. In Tsc2-deficient neurons, the expression of stress markers such as CHOP and HO-1 is increased, and this increase is completely reversed by the mTOR inhibitor rapamycin both in vitro and in vivo. Neurons lacking a functional TSC1/TSC2 complex have increased vulnerability to ER stress-induced cell death via the activation of the mitochondrial death pathway. Importantly, knockdown of CHOP reduces oxidative stress and apoptosis in Tsc2-deficient neurons. These observations indicate that ER stress modulates mTOR activity through the TSC protein complex and that ER stress is elevated in cells lacking this complex. They also suggest that some of the neuronal dysfunction and neurocognitive deficits seen in TSC patients may be attributable to ER and oxidative stress and therefore potentially responsive to agents moderating these pathways.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the growth of benign tumors called hamartomas in multiple organs, including the brain (Crimo et al., 2006). TSC patients suffer from epilepsy, autism, and developmental delay. Within the CNS, TSC is associated with cortical tubers, made up of giant cells, dysmorphic neurons, and astrocytes. TSC is caused by mutations in either the TSC1 or TSC2 genes. Proteins encoded by TSC1 or TSC2 genes interact with each other to form the TSC1/TSC2 complex. One of the major cellular functions of the TSC1/TSC2 complex is to limit protein synthesis and regulate cell size by inhibiting the Rheb–mammalian target of rapamycin (mTOR) pathway (Kwiatkowski and Manning, 2005). Mutations in either TSC1 or TSC2 lead to constitutive activation of mTOR, which phosphorylates substrates such as S6 kinase (S6K) and 4E-BP1, ultimately increasing protein synthesis.

Recently, embryonic fibroblasts and kidney tumors from Tsc2-deficient mice were shown to have increased endoplasmic reticulum (ER) stress (Ozcan et al., 2008). ER stress can be caused by excessive protein synthesis, perturbation in calcium homeostasis, or nutrient deprivation (Ron and Walter, 2007). Under normal conditions, the ER stress sensor GRP78 has an inhibitory role on the effectors [PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1)] of the unfolded protein response (UPR), which is the cellular response to ER stress (Dorner et al., 1992; Liu et al., 2000). During ER overload, GRP78 releases its inhibition of PERK, ATF6, and IRE1 (Mori, 2000) and activates the UPR. The UPR leads to three distinct specific cascades: (1) the PERK/eIF2α pathway reduces protein synthesis by inhibiting translation; (2) the ATF6 pathway activates transcription of chaperone proteins increasing folding capacity; (3) the IRE/XBP-1 pathway promotes proteosome-dependent protein degradation to remove proteins from the ER (Bertolotti et al., 2000; Mori, 2000; Liu et al., 2003; Rutkowski and Kaufman, 2004). Ultimately, the UPR response results in either the successful elimination of ER overload or, if unsuccessful, in ER stress-induced cell death via caspase activation and induction of the proapoptotic transcription factor CHOP (C/EBP homologous protein, GADD153) (Oyadomari and Mori, 2004).

Although ER stress has been demonstrated in Tsc-deficient mouse embryonic fibroblasts (MEFs) and kidney tumors (Ozcan et al., 2008), it remains unclear whether TSC deficiency leads to ER stress in neurons, what role mTOR pathway plays in neuronal stress response, and whether similar dysfunctions are present in TSC patients.
investigated the role of the TSC1/TSC2 complex during ER stress in greater detail and examined the effects of TSC deficiency on neuronal stress pathways. We demonstrate that TSC2 is initially inactivated in neurons during ER stress and later activated, as part of an apparent regulatory mechanism to limit mTOR activity. Lack of a functional TSC1/TSC2 complex abolishes this regulation, resulting in increased ER stress and vulnerability to neuronal damage. Furthermore, Tsc-deficient neurons have increased accumulation of reactive oxygen species (ROS) and oxidative stress. Similar dysfunctions were identified in TSC brain lesions in vivo, identifying a new role for the TSC1/TSC2 complex in the neuronal stress response.

Materials and Methods

Animals. All experimental procedures were performed in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at Children’s Hospital (Boston, MA). The Tsc1−/− Syn-Cre+ mice used in this study were described previously (Meikle et al., 2007). For rapamycin treatment, mice were injected intraperitoneally at 6 mg/kg every other day from postnatal day 9 (P9) to P33. Mice subjected to the On/Off treatment were on rapamycin treatment (6 mg/kg) every other day from P9 to P30, followed by no treatment until P45 (On/Off) (Meikle et al., 2008).

Neuronal cultures. Neuronal cultures were prepared as published previously (Sahin et al., 2005). Briefly, hippocampi from 18- to 24-day-old rat embryos (CD1; Charles River) were isolated under the microscope and collected in HBSS containing 10 mM MgCl2, 1 mM kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. After 5 min dissociation at 37°C in 30 U/ml papain (Worthington), neurons were mechanically triturated and plated in Neurobasal (NB) medium containing B27 supplement, 2 mM l-glutamine, and penicillin/streptomycin (Invitrogen). For biochemical analysis, cells were plated at 1 × 106 cells per well onto six-well plates coated with 20 μg/ml poly-D-lysine (PDL) and 2.5 × 106 cells per plate for immunofluorescent (IF) studies onto PDL–laminin-coated glass coverslips in 24-well plates.

