Aberrant mitochondrial fission in neurons induced by protein kinase Cδ under oxidative stress conditions in vivo

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INTRODUCTION

Mitochondria are critical for cell survival; mitochondrial dysfunction reduces ATP production, impairs calcium homeostasis, and enhances generation of reactive oxygen species (ROS), which, if left unchecked, can lead to cell death (DiMauro and Schon, 2008). Mitochondria are highly dynamic organelles that constantly change shape and number by fusion and fission in response to different stimuli and to changes in metabolic demands of the cell (Chen and Chan, 2005; Chan, 2006). These mitochondrial dynamic processes are required to preserve proper functioning of the cells; they enable mitochondrial recruitment to critical subcellular compartments, content exchange between mitochondria, control of mitochondrial shape and number, mitochondrial communication with the cytosol, and mitochondrial quality control (Chen and Chan, 2009). Neurons are particularly sensitive to changes in mitochondrial dynamics due to their high energy demands (Chen and Chan, 2009), and recent studies have highlighted a causal role of impaired mitochondrial dynamics (fusion and fission) in neuronal dysfunction and death (Frank et al., 2001; Youle and Karbowski, 2005; Bansour et al., 2006; Cheung et al., 2007). Therefore drugs that correct aberration in mitochondrial dynamics may serve as new therapeutics for diverse neurological diseases.

At least two proteins—dynamin-related protein 1 (Drp1) and the mitochondrial outer membrane protein Fis1—are required for mitochondrial fission in mammalian cells (Labrousse et al., 1999; Smirnova et al., 2001; James et al., 2003; Yoon et al., 2003). Similar to other membrane mechanoenzymes, Drp1 is a large GTPase located mostly in the cytosol. Upon activation, a pool of Drp1 translocates to the mitochondria, where it binds to Fis1, thus assembling future fission sites; Drp1 then enables severing of the mitochondrial

ABSTRACT

Neuronal cell death in a number of neurological disorders is associated with aberrant mitochondrial dynamics and mitochondrial degeneration. However, the triggers for this mitochondrial dysregulation are not known. Here we show excessive mitochondrial fission and mitochondrial structural disarray in brains of hypertensive rats with hypertension-induced brain injury (encephalopathy). We found that activation of protein kinase Cδ (PKCδ) induced aberrant mitochondrial fragmentation and impaired mitochondrial function in cultured SH-SY5Y neuronal cells and in this rat model of hypertension-induced encephalopathy. Immunoprecipitation studies indicate that PKCδ binds Drp1, a major mitochondrial fission protein, and phosphorylates Drp1 at Ser 579, thus increasing mitochondrial fragmentation. Further, we found that Drp1 Ser 579 phosphorylation by PKCδ is associated with Drp1 translocation to the mitochondria under oxidative stress. Importantly, inhibition of PKCδ, using a selective PKCδ peptide inhibitor (δV1-1), reduced mitochondrial fission and fragmentation and conferred neuronal protection in vivo and in culture. Our study suggests that PKCδ activation dysregulates the mitochondrial fission machinery and induces aberrant mitochondrial fission, thus contributing to neurological pathology.
PKCδ induces excessive mitochondrial fission

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RESULTS

PKCδ inhibition reduces mitochondrial ultrastructural damage in hypertensive rat brains

We induced pathological hypertension by keeping Dahl salt-sensitive (DS) rats on an 8% high-salt diet. These rats developed HTNE between the ages of 11 and 15 wk (Qi et al., 2008). Major neurological symptoms included seizures, head and forelimb repetitive twitching, forelimb and hind limb paralysis, and severe lethargy (Qi et al., 2008). To determine the extent of neuronal damage in these rat brains, we used transmission electron microscopy (TEM) and examined brain cortex sections of hypertensive rats treated with the PKCδ inhibitor 8V1-1 or with the control peptide carrier TAT47-57, a component of 8V1-1 that is used for the delivery of the peptide across cell membranes (Chen et al., 2003). The mitochondria were scored and accounted as tubular/connected versus fragmented by a viewer blinded to treatment group. At least

Quantitative data from three rats in each group are provided in the histogram. *p < 0.05 vs. normotensive rats (Nor); # p < 0.05 vs. TAT-treated hypertensive rats. (D) Human SH-SY5Y cells were treated with control peptide TAT or 8V1-1-TAT (1 µM each), 15 min before treatment with H2O2 (200 µM) or Ang II (1 µM). The levels of Drp1 and PKCδ in the mitochondrial fractions were determined in three independent experiments. VDAC and Tom20 were used as internal loading controls. *p < 0.05 vs. control cells; #p < 0.05 vs. TAT-treated cells.