Lentivirus infection. Viral stocks for lentiviral infection were prepared as described previously (Mostoslavsky et al., 2005), except that the four packaging vectors (kindly provided by Dr. R. C. Mulligan, Department of Genetics, Harvard Medical School, Boston, MA) were cotransfected into HEK293 T cells with the plasmid to be coexpressed using Lipofectamine 2000 according to the instructions of the manufacturer. Viral particles were collected 48 and 72 h after transfection and filtered through a 0.45 μm membrane. Hippocampal neurons were infected at 1 day in vitro (1 DIV) in the presence of polybrene at 0.6 μg/ml. Six hours after infection, the virus-containing medium was replaced by fresh NB/B27 medium. After infection, neurons were kept in culture for an additional 10 d. Control short hairpin RNA (SHRNA) construct against the luciferase gene (here referred to as GL3-SH) was described previously (Flavell et al., 2006). The sequence for Tsc2 gene targeting was the following: 5′-GGTTGAGGAGGACCCGATCCA-3′.

Semiquantitative and real-time quantitative PCR. Total RNA was prepared with an RNAeasy kit (Qiagen) following the instructions of the manufacturer and quantified by a spectrophotometer. A total of 2 μg of poly(A) mRNA was used for reverse transcription using the SuperScript RT system (Invitrogen). Semiquantitative PCR reactions were performed using Taq Polymerase (PerkinElmer Life and Analytical Sciences). Quantification of the semiquantitative PCR was performed by densitometry scans, and values were normalized against total β-actin. Real-time PCRs were performed using SYBG Green PCR Master Mix (Applied Biosystems). All quantitative PCR (qPCR) reactions were performed in triplicate and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis was performed using 7300 System SDS Software on a 7300 Real Time PCR System. The sequences of the primers for both semiquantitative and qPCRs are listed in the supplemental data (available at www.jneurosci.org as supplemental material).

In all cases, data were expressed as means ± SE of at least three independent experiments. Statistical analysis was performed by unpaired two-tailed Student’s t test and considered significant at p < 0.05.

CHOP knockdown. CHOP SHRNA (CHOP-Sh) and control CHOP RNAi (CHOP-C) were purchased from Sigma, and the sequences are as follows: CHOP-Sh, 5′-GAAAAGAAGGAGAAGATCA-3′; CHOP-C 5′-CGGAAGTGTCACCAGCAC-3′.

Antibodies and reagents. Antibodies used for this study included the following: rabbit polyclonal anti-phospho-S6 (Ser235/236) (catalog #2211), mouse monoclonal anti-total S6 (catalog #2317), rabbit polyclonal anti-phospho-Akt (Ser473) (catalog #9271), rabbit polyclonal anti-S6K (catalog #9202), rabbit polyclonal anti-phospho-S6K (Thr389) (catalog #9234), rabbit polyclonal anti-Tsc1 (catalog #4906), and rabbit polyclonal anti-Tsc2 (Thr1462) (catalog #3611) (all from Cell Signaling Technology); rabbit polyclonal anti-Tsc2 (sc-893), mouse monoclonal anti-GADD153 (CHOP) (sc-7351), and goat polyclonal anti-Akt (sc-1618) (all from Santa Cruz Biotechnology); and rabbit polyclonal anti-GRP78 (SPA-826) and mouse monoclonal anti-heme oxygenase-1 (HO-1) (OSA-110) (from Stressgen). HRP-conjugated secondary antibodies were from VWR.

Western blot. Details can be found in the supplemental data (available at www.jneurosci.org as supplemental material).

ER stress induction. Thapsigargin (Tg) and Tunicamycin (Tm) were purchased from Sigma and used at a final concentration of 0.5 μM and 4 μg/ml, respectively. Stocks of drugs were made in DMSO and freshly diluted in NB media at 20X of the final concentration before performing each experiment. The same amount of DMSO was used as vehicle-only control. Before ER stress induction, NB/B27 media was replaced with NB in the presence of penicillin/streptomycin for 4 h, and drugs were then added for an additional 3, 6, and 24 h. When indicated, rapamycin was used for 24 h in NB media at a final concentration of 20 nM.

Apoptosis quantification. The number of apoptotic cells was determined by Hoechst staining and trypan blue exclusion test. Embryonic day 17 rat neurons were plated on coverslips at a density of 10 × 104 cells/ml and infected with lentivirus as described above. After 10 DIV, neurons were left untreated or treated for ER stress induction. For Hoechst quantification, neurons were fixed and stained with 5 μg/ml Hoechst (Invitrogen) for 5 min at room temperature. Neurons were then washed in PBS, mounted, and analyzed with a Leica DM RXA microscope equipped with epifluorescence. Apoptotic nuclei were counted under a 20X objective and expressed as the percentage of the total number of infected cells in the same field. Data are expressed as means ± SE from at least three different experiments, and statistical analysis was performed by Student’s t test. For trypan blue exclusion test, neurons were harvested as described in flow cytometric analysis and resuspended in a 0.2% trypan blue solution (Sigma) prepared in HBSS for 5 min at room temperature. Apoptotic cells were evaluated under bright-field microscopy by counting nonviable cells (dye-positive) and viable cells (dye-negative), using hemocytometer grid counts.

For quantification of cell death at the single-cell level, the number of apoptotic cells was determined by counting cleaved caspase 3 (c3) (Cell Signaling Technology catalog #9664) positively stained neurons after immunofluorescent microscopy using a 20X objective. Data were expressed as a percentage of the total number of infected cells. The experiment was performed in triplicate, and at least 300 cells per experiment were counted. Statistical analysis was performed by unpaired two-tailed Student’s t test and considered significant at p < 0.05.

Immunocytochemical analysis. Details can be found in the supplemental data (available at www.jneurosci.org as supplemental material).

Mitochondrial ROS. Rat hippocampal neurons were cultured in NB media for 24 h, followed by incubation with 100 mM MitoTracker Red CM-H2Xros dye (MT-Red) (Invitrogen) for 30 min before being processed for immunofluorescence and stained with Hoechst. Oxidative stress was quantified by counting the number of MT-Red-labeled cells under an epifluorescent microscope with a rhodamine filter and expressed as the average percentage of MT-Red-labeled cells from three independent experiments.

Flow cytometric analysis. Neurons cultured in NB media for 24 h were harvested by 5 min incubation at 37°C with 15 U/ml papain (Worthington) made in HBSS containing 10 mM MgCl2, 1 mM kynurenic acid, 10 mM HEPES, and penicillin/streptomycin. Before dissociation, a solution of 7 mg/ml trypsin inhibitor (Sigma) was added to stop the reaction.
Neurons were then collected by centrifugation, washed, and resuspended in NB media at $2 \times 10^5$ cells/ml. Neurons were divided into two aliquots, which were incubated in the absence or presence of 100 nM MitoTracker Red CM-H$_2$XRos dye (Invitrogen). After 20 min at 37°C, neurons were collected by centrifugation, rinsed, and fixed in 4% paraformaldehyde made in PBS for 15 min at room temperature. After fixation, neurons were washed and resuspended in 200 µl for analysis. Flow cytometric analysis was performed with Dako MoFlo equipped with Spectra-physics laser model 177 with an emission at 488 and a strength of 100 mW. Data were analyzed with Summit 4.3 software (Dako). Gating was performed before the collection of data to remove apoptotic cells and cellular debris. Mean fluorescence intensity of MT-Red was calculated by subtracting for each sample the fluorescence-activated cell sorting (FACS) measurement obtained in the absence of the dye (background) to the measurement obtained in the presence of the dye.

**Results**

**TSC and mTOR are dynamically regulated under ER stress**

Because some of the most severe manifestations of TSC disease are in the CNS, we investigated the role of the TSC1/TSC2 complex in the neuronal response to ER stress. We treated rat hippocampal neurons with two widely used ER stress inducers: the ER-Ca$^{2+}$-ATPase blocker Tg and the N-glycosylation inhibitor Tn (Li et al., 2000; Urano et al., 2000). To determine the optimal doses for these ER stress-inducing chemicals in neurons, we performed dose/response curves (0.1–5 µM for Tg; 1–12 µg/ml for Tn) using wild-type rat hippocampal neurons and assessed cell death as the outcome. Expression of UPR-regulated genes GRP78 and CHOP confirmed ER stress induction already at the lowest concentrations used for both drugs (supplemental Fig. 1A–C, available at www.jneurosci.org as supplemental material). As expected, cell death assessed by Hoechst staining (supplemental Fig. 1D, E, available at www.jneurosci.org as supplemental material) and trypan blue exclusion assay (supplemental Fig. 1F, available at www.jneurosci.org as supplemental material) showed that the percentage of apoptotic neurons increased in a dose-dependent manner for both Tg and Tn. For the purpose of this study, we decided to use 0.5 µM for Tg and 4 µg/ml for Tn because, at these doses, we observed a robust ER-stress-induced UPR activation and ER-stress-induced cell death of at least 50–60% neuronal cells.

When assessing the effect of ER stress on the Akt/mTOR pathway, we found a response that varied with duration of treatment. Tg treatment led to an initial activation of mTOR as evidenced by increased phosphorylation of S6 ribosomal protein (phospho-S6 Ser235/236) (Fig. 1A, D). In contrast, longer exposure to Tg (24 h) correlated with a progressive decrease in Akt activity (phospho-Akt Ser473) and in the phosphorylation of Tsc2 at Thr1462 (Fig. 1A, C, E), a known Akt phosphorylation site (Inoki et al., 2002; Potter et al., 2002). Thr1462 phosphorylation is known to inhibit TSC complex activity (Inoki et al., 2002; Manning et al., 2002). Accordingly, we observed inhibition of the downstream mTOR pathway, as indicated by decreased phosphorylation of S6 at 24 h (Fig. 1A, D, E). ER stress was confirmed by the time-dependent increase in the expression of the UPR-regulated gene, GRP78. Prolonged ER stress (24 h) correlated with expression of the proapoptotic UPR regulated gene CHOP with apoptosis as shown by activated (cleaved) caspase 3.

**Tsc2-deficient neurons show increased basal and ER-stress-induced cell death**

Although ER-stress-activated signaling is a protective cellular response to reduce ER load, prolonged ER stress often leads to cell death by apoptosis (Rao et al., 2001). To assess the effects of activating UPR response, we treated Tsc2 knockout and control neuronal cultures with the ER stress inducers Tg or Tn for 3, 6, and 24 h. Knockdown of Tsc activity was confirmed by the reduced Tsc2 protein level and constitutively high S6 and S6K phosphorylation (Fig. 3A, B). Exposure to either Tg or Tn induced cellular ER stress as seen by the gradual increase in the expression of the ER stress sensor GRP78. Western blot analysis showed that Tsc2 knockout neurons had a small but significant
increase in the levels of CHOP protein expression at baseline compared with control-infected neurons, which was consistent with the observed increased basal transcription of CHOP mRNA. No differences were observed in the baseline and in the ER-stress-induced GRP78 levels between the control and Tsc-deficient neurons (supplemental Fig. 3A, B, available at www.jneurosci.org as supplemental material).