Note that either TAT or 8V1-1 treatment in control rats has no effect on mitochondrial structure (unpublished data). (B) The percentage of fragmented mitochondria relative to the total number of mitochondria (tubular and fragmented mitochondria) is presented as the mean ± SE of three rats in each group. *p < 0.05. At least 16 fields of cerebral cortex in each rat were analyzed, and more than 500 of mitochondria in each animal were scored and accounted. (C) Mitochondrial and cytosolic fractions of cerebral cortex were subjected to Western blot analysis. The levels of Drp1 and PKCδ in the mitochondrial and cytosolic fractions were determined. VDAC and enolase (markers of mitochondria and cytosol, respectively) were used as internal loading controls. Quantitative data from three rats in each group are provided in the histogram. *p < 0.05 vs. normotensive rats (Nor); #p < 0.05 vs. TAT-treated hypertensive rats. Major neurological symptoms included seizures, head and forelimb repetitive twitching, forelimb and hind limb paralysis, and severe lethargy (Qi et al., 2008). To determine the extent of neuronal damage in these rat brains, we used transmission electron microscopy (TEM) and examined brain cortex sections of hypertensive rats treated with the PKCδ inhibitor 8V1-1 or with the control peptide carrier TAT47-57, a component of 8V1-1 that is used for the delivery of the peptide across cell membranes (Chen et al., 2003). The mitochondria were scored and accounted as tubular/connected versus fragmented by a viewer blinded to treatment group. At least

membranes through a GTP hydrolysis-dependent mechanism (Smirnova et al., 2001; James et al., 2003; Yoon et al., 2003). Cell culture studies demonstrated that excessive mitochondrial fission is associated with apoptosis, neuronal dysfunction, and cell death (Frank et al., 2001; Youle and Karbowiak, 2005; Barsoum et al., 2006; Cheung et al., 2007). Inhibition of Drp1 by either expressing a Drp1 dominant mutant or RNA interference leads to increased length and interconnectivity of mitochondrial tubules, thereby inhibiting the fission process and preventing cell death (Frank et al., 2001; Jagasia et al., 2005). Recently, an infant patient with a dominant negative Drp1 allele (Waterham et al., 2007) and mice lacking Drp1 (Ishihara et al., 2009; Wakabayashi et al., 2009) were found to have a wide range of brain developmental abnormalities. These findings collectively suggest a critical role of mitochondrial fission in the central nervous system (CNS). However, the signaling enzymes that regulate mitochondrial dynamics, the mechanisms by which excessive mitochondrial fragmentation and dysfunction are induced, and the roles of these processes in human diseases have not been identified.

Hypertensive neuroencephalopathy (HTNE) is a neurological disease associated with cognitive and physical disabilities that can lead to death in patients with severe hypertension (Schwartz, 2002). We recently reported that a 4-wk treatment of hypertensive rats with 8V1-1, a protein kinase Cδ (PKCδ)–selective peptide inhibitor (Chen et al., 2001a), reduced mortality from 50% in control-treated hypertensive rats to 8% (Qi et al., 2008). We showed that 8V1-1 treatment did not reduce blood pressure, but it improved blood-brain barrier (BBB) function (Qi et al., 2008). Notably, we observed excessive mitochondrial fission and fragmentation, which was associated with ultrastructural damage of the organelle in brains of hypertensive rats with HTNE symptoms as well as in the brains of humans who died of hypertension-induced neurological complications. In the animal model, these mitochondrial aberrations were prevented when the animals were treated with the PKCδ peptide inhibitor. Here we set out to identify the molecular basis of PKCδ-mediated regulation of mitochondrial fission and its role in neuropathogenesis.

FIGURE 1: Inhibition of PKCδ reduces excessive mitochondrial fission induced by HTNE. (A) Top: Male Dahl salt-sensitive (DS) rats were fed a high-salt diet (8% NaCl) from 6 to 15 wk of age and treated for 4 wk (beginning at age 11 wk) with either the control cell permeable carrier peptide, TAT47-57, or the PKCδ-inhibitor (8V1-1 coupled to TAT for cell delivery), using subcutaneously implanted osmotic Alzet pumps that deliver 1.0 mg·kg·d. Bottom: Transmission electron microscope (TEM) images of cerebral cortex samples from 13-wk-old control and hypertensive rats with HTNE. Scale bar is 0.2 µm. Arrowhead indicates mitochondria undergoing fission. The arrow indicates fragmented mitochondria. The data are representative EM imaging. Note that either TAT or 8V1-1 treatment in control rats has no effect on mitochondrial structure (unpublished data). (B) The percentage of fragmented mitochondria relative to the total number of mitochondria (tubular and fragmented mitochondria) is presented as the mean ± SE of three rats in each group. *p < 0.05. At least 16 fields of cerebral cortex in each rat were analyzed, and more than 500 of mitochondria in each animal were scored and accounted. (C) Mitochondrial and cytosolic fractions of cerebral cortex were subjected to Western blot analysis. The levels of Drp1 and PKCδ in the mitochondrial and cytosolic fractions were determined. VDAC and enolase (markers of mitochondria and cytosol, respectively) were used as internal loading controls. Quantitative data from three rats in each group are provided in the histogram. *p < 0.05 vs. normotensive rats (Nor); #p < 0.05 vs. TAT-treated hypertensive rats. (D) Human SH-SY5Y cells were treated with control peptide TAT or 8V1-1-TAT (1 µM each), 15 min before treatment with H2O2 (200 µM) or Ang II (1 µM). The levels of Drp1 and PKCδ in the mitochondrial fractions were determined in three independent experiments. VDAC and Tom20 were used as internal loading control. *p < 0.05 vs. control cells; #p < 0.05 vs. TAT-treated cells.

 noteworthy for its role in neuropathogenesis.
PKCδ inhibition prevents HTNE-induced excessive mitochondrial fission

Translocation of Drp1 to the mitochondria is required for the division (fission) of this organelle (Labrousse et al., 1999; Smirnova et al., 2001). To confirm our observations from TEM, we determined the subcellular localization of Drp1 in hypertensive rat brains. Western blot analysis revealed a threefold increase in the level of Drp1 in mitochondria-enriched fractions of hypertensive rat brains treated with control peptide TAT. (Note: We previously determined that TAT has no biological effects in various tide TAT. (Note: We previously determined extensive rat brains treated with control peptide, mitochondria-enriched fractions of hypertensive rat brains. Western blot analysis revealed a localization of Drp1 in hypertensive rat brains. The number of fragmented mitochondria was increased threefold as compared with that in the normotensive rat brains (Figure 1B). Moreover, mitochondria undergoing fission as well as mitochondria containing fewer cristae were evident. These mitochondrial ultrastructural abnormalities were greatly reduced in the δV1-1-treated hypertensive rats (Figure 1, A, right panel, and B).

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Because the effect of δV1-1 in vivo could reflect the overall protection of the BBB in the hypertensive rats (Qi et al., 2008) rather than a direct effect of PKCδ on the neurons, we next determined the effect of PKCδ inhibition on mitochondrial structure in cultured human neuroblastoma SH-SYSY cells. Similar to our findings in vivo, we found that in SH-SYSY cells treated with hydrogen peroxide (H2O2, an oxidative stress inducer) or with angiotensin II (Ang II, a hormone that is elevated under hypertensive conditions), the translocation of both PKCδ and Drp1 from cytosol to the mitochondria was blocked by δV1-1 treatment (Figure 1D) without affecting total levels of the two proteins (data not shown). In contrast, in glial CCF-1 cells (where Drp1 and PKCδ levels are very low), there was no change in the mitochondrial morphology in these conditions (Supplemental Figure 2). Thus there are likely distinct regulatory pathways for mitochondrial fission in different cell types. Together, these data suggest that Drp1 is overactivated in response to stimuli related to hypertensive brain injuries and that association of Drp1 with the mitochondria might be regulated by PKCδ activation.