The effects of ER stress induction on neuronal viability were then monitored using an antibody for cleaved (active) caspase 3 on Western blots. In control neurons, Tg or Tn treatments induced cleavage of caspase 3 only after 24 h, whereas in Tsc2-deficient neurons, cc3 was already detectable at baseline and further increased shortly after (3 h) ER stress induction (Fig. 3A, B). Similarly, as assessed by Hoechst staining, a higher proportion of Tsc2 knockdown neurons showed a significant increase in apoptotic nuclei at baseline and after short Tg (3 h) (Fig. 3C) or Tn (3 and 6 h) treatment (Fig. 3E). Similar results were obtained when assessing baseline cell death by trypan blue exclusion assay for Tg (Fig. 3D) and Tn (Fig. 3F). To further confirm induction of apoptosis in Tsc2 knockdown cells, we assessed cytoplasmic levels of cytochrome c, a marker of early apoptosis (Ferri and Kroemer, 2001; Rao et al., 2001). Consistent with the higher level of baseline apoptosis, we detected cytochrome c release in the cytosolic fraction of Tsc2-deficient neurons only (Fig. 3G). Denitometric quantification of cytosolic and mitochondrial cytochrome c levels was performed on three independent experiments and revealed a 4.9-fold increase in the cytosolic cytochrome c release in Tsc2-deficient neurons compared with controls (*p < 0.01 by t test). In cells under ER stress, the inositol requiring enzyme 1 (IRE1) pathway is responsible for the alternative splicing of the XBP-1 transcript (Lee et al., 2002). Interestingly, compared with neurons infected with control virus, Tsc2-deficient neurons had a more robust ER stress-induced activation of the IRE1 pathway during both Tg and Tn treatment, as shown by XBP-1 splicing (supplemental Fig. 4A, B, available at www.jneurosci.org as supplemental material). Together, these findings suggest that lack of Tsc activity in cultured hippocampal neurons correlates with increased ER-stress-induced cell death via activation of the mitochondrial death pathway.

Tsc-deficient neurons have increased oxidative stress and undergo cell death via a CHOP-dependent mechanism

CHOP is a proapoptotic transcription factor that promotes apoptosis by modulat-
ing the expression of proteins that regulate cell survival and death pathways (Oyadomari and Mori, 2004). In particular, CHOP has been found to affect expression and localization of bcl2 family members and influence the cellular redox status (McCullough et al., 2001; Marciniak et al., 2004). To determine which of these CHOP targets were affected in Tsc-deficient neurons, we compared the mRNA levels of survival and oxidative stress regulated genes by qRT-PCR (Fig. 4A). Tsc2 knockdown did not change the expression of the prosurvival factor bcl2 or of the cellular antioxidant defense gene thioredoxin 2 (Trx-2). Instead, we observed a significant mTOR-dependent increase in the expression (3.5-fold) of the antioxidant enzyme heme oxygenase-1 (HO-1) and of the ER oxidoreductase enzyme ERO1α. HO-1 protein levels were also increased in Tsc2-deficient neurons, and, during rapamycin treatment, this increase was blocked (Fig. 4B,C). However, under the same conditions, rapamycin treatment was not sufficient to prevent cell death.

Figure 2. Regulation of UPR genes in Tsc-deficient neurons at baseline and after ER stress induction. A, Real-time qRT-PCR of total RNA from rat hippocampal neurons infected with GL3-Sh (control) and Tsc2-Sh RNAi lentivirus. The ER stress regulated genes CHOP, ATF4, and GRP78 are increased in the Tsc2-deficient neurons in an mTOR-dependent manner as shown by a direct comparison of the effect of rapamycin (Rap.; 20 nm for 24 h) in each genotype. Significant p values are as follows: *p < 0.01 and **p < 0.05. Values normalized against GAPDH represent means of at least three independent experiments, and error bars represent SE. B, IF analysis of control and Tsc2-Sh infected neurons stained with CHOP antibody (red). GFP fluorescence (green) was used to identify infected neurons. Scale bar, 50 μm. Higher magnification of CHOP-positive neurons in the Tsc2-infected cultures (white box) is shown in the bottom panel. Scale bar, 20 μm. C, Quantification of IF analysis shows a significant increase in CHOP nuclear and cytosolic staining in Tsc2-deficient neurons (*p < 0.05). Data represent means of three different experiments, and error bars represent SE. At least 300 cells were counted in each experiment, and statistical analysis was performed by the Student’s t test. D, Representative IF images of control GL3-Sh and Tsc2-deficient neurons treated with DMSO (vehicle) or Tg for 3, 6, and 24 h and stained with CHOP antibody. In the merged panels, infected neurons are in green, CHOP antibody in red, and Hoechst staining in blue. Scale bar, 50 μm. E, Quantification of nuclear expression from at least three different experiments per time point. Values are expressed as means, and error bars represent SE. p values determined by Student’s t test are as follows: *p < 0.05, **p < 0.01.