![Figure 2](image-url)
PKCδ mediates mitochondrial fission by interacting with Drp1

Because 8V1-1 abolished Drp1 translocation to the mitochondria in hypertensive rats, we next determined whether Drp1 and PKCδ interact directly. To minimize potential nonspecific interactions, mitochondria isolated from rat brains were solubilized with detergent (1% Triton X-100) before immunoprecipitation with anti-Drp1 antibodies. We found that Drp1 associated with PKCδ in the mitochondrial fractions from hypertensive rat brains treated with the control peptide TAT, but not from hypertensive rats treated with 8V1-1 (Figure 2A, left panel). Conversely, PKCδ and Drp1 coimmunoprecipitated from the cytosolic fractions of normotensive rat brains, but this association decreased in hypertensive rat brains treated with TAT (Figure 2A, right panel). Note that 8V1-1 treatment decreased the levels of PKCδ/Drp1 complex even in the cytosolic fractions (Figure 2) and that 8V1-1 alone did not disrupt the interaction between PKCδ and Drp1 under normal conditions (Supplemental Figure 3). Because the total levels of both proteins do not change (Supplemental Figure 1), it is possible that 8V1-1 treatment might trigger translocation of the complex to another cellular compartment, the identity of which has yet to be determined.

We found that Drp1 association with PKCδ was specific; PKCe, another PKC isozyme that is highly expressed in the brain, did not coimmunoprecipitate with Drp1 in either fraction (Figure 2A). Moreover, Drp1 did not bind to the mitochondrial inner membrane protein, adenine nucleotide translocator (ANT), or the mitochondrial matrix protein glucose-regulated protein 75 (GRP75), although it was slightly associated with the mitochondrial outer membrane protein voltage-dependent anion channel (VDAC) (Figure 2B). Thus it appears that PKCδ and Drp1 selectively interacted in the cytosol and that this complex translocates to the mitochondria in response to hypertensive brain injury. A reduced association of the PKCδ/Drp1 complex with mitochondria in the presence of the PKCδ inhibitor 8V1-1 was then confirmed in the cultured SH-SYSY neurons exposed to H2O2 and Ang II (Figure 2C).

Further, we found that inhibition of PKCδ by 8V1-1 treatment blocked phosphorylation of mitochondrial-associated Drp1 on serine/threonine residues in hypertensive rat brains and in vitro–cultured SH-SYSY cells (Figure 3A, left panel). However, we did not find increased phosphorylation of Drp1 in the cytosolic fractions in response to HTNE (Figure 3A, right panel). Next, an in vitro kinase assay confirmed that recombinant PKCδ can directly phosphorylate recombinant Drp1 in the presence of PKC activators (Figure 3B).

PKCδ phosphorylation of Ser 579 in the variable domain of Drp1 is required for Drp1-mediated fission of mitochondria

We mapped the PKCδ phosphorylation site on Drp1. Mass spectroscopy analysis revealed that Ser 579 of Drp1 is the only site that is phosphorylated by PKCδ in the presence of the PKC activators (Figure 3C and Supplemental Figure 4). This Ser site is highly conserved among species (Figure 3C), and it is located at the tip of the variable domain of Drp1 (Figure 3, C and D), which renders this site more accessible for phosphorylation.

Next, using a specific phospho-antibody for Drp1 (anti-Drp1 phospho-Ser 616; note that Ser 616 in Drp1 isomorf 1 corresponds to Ser 579 in Drp1 isomorf 3) (Figure 3C), we found that inhibition of PKCδ by the peptide inhibitor 8V1-1 inhibited Drp1 Ser 579 phosphorylation in the mitochondrial fractions isolated from brains of rats with HTNE (Figure 4A) or cultured SH-SYSY cells exposed to H2O2 (Figure 4B). Similarly, down-regulation of PKCδ by small interfering RNA (siRNA) abolished Drp1 phosphorylation at the mitochondria of cultured cells (Figure 4B). Cyclin-dependent kinase 1 (CDK1) has
been reported to phosphorylate Drp1 at the same site as the one reported here (Taguchi et al., 2007). However, treatment with a specific CDK1 inhibitor, R03306 (9 µM), did not block Drp1 Ser 579 phosphorylation in mitochondrial fractions isolated from cells exposed to H₂O₂, although it abolished the phosphorylation of histone H3 (Supplemental Figure 5), a substrate of CDK1 (Lee and Song, 2008).