HO-1 is a member of the heat shock family (Hsp32), and its expression is induced when cells experience oxidative stress (Takahashi et al., 2004), whereas the ERO1α is a glycosylated flavoenzyme implicated in oxidative protein folding and ROS production in the ER by promoting disulphide bond formation (Harding et al., 2003; Sevier and Kaiser, 2008). Therefore, we asked whether Tsc deficiency would induce increased production of ROS. Control and Tsc2-deficient rat hippocampal neurons were treated in culture with MT-Red (Fig. 4D,E). MT-Red generates fluorescence only during oxidation by superoxide produced by mitochondria (Kim et al., 2002). Quantification of MT-Red-labeled cells under an epifluorescent microscope was performed from three independent experiments and revealed a 2.6-fold increase in the percentage of Tsc2-deficient-positive neurons compared with control-infected cells (control, 14.3 ± 2.5% vs Tsc2-deficient neurons, 37.4 ± 1.4%; n = 250 cells per experiment; *p < 0.001 by t test). MT-Red accumulation, as as-
and trypan blue exclusion test (two-tailed), and error bars represent SE. Values were normalized to total Akt level, and statistical analysis was performed by a two-tailed t test with an adjusted significant *p value < 0.0125 after Bonferroni's correction for multiple pairwise comparisons. C–F, Quantification of cell death after Tg (C, D) and Tn (E, F) treatments of control and Tsc2-Sh neurons by Hoechst staining (C, E) and trypan blue exclusion test (D, F). For each panel, data are expressed as means ± SE from three independent sets of experiments per time point. Statistical analysis was performed with the Student’s t test (*p < 0.05, **p < 0.005, ***p < 0.0005 in D; *p < 0.05, **p < 0.01 in E; *p < 0.05 in F). G, Neuronal fractionation of control and Tsc2-deficient neurons shows increased cytochrome c release into the cytosolic fraction of Tsc2-Sh neurons, indicating activation of the mitochondrial death pathway. Cytochrome c oxidase IV (COX IV) and total Akt were used as mitochondrial (Mito.) and cytosolic (Cyt.) fraction markers, respectively.

Figure 3. Lack of Tsc activity correlates with increased CHOP expression. Representative Western blots of protein lysates from GL3-Sh- and Tsc2-Sh-infected hippocampal neurons after ER stress induction. Neurons were left untreated (−), treated with vehicle (DMSO), with 0.5 μM Tg (A), or with 4 μg/ml Tn (B) for 3, 6, and 24 h. At baseline, Tsc2-deficient neurons have increased cleaved caspase 3. The densitometric quantification of CHOP protein induction represents means of three independent experiments, and error bars represent SE. Values were normalized to total Akt level, and statistical analysis was performed by a two-tailed t test with an adjusted significant *p value < 0.005, **p value < 0.0005 in B. We found that, compared with CHOP-C virus, CHOP-Sh virus efficiently reduced the baseline and the Tg-stress-induced HO-1 expression at both the mRNA and protein levels in Tsc2-deficient neurons (Fig. 5C). Consistent with its role in promoting ERO1α activation (Marciniak et al., 2004), CHOP knockdown reduced ERO1α expression in Tsc2-deficient neurons (supplemental material). A significant reduction was also identified in the oxidative stress response of Tsc2-deficient cultures infected with CHOP-Sh compared with those infected with CHOP-C virus (Fig. 5D, available at www.jneurosci.org as supplemental material). Quantification of MT-Red-positive neurons was performed on immunofluorescent images from three independent experiments after incubation with 100 nM MitoTracker Red CM-H2XRos (at least 200 cells per experiment; those infected with CHOP-Sh compared with those infected with CHOP-C virus, 36.9 ± 2.0 vs Tsc2-Sh cultures infected with CHOP-Sh virus, 17.0 ± 5.0; *p < 0.05 by t test). Most importantly, CHOP silencing reduced cell death in Tsc2-deficient neurons by cleaved caspase staining by both Western blotting and at the single-cell level (Fig. 5B, C, E, F). Together, these data indicate that, in the setting of Tsc deficiency, CHOP upregulation plays a major role in both oxidative stress response and cell death induction.

Previous studies have shown that loss of TSC1/TSC2 complex activity correlates with an mTOR-dependent negative feedback on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which in turn results in reduced Akt activation (Zhang et al., 2006). To investigate whether a similar inhibition was occurring in Tsc-deficient neurons, we analyzed Akt at the phospho-Ser473 activation site using Western blot. When compared with control