To further determine the functional importance of Ser 579 phosphorylation, we generated a Ser-579-to-alanine mutant of Drp1 (Drp1 S579A). We first reduced the levels of the endogenous Drp1 using siRNA in SH-SY5Y cells, followed by transfection of either wild-type Drp1 (Drp1 wt) or mutant Drp1 (Drp1 S579A; Figure 4C, left panel). Treatment with H₂O₂ led to an increase in Ser 579 phosphorylation of Drp1 at the mitochondria of the cells expressing Drp1 wt, whereas Drp1 phosphorylation under the same conditions failed to occur in cells expressing the mutant Drp1 S579A (Figure 4C, right panel). Moreover, the translocation of Drp1 to the mitochondria in cells expressing Drp1 S579A was abolished in response to H₂O₂, when compared with that in the cells expressing Drp1 wt. Furthermore, in vitro phosphorylation of Drp1 by PKCδ was significantly reduced in Drp1 S579A mutant compared with that in Drp1 wt (Figure 4C, left panel). These data strongly support that phosphorylation of Drp1 by PKCδ occurs predominantly at Ser 579.

Next, we found that the expression of the Drp1 S579A mutant reduced H₂O₂ and Ang II–induced mitochondrial fragmentation in the SH-SY5Y cells. In cells treated with control siRNA and in cells expressing Drp1 wt, treatment with H₂O₂ and Ang II for 6 h resulted in fragmented mitochondria (Figure 5, A, top, and B). siRNA of Drp1 decreased mitochondria fragmentation (Figure 5A). Similarly, cells expressing Drp1 S579A reduced mitochondrial fragmentation after exposing to both stressors (Figure 5, A, bottom, and B; see cells framed with a dashed line). Taken together, these data demonstrate that Drp1 is a substrate of PKCδ and that phosphorylation of Drp1 at Ser 579 is required for Drp1-mediated mitochondrial fission.

Translocations of PKCδ and Drp1 to the mitochondria are interdependent

To directly determine whether the translocations of PKCδ and Drp1 to the mitochondria are dependent on each other, we reduced the cellular levels of PKCδ or Drp1 by transfecting human neuronal SH-SYSY cells with siRNA for PKCδ or for Drp1, respectively (Figure 6A).

Translocation of Drp1 to the mitochondria after 30 min of Ang II treatment was abolished when PKCδ levels were reduced by PKCδ siRNA, as compared with control siRNA–transfected cells (Figure 6B and C). Conversely, PKCδ translocation to the mitochondria was completely abolished in cells expressing Drp1 siRNA when exposed to the same treatment (Figure 6, B and C). These data show that Drp1 and PKCδ are both required for the translocation of either protein to the mitochondria, further supporting our findings that PKCδ and Drp1 move to the mitochondria as a preformed complex.

PKCδ and Drp1 complex is associated with mitochondrial fragmentation, leading to neuronal cell death in response to stimuli related to HTNE

To determine the functional consequence of PKCδ- and Drp1-mediated mitochondrial fission impairment, we next determined the mitochondrial morphology and neuronal cell death in cultured SH-SYSY cells treated with H₂O₂ and Ang II. As shown in Figure 6, consistent with previous studies (Barsoum et al., 2006; Cheung et al., 2007; Han et al., 2008; Brooks et al., 2009), mitochondria in control cells were filamentous with a tubular or threadlike staining pattern. The mitochondria in these cells were interconnected to form a reticulum. However, increased oxidative stress in the cultured cells by Ang II or H₂O₂ treatments disrupted the mitochondrial network, and these mitochondria were fragmented into short rods or spheres (Figure 7A). Further, the number of cells with fragmented mitochondria was increased by more than 10-fold at 6 h of Ang II or H₂O₂ treatment (Figure 7B). Importantly, inhibition of PKCδ (by 8V1-1 treatment; Figure 7, A and B) or decreased levels of Drp1 (by siRNA treatment; Figure 7C) significantly reduced mitochondrial fragmentation under the same conditions. Finally, we found that inhibiting PKCδ or knocking down Drp1 increased cell survival in response to Ang II or H₂O₂ treatment (Figure 7, D and E).

DISCUSSION

In this study, we demonstrated for the first time that PKCδ is a critical regulator of mitochondrial fission in a CNS disease. We show that PKCδ activation impairs neuronal mitochondrial morphology and increases
neuronal cell death, at least in part, by inducing Drp1-dependent fission and fragmentation of the mitochondria under oxidative stress conditions (Figure 8).

Hypertension, which can cause vascular dementia in humans (Moretti et al., 2008), has been found to lead to oxidative stress and increased ROS production in neuronal cells (Iadecola and Davison, 2008), which trigger PKCδ activation (Cieslak and Lazou, 2007; Qi et al., 2009). In a previous study, we showed that inhibition of PKCδ by sustained treatment with V1-1 for 4 wk increases survival of hypertensive rats with HTNE symptoms partly by reversing BBB failure (Qi et al., 2008). Here we identified an additional PKCδ-mediated pathological mechanism, involving mitochondrial fission impairment in the neurons of hypertensive rat brains. Further, the selective mitochondrial damage in neurons (and lack of effect on glia, for example; Supplemental Figure 2) is consistent with the observation that neurons are particularly vulnerable to changes in mitochondrial dynamics because of their unique requirement for high levels of energy (Chen and Chan, 2005; Cheung et al., 2007; Knott et al., 2008). Because prolonged treatment of normal rats with the PKCδ peptide inhibitor has no apparent adverse effects in any organ, including the CNS (Qi et al., 2008), and because treatment with V1-1 in neurons under basal conditions has no effect on mitochondrial morphology (Figure 7, A and B), it is possible that PKCδ inhibition may provide a new means to reduce mitochondrial dysfunction and the resulting neuronal injury in hypertensive subjects.

In this study, we reported that PKCδ-mediated phosphorylation of Drp1 at Ser 579 promotes mitochondrial fragmentation under pathological conditions related to hypertensive brain injury. Phosphorylation of Drp1 at this site (Ser 579) by Cdk1 in the early mitotic phase of HeLa cells has a similar effect (Taguchi et al., 2007). Because neurons are postmitotic cells and the levels of Cdk1 in these cells are very low (Gompel et al., 2004), Cdk1 is unlikely to mediate mitochondrial fission under these pathological conditions. Indeed, we found that treatment with a CDK1 inhibitor has no effect on Drp1 phosphorylation under oxidative stress in cultured SH-SY5Y cells (Figure 4B and Supplemental Figure 5), further supporting our hypothesis that PKCδ-induced Drp1 phosphorylation is likely to be a distinct pathway from that of CDK1. In addition, calcium/calmodulin-dependent kinase I (CaMKI)–mediated Drp1 phosphorylation at another serine, Ser 600, was found to induce mitochondrial fission in neurons in response to high potassium (Han et al., 2008). Phosphorylation of Drp1 by cAMP-dependent protein kinase A at the same residue (ser 600) as CaMKI appears to have opposing effects on mitochondrial morphology, as seen in PC12 cells (Cribbs and Strack, 2007; Cereghetti et al., 2008). However, none of these studies determined the role of that protein kinase on mitochondrial function and neuronal integrity in animal models, as we report in the current study. If these kinases modify disease state in vivo, it should be determined whether they act synergistically to regulate mitochondrial dynamics under pathological conditions.

Because mitochondrial morphology is regulated by a balance between fission and fusion (Chan, 2006), we cannot rule out the possibility that hypertension-induced mitochondrial fragmentation is associated with a disruption of mitochondrial fusion as well. However, using a variety of biochemical and molecular biological tools, we concluded that the fission process may be the main target of activated PKCδ in the mitochondria in response to oxidative stresses. We showed that activated PKCδ directly binds to and phosphorylates Drp1 in the mitochondria of neuronal cells, leading to mitochondrial fragmentation and subsequent neuronal cell death. Thus, the mitochondrial fission impairment induced by HTNE in a hypertensive rat model is, at least in part, due to activation of PKCδ.