Tsc-deficient neurons undergo cell death via a CHOP-dependent mechanism

The identification of increased cell death and ROS production in Tsc-deficient neurons led us to ask whether CHOP activity was necessary for these effects. To investigate this question, we silenced CHOP expression using RNAi. Rat hippocampal neurons were first infected with either GL3-Sh virus or Tsc2-Sh virus, and, after 6 d, they were reinfected with lentiviral vectors expressing either a CHOP-Sh or control CHOP-C constructs. GL3-Sh- and Tsc2-Sh-infected neuronal cultures were then either left untreated or treated with Tg for ER stress induction. CHOP-Sh RNAi efficiently reduced baseline and Tg-induced CHOP expression at both the RNA and the protein level in Tsc-deficient neurons and in Tg-treated GL3-Sh cultures (Fig. 5A, B). We found that, compared with CHOP-C virus, CHOP-Sh virus efficiently reduced the baseline and the ER-stress-induced HO-1 expression at both the mRNA and protein levels in Tsc2-deficient neurons (Fig. 5C). Consistent with its role in promoting ERO1α activation (Marciniak et al., 2004), CHOP knockdown reduced ERO1α expression in Tsc2-deficient neurons (supplemental material). A significant reduction was also identified in the oxidative stress response of Tsc2-deficient cultures infected with CHOP-Sh compared with those infected with CHOP-C virus (Fig. 5D, available at www.jneurosci.org as supplemental material). Quantification of MT-Red-positive neurons was performed on immunofluorescent images from three independent experiments after incubation with 100 nM MitoTracker Red CM-H2XRos (at least 200 cells per experiment; Tsc2-Sh cultures infected with CHOP-C virus, 36.9 ± 2.0 vs Tsc2-Sh cultures infected with CHOP-Sh virus, 17.0 ± 5.0; *p < 0.05 by t test). Most importantly, CHOP silencing reduced cell death in Tsc2-deficient neurons by cleaved caspase staining by both Western blotting and at the single-cell level (Fig. 5B, C, E, F). Together, these data indicate that, in the setting of Tsc deficiency, CHOP upregulation plays a major role in both oxidative stress response and cell death induction.

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In vivo reduce both cell death and oxidative stress.

Deficient neurons, knockdown of CHOP alone is sufficient to regulated Akt might contribute to increased apoptosis in Tsc-deficient neurons is CHOP independent, and, although down-expression in neurons and display neurological decline with mTOR-dependent oxidative stress response.

**Figure 4.** Lack of Tsc activity correlates with mTOR-dependent oxidative stress response. A, Real-time qRT-PCR from RNA samples of GL3-Sh- and Tsc2-Sh-infected neurons untreated (−) or treated (+) with 20 nm rapamycin for 24 h. Data are normalized to GAPDH level and are expressed as means ± SE of at least three different experiments (⁎p < 0.01). B, Representative Western blot of protein lysates from control and Tsc2-Sh hippocampal neurons untreated (−) or treated (+) with 20 nm rapamycin (Rap.) for 24 h. Increased HO-1 protein expression but not cleaved caspase 3 levels are reversed by rapamycin treatment in Tsc2-deficient neurons. Phospho-S6 was used to confirm mTOR downregulation by rapamycin, and total S6 was used as a loading control. C, Quantification of HO-1 protein induction. Data are averages ± SE from three different experiments. Statistical analysis was performed by a two-tailed t test with adjusted significant values in the absence of rapamycin and in the presence of rapamycin (⁎p < 0.025 after Bonferroni's correction for multiple pairwise comparisons). D, E, ROS production is increased in Tsc-deficient neurons. Representative II images of control (B) and Tsc2-deficient (B) rat hippocampal neurons in the absence and in the presence of 100 nm MT-Red. Scale bar, 20 μm. F, Representative distribution of the MT-Red FMI detected in control GL3-Sh- and Tsc2-Sh-infected cultures by flow cytometric analysis. The shift to the right on the MT-Red fluorescence distribution in Tsc2-Sh cultures indicates increased FMI.

Cultures, Tsc2-Sh-infected neurons had indeed lower basal levels of Akt activation that did not change after CHOP-Sh RNAi (Fig. 5B). These data indicate that reduced Akt activity in Tsc2-deficient neurons is CHOP independent, and, although down-regulated Akt might contribute to increased apoptosis in Tsc2-deficient neurons, knockdown of CHOP alone is sufficient to reduce both cell death and oxidative stress.

**In vivo identification of stress response in brains from Tsc1−/− SynCre+ mice and in the tuber of a TSC patient**

Our data demonstrated increased oxidative stress in Tsc2-deficient hippocampal cultures in vitro. To determine whether a similar stress response occurs in vivo, we assessed levels of expression for CHOP and HO-1 in total brain lysates from Tsc1−/− SynCre+ mice (neuronal Tsc1 knock-out) (Meikle et al., 2007). Tsc1−/− SynCre+ mice experience near-complete loss of Tsc1 expression in neurons and display neurological decline with median survival of 35 d. Both the neurological abnormalities and the median survival are markedly improved when mutant mice are treated with rapamycin from P7 to P33 (Meikle et al., 2008). To investigate ER stress responses in vivo, we used Tsc1−/− SynCre+ mice either untreated or treated with rapamycin. Both CHOP and HO-1 protein levels were increased in Tsc1−/− SynCre+ brain lysates and were reduced in mice treated with rapamycin (Fig. 6A,B). Interestingly, Tsc1−/− SynCre+ mice taken off rapamycin after 3 weeks of treatment showed recurrence of HO-1 expression (supplemental Fig. 6, available at www.jneurosci.org as supplemental material).

To extend these findings at the cellular level, immunohistochemical analysis for HO-1 was performed on control and Tsc1−/− SynCre+ brains. Costaining with phospho-S6 antibody was used to identify neurons with increased mTOR activity. Phospho-S6-positive dysplastic cells identified in the hippocampus and the red nucleus of Tsc1−/− SynCre+ brains were also positive for HO-1 (Fig. 6C,D). Furthermore, rapamycin treatment decreased phospho-S6 staining and HO-1 expression in Tsc1−/− SynCre+ brains to levels comparable with controls (Fig. 6D).