Because mitochondrial dynamics is necessary for neuronal functions such as synaptic maintenance (Li et al., 2004), neuronal energy generation (Barsoum et al., 2006), and brain development (Waterham et al., 2007; Ishihara et al., 2009), aberrant mitochondrial fission...
over time could lead to greater mitochondrial dysfunction and to
deficits, which in turn impair neuronal dysfunction. Cell cul-
ture studies have recently suggested that impaired mitochondrial
dynamics and excessive mitochondrial fission are connected to a
number of neurological diseases, such as Parkinson’s diseases (Deng
et al., 2009). Therefore PKCδ, 2009). Our findings of aberrant mitochondrial fission in neu-
rons of hypertensive rats and its dependence on PKCδ activation suggest that PKCδ is a component of the mitochondrial fission machinery, at least under pathological conditions. It also provides the first evidence that the signal pathway could regulate mitochondrial dynamics in an animal model of neurological disorder. Thus PKCδ-induced aberrant mitochondrial fission may represent a common mechanism contributing to the pathology of these diseases. Moreover, analysis of human brains supports our findings that impaired mitochondrial dynamics is associated with brain disorders (Cho et al., 2009). Therefore a PKCδ-selective inhibitor, such as 8V1-1, may be a useful treatment for the diseases in which impairment of mitochondrial dynamics occurs.

MATERIALS AND METHODS

Materials

Ang II, hydrogen peroxide, phospholipids, protease inhibitor cock-
tail, and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Glutathione S-transferase (GST)–Drp1
was from Abnova (Walnut, CA), GST-PKC δ was from Cell Signaling Biotechnology (Danvers, MA), and antibodies for PKCδ, PKCε, Tom20, GRP75, and enolase were from Santa Cruz Biotechnology (Santa Cruz, CA). Drp1 (DLP1) was from BD Biosciences (Rockville, MD). VDAC was from MitoSciences (Eugene, OR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) anti-
tibody, clone 6C5, was from Advanced Immunochemical (Long Beach, CA). Antibodies to phosphorylated serine and thre-
onine and Drp1 phospho-Ser616 were from Cell Signaling Biotechnology. Anti-
mouse immunoglobulin G (IgG) and anti-rabbit IgG, peroxidase-linked species-
specific antibodies, were from GE Healthcare (Piscataway, NJ). The PKCδ-specific antagonist peptide 8V1-1 (PKCδ inhibitor, amino acids 8–17) was synthe-
sized by American Peptide (Sunnyvale, CA) and conjugated to TAT-carrier peptide (amino acids 47–57) via a cysteine-cysteine
S–S bond at their N termini, as previously described (Chen et al., 2001a).

Methods

Rat model of HTNE. As described in our
previous study (Qi et al., 2008), 4–5-wk-
old male DS rats were obtained from Harlan (Indianapolis, IN). The rats were fed with a high-salt diet containing 8% NaCl
from the age of 6 wk. Within a few days,
their blood pressure increased from
around 150 to 180 mm Hg (Payne and
Smeda, 2002; Qi et al., 2008). From the
age of 11 to 15 wk, the rats were treated
with the control carrier peptide TAT47–57 (Chen et al., 2001a) or
with 8V1-1 conjugated to TAT47–57 (1.0 mg/kg/d) using an osmotic
pump (Alzet Osmotic Pump, Palo Alto, CA) implanted subcutaneously on their backs. Major neurological findings and
symptoms included seizures, head and forelimb repetitive
 twitching behavior, forelimb and hind limb paralysis, and severe lethargy. If one of these symptoms occurred, they were regarded as a sign of HTNE. Animal protocols were approved by the
Stanford University Institutional Animal Care and Use Committee.

Cell culture. Human neuroblastoma SH-SY5Y cells were maintained
in DMEM (50%) and F12 medium (50%) supplemented with 10%
heat-inactivated fetal calf serum. All cultured cells were maintained
at 37°C in 5% CO2–95% air.

Drp1 structure predication. Structure modeling was conducted
using the I-TASSER server (Wu et al., 2007; Zhang, 2007, 2008), the
winner of the two latest Critical Assessment of Techniques for Protein Structure Prediction competitions. The high confidence score of 0
(ranging from −5 to 2) to the predicted Drp1 protein structure indicates the reliability of our model. PyMOL was applied to
subsequent graphics processing.