To determine whether our findings could be extended to human TSC disease, we performed immunohistochemical analysis on sections from a tuber of a 4-year-old TSC patient. Giant cells with increased mTOR activity were identified in the human tuber by phospho-S6 antibody staining (Fig. 6E). CHOP and HO-1 colabeling was observed in 44% (80 of 115 counted) and 57% (70 of 123 counted) of the phospho-S6-positive cells, respectively. No CHOP or HO-1 labeling was observed in the perituber brain regions (Fig. 6F) or in the brain of a non-TSC patient with focal dysplasia (Fig. 6G). In agreement with previous reports, balloon cells that are typically found in focal dysplasias showed some phospho-S6 and SM1-311 staining (Lurton et al., 2002; Baybis et al., 2004). Together, these findings strongly suggest that the ER...
Figure 5. Silencing of CHOP expression in GL3-Sh and Tsc2-Sh neurons. A, B, Control and Tsc2-Sh neurons infected with CHOP-C and CHOP-Sh RNAi lentivirus. Neurons were left untreated (−), treated with DMSO-vehicle (D), or treated with 0.5 μM Tg for 24 h. Total RNA and protein lysates were prepared for RT-PCR (A) and Western blot analysis (B), respectively. A, CHOP knockdown reduces oxidative stress response as shown by HO-1 expression at the RNA level. A downstream target of CHOP, advillin (Wang et al., 1998), was used to confirm inhibition of CHOP activity, and β-actin was used as a loading control. B, In Tsc2-deficient neurons, CHOP-Sh RNAi abrogates HO-1 expression and partially rescues cell death. Increased S6 phosphorylation, as detected by Western blot analysis, confirms mTOR activation in Tsc2-deficient neurons. Total Akt confirms equal loading.

Discussion

Despite recent progress identifying the genetic mutations and the signaling pathways associated with TSC pathology, the pathogenesis of the diverse neurological symptoms present in this disease remain poorly understood, and treatments are elusive. Here, we demonstrate that Tsc deficiency correlates with the upregulation of specific stress-related cellular responses both in vitro and in vivo (summarized in Fig. 6F). First, we detected ER overload and oxidative damage in Tsc2-deficient hippocampal neurons, in brains from Tsc1<sup>−/−</sup> Syn<sup>Cre</sup> mice and in human TSC tissue. Second, we demonstrated that these cellular abnormalities are the consequence of constitutive mTOR activation because rapamycin treatment abolished stress responses both in vitro and in vivo. Third, we showed that neuronal stress responses in vitro increased vulnerability to cell death via activation of the mitochondrial death pathway and that silencing CHOP reduced apoptosis. The identification of similar stress responses in primary rodent hippocampal neurons with nearly complete Tsc2 gene silencing and in the human TSC brain highlight the damaging neuronal responses that result from mTOR hyperactivity.

We have shown recently that components of the TSC/mTOR pathway are differentially localized during the development of neuronal polarity, as defined by the elaboration of a single neuron and multiple dendrites (Choi et al., 2008). This fine regulation of TSC activity in neurons during the neuronal polarization process, together with the identification of multiple axon formation in neurons lacking Tsc, indicates a critical role for the TSC/mTOR pathway in axonal specification and connectivity. These findings, along with the identification of a critical role for TSC pathway in dendritic structure (Tavazoie et al., 2005), have highlighted the neuronal defects contributing to the neurological symptoms. In vivo studies using knock-out mice have indeed shown that loss of TSC in neurons correlates with anatomical brain abnormalities and neurological defects (Meikle et al., 2007).

In previous reports, loss of TSC1/TSC2 complex has been implicated in increased ER stress in MEFs from Tsc1 and Tsc2 knock-out mice (Ozcan et al., 2008). Al-
though MEFs display increased expression of the ER stress chaperone GRP78 and activation of the PERK signaling pathway, we did not detect any changes in GRP78 protein level (Fig. 3A) (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) or PERK activation (data not shown) in neurons. Such differences may represent cell-type-specific responses. Our study suggests that neurons lacking Tsc have a basal activation of CHOP via the canonical ATF4 pathway (Fawcett et al., 1999; Harding et al., 2000; Ma et al., 2002). Because many extrinsic factors such as hypoxia, hypoglycemia, and exposure to natural and experimental toxins can lead to ER stress (Koumenis, 2006; Zhang and Kaufman, 2006), Tsc-deficient neurons are more likely to be vulnerable to such insults.

Implications of ER stress for neurological manifestations of TSC

Epilepsy is by far the most common medical condition associated with TSC, occurring in 80–90% of patients. The relationship between ER stress and epilepsy is just starting to be investigated. For example, kainate-induced seizures in rats and depolarization in cultured rat hippocampal neurons lead to ER stress (Sokka et al., 2007). Moreover, increased UPR has been observed in hippocampi resected from patients with temporal lobe epilepsy (Yamamoto et al., 2006). Finally, ER overload attributable to abnormal trafficking of misfolded proteins has been proposed to occur in several epilepsy-related “channopathies” (Hirose, 2006). Together,
these findings indicate that seizures can exacerbate ER stress and underlying ER stress could potentially contribute to seizures by misfolding of synaptic proteins. This is particularly important in TSC disease because the vast majority of patients experience seizures and many of the cases are medically intractable. A better understanding of the relationship between the TSC/mTOR pathway, ER stress, and seizures may help to uncover novel therapies for intractable epilepsy in patients.