DNA construction. The wild-type Drp1 (human isoform 3) plasmid
was provided by Alexander M. van der Bliek (University of California,
Los Angeles) and Zheng Dong (Medical College of Georgia,

### Figure 6: PKCδ and Drp1 are interdependent. Human neuroblastoma SH-SY5Y cells were transfected with control siRNA, PKCδ siRNA, or Drp1 siRNA. After 48 h, cells were treated with Ang II (1 µM) for 30 min. (A) Total cell lysates were analyzed by Western blot to confirm knockdown of PKCδ and Drp1 in the mitochondrial fractions were analyzed by Western blot at the indicated groups. (B) The levels of PKCδ and Drp1 in the mitochondrial fractions were analyzed by Western blot at the indicated groups. (C) Histograms depicting the amount of Drp1 (left) and PKCδ (right) associated with the mitochondria of SH-SY5Y cells. The data are expressed as mean ± SE of three independent experiments. *p < 0.05, **p < 0.01 vs. control group; # p < 0.05, #p < 0.01 vs. Ang II-treated group.
confluence were transfected for 48 h with siRNA of Drp1, PKCδ, or control siRNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

**Mitochondrial isolation.** SH-SYSY cells were washed with cold phosphate-buffered saline (PBS) and incubated on ice in lysis buffer (250 mM sucrose, 20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, protease inhibitor cocktail, phosphatase inhibitor cocktail) for 30 min. Cells were scraped and then disrupted 10 times by repeated aspiration through a 25-gauge needle, followed by a 30-gauge needle. Brain tissue was minced and ground by pestle in lysis buffer. The homogenates were spun at 800 × g for 10 min at 4°C, and the resulting supernatants were spun at 10,000 × g for 20 min at 4°C. The pellets were washed with lysis buffer and spun at 10,000 × g again for 20 min at 4°C. The final pellets were suspended in lysis buffer containing 1% Triton X-100 and used as markers and loading controls.

**Immunofluorescence.** Cells cultured on eight-well glass chambers were washed with cold PBS fixed in 4% formaldehyde. For mitochondrial staining, the cells were incubated with 100 nM Mitotracker (Invitrogen) for 30 min at 37°C and imaged by fluorescence microscopy (Leica 2000).

**Electron microscopy.** Brain tissues were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (pH 7.4) and postfixed in 2% OsO4 before being embedded. Analysis was performed by a TEM (Carl Zeiss MicroImaging, Thornwood, NY) on ultrathin sections stained with uranyl acetate and lead citrate. For mitochondrial measurements, digital images of multiple samples were collected from at least 16 random fields during EM analysis. To determine the morphology of the mitochondria, the EM images were analyzed with Photoshop, using the counting and area analysis function, in an approach similar to that reported by other investigators (Shen et al., 2004; Chen et al., 2009). Scores were given in a blinded fashion.

**Immunoprecipitation.** Mitochondrial or cytosolic fractions of rat brain or cultured cell homogenates (200 µg protein) were incubated with the indicated antibodies for 3 h at 4°C, followed by incubation with protein A/G agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. The immunoprecipitates were separated on SDS–PAGE.
supernatants correspond to the total cell lysates. Homogenates were spun at 14,000 rpm for 20 min at 4°C. The phosphatase inhibitor cocktail. After 20 min of incubation on ice, 1 mM EGTA, 1% Triton X-100, protease inhibitor cocktail, following lysis buffer: 10 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, Tris-HCl, 20 mM MgCl₂, 1 µM dithiothreitol, 25 µM ATP, 1 mM CaCl₂).

Measurement of cell viability. Human SH-SY5Y cells were treated with PKCδ inhibitor δV1-1 or siRNA for Drp1, followed by Ang II (1 µM for 48 h) or hydrogen peroxide (H₂O₂, 200 µM for 24 h) treatment. The cell viability was measured using an invitro toxicity assay MTT-based kit (Sigma), according to the manufacturer’s instruction.

Western blot analysis. Protein concentrations were determined by Bradford assay, and 10 µg of proteins was resuspended in Laemmli buffer, loaded on SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibody followed by visualization by ECL.

Tissue total lysate preparation. Samples were processed in the following lysis buffer: 10 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitor cocktail, phosphatase inhibitor cocktail. After 20 min of incubation on ice, homogenates were spun at 14,000 rpm for 20 min at 4°C. The supernatants correspond to the total cell lysates.

Statistical methods. Data are expressed as mean ± SE. Unpaired t test for differences between two groups, one-factor ANOVA with Fisher’s test for differences among more than two groups, and Fisher’s test for categorical data were used to assess significance (p < 0.05).

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REFERENCES