Implications of oxidative damage in Tsc mutant brains

Accumulating evidence has revealed a crosstalk between ER and oxidative stress responses, such that excessive ROS production can contribute to UPR induction and vice versa (Yokouchi et al., 2008). For instance, UPR-regulated genes can create an imbalance in the cellular redox status and release free radicals such as superoxide anions, leading to damage of ER-resident proteins (Verkhratsky and Petersen, 2002). The combined cellular insult that may arise from ER and oxidative stress has been proposed to further contribute to cell death by increasing the accumulation of ROS (Haynes et al., 2004). Our identification of an altered redox balance in Tsc-deficient neurons is consistent with previous reports of increased basal and growth factor-stimulated ROS in Tsc2+/− MEs (Finlay et al., 2005). In Tsc-deficient neurons, we found that silencing of CHOP was sufficient to reduce the oxidative stress response, thus indicating a tight-linked connection between these two cellular stress pathways.

The brain is highly sensitive to oxidative stress, which has been correlated with the pathogenesis of several neurological disorders (Reynolds et al., 2007). In particular, the cellular toxicities resulting from oxidative stress, such as massive calcium overload, energy depletion, and ROS production, are thought to affect neuronal function by lowering the cellular capacity to respond to stress. A number of studies have suggested that, although the response to stress offers homeostatic control of cellular function, a prolonged stress response itself can be toxic (Kaufman, 2002). For example, the expression of HO-1, the rate-limiting enzyme for the degradation of the heme, is overall considered to be beneficial (Schipper, 2004b). However, excessive HO-1 can also contribute to the increase of carbon monoxide (CO) and/or free iron levels, which can have toxic effects on mitochondrial function (Ryter and Tyrrell, 2000; Barañano and Snyder, 2001; Schipper, 2004a). Expression of HO-1 in phospho–S6-positive ectopic neurons of Tsc1Pagger–SynCre− mice and in the dysplastic cells of human tubers indicates a concomitant increase in mTOR activity and occurrence of oxidative stress in vivo. Importantly, mTOR inhibition in vivo reduced CHOP and HO-1 expression. mTOR inhibitors have already been successfully used in several brain-specific TSC mouse models and shown to efficiently improve survival and neurological phenotypes (Ehnninger et al., 2008; Meikle et al., 2008; Zeng et al., 2008). These results together with our study addressing the molecular targets affected by rapamycin treatment in vivo provide new insights into the cellular basis of the neuronal dysfunction in TSC.

In the future, it will be important to investigate the damaging effects that could arise from HO-1 overproduction in the TSC brain, such as iron deposition or CO accumulation (Patel et al., 1996). For instance, a highly detrimental effect of iron deposition is the accumulation of free iron in the mitochondria as a result of increased oxidative stress. This would eventually cause mitochondrial dysfunction and energy production failure, affecting several ATP-dependent processes such as the uptake of excitotoxic neurotransmitters (Beal, 1998; Trushina and McMurray, 2007). Such neuronal insults can contribute to glutamate excitotoxicity, which has been implicated in the etiology of seizure-related disorders (Patel, 2002; Schipper, 2004b). In addition, excess CO production could cause dysfunction in synaptic plasticity and consequently lead to defects in cognitive development (Stevens and Wang, 1993; Zhuo et al., 1993). Similarly, the increased ERO1α expression identified in Tsc-deficient neurons could potentially exacerbate neuronal function by enhancing ROS production (Harding et al., 2003; Marciniak et al., 2004). Therefore, the combination of ER and oxidative stress detected in Tsc-deficient neurons may contribute not only to epilepsy but also to neurodevelopmental disabilities in TSC patients.

TSC1/TSC2 as key regulators of cellular stress responses

Here we demonstrate a dynamic regulation of TSC1/TSC2 complex activity downstream of the PI3K/Akt pathway in cells under ER stress. We found that, under short exposure to ER stress, both Akt and mTOR are active, whereas the TSC1/TSC2 complex is inhibited. In contrast, under persistent ER stress, mTOR is inhibited in a Tsc-dependent manner. When cells undergo ER stress, the UPR is initially activated as part of a cellular protective mechanism to circumvent ER overload and reestablish proper ER function (Zhang and Kaufman, 2006). However, if ER stress persists, it results in cell death. Thus, cellular fate under ER stress is a balance between survival and apoptotic signals. Previous studies in cell lines from breast, lung, and prostate cancer have shown that the PI3K/Akt pathway can be differentially regulated to modulate cellular ER stress (Hu et al., 2004; Hosoi et al., 2007). We now show the TSC1/TSC2 plays a crucial role in the regulation of ER stress by PI3K/Akt. It is generally thought that the PI3K/Akt pathway is regulated by extrinsic signals such as the activation of growth factor receptor tyrosine kinases. Our data indicate that cells under stress can intrinsically modulate the Akt/TSC/mTOR pathway. Recent work has shown that neuronal injury, such as axotomy, can suppress mTOR and decrease protein synthesis (Park et al., 2008). Based on our findings, one possible mechanism underlying this effect may be through ER-stress mediated regulation of Akt and TSC. Exploring the cell-intrinsic upstream mechanisms regulating Akt/TSC/mTOR pathway will be important in understanding the biology of cellular stress and TSC disease. This has implications not only for TSC but also for the spectrum of neurological disorders in which either genetic mutations or environmental insults perturb ER function.

References


References


