Insulin Receptor-Protein Kinase C-\(\gamma\) Signaling Mediates Inhibition of Hypoxia-Induced Necrosis of Cortical Neurons

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ABSTRACT

Ischemic stress causes neuronal death and functional impairment. Evidence has suggested that cells in the ischemic core first lose viability due to the decline in blood flow and cellular energy metabolism and then die by necrosis. Although inhibition of necrosis could be a potent therapeutic target for brain ischemia, known neurotrophic factors are ineffective for neuronal necrosis. We previously reported that insulin, but not brain-derived neurotrophic factor or insulin like-growth factor-1, inhibited neuronal necrosis under serum-free starvation stress. Although insulin receptors are abundant in the central nervous system as well as in peripheral tissues, neurons are not dependent upon insulin for their glucose supply, indicating that insulin receptors have other roles in the central nervous system. In the present study, by using hypoxia-reperfusion stress, we showed that cortical neurons rapidly die by necrosis as evaluated by propidium iodide staining and transmission electron microscopic analysis. As expected, insulin treatment significantly inhibited neuronal necrosis, although this effect was blocked by pretreatment with an antisense oligonucleotide for the insulin receptor. Furthermore, an inhibitor of protein kinase C (PKC) eliminated the insulin-induced antinecrotic effect. The addition of insulin induced significant translocation of only the PKC-\(\gamma\) isoform, whereas antisense oligonucleotide treatment for this isoform abolished the insulin-induced inhibition of necrosis. Together, these results suggest that insulin mediates inhibition of neuronal necrosis through a novel mechanism involving PKC-\(\gamma\) activation.

Insulin receptors (IRs) are found in the central nervous system as well as in peripheral tissues, being particularly abundant in the olfactory bulb, cerebral cortex, hypothalamus, cerebellum, and choroid plexus (Schulingkamp et al., 2000). Of great importance is that insulin is synthesized in and secreted by neurons (Devaskar et al., 1994; Rulifson et al., 2002). Although it is well known that insulin stimulates glucose uptake in peripheral tissues, such as adipose tissue, liver, or muscle, through redistribution of the glucose transporter GLUT4 (Khan and Pessin, 2002), the roles of brain insulin and its receptor in glucose transportation remain to be clarified, since neurons are not fully dependent on insulin for their glucose supply (Shulingkamp et al., 2000; Craft and Watson, 2004).

Accumulating evidence suggests that insulin has various functions in the central nervous system apart from its glucose uptake activity, such as increasing the expression of postsynaptic GABA\(_{\text{A}}\) receptors (Wan et al., 1997), axon guidance during development of the visual system (Song et al., 2003), and reducing intracellular amyloid \(\beta\) and the level of tau phosphorylation in Alzheimer’s disease (Hong and Lee, 1997; Gasparini et al., 2001). In addition, similar to other known neurotrophic factors, insulin can inhibit neuronal apoptosis (Ryu et al., 1999; Barber et al., 2001). However, unlike other known neurotrophic factors, we previously reported that insulin can protect neurons from necrosis under conditions of serum-free starvation stress (Hamabe et al., 2003). Since neuronal necrosis develops in the ischemic core region and is propagated in its vicinity, inhibition of necrosis...
should be important for minimizing ischemic injury. Here, we attempt to characterize the nature of insulin-induced inhibition of necrosis by using an ischemic hypoxia-reperfusion model in culture.

**Materials and Methods**

**Cell Culture.** Primary culture of the cerebral cortex from 17-day-old embryonic rats was performed according to a previously reported protocol (Hamabe et al., 2000; Fujita and Ueda, 2003a,b). Briefly, cortical tissues were minced into pieces in sterile PBS, pH 7.4, and dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA) and 0.01% DNase I (Sigma-Aldrich, St. Louis, MO) in PBS for 12 min at 37°C. The reaction was terminated by the addition of 0.25% soybean trypsin inhibitor (Sigma-Aldrich), and the cell suspension was centrifuged at 1000g for 5 min. The pellet was resuspended in serum-supplemented D/F medium [1:1 Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium (Invitrogen) containing 5% horse serum (HS), 5% fetal bovine serum (FBS), and 1% 2-mercaptoethanol (Invitrogen)]. Dissociated neurons were seeded at a density of 0.5 \( \times 10^5 \) cells/cm\(^2\) onto 96-well plates for WST-8 reduction assays and eight-well Lab-Tek (Naperville, IL) chamber slides for morphological assessment, all of which had been precoated with poly-DL-ornithine (Sigma-Aldrich), and subsequently cultured at 37°C in a 5% CO\(_2\) atmosphere. Cytosine \( \beta \)-arabinofuranoside ( Ara-C; Sigma-Aldrich) at 0.5 \( \mu \)M was added to the culture at 24 h after seeding, followed by another 48 h of culture.

**Hypoxia-Reperfusion Stress Model.** After culture for 3 days as indicated above, neurons were washed twice with glucose-free balanced salt solution (BSS; 116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), and 1 mM NaH\(_2\)PO\(_4\), pH 7.3), which had been deaerated using a vacuum. After the replacement of the BSS with fresh BSS containing 17.5 mM glucose, neurons were exposed to hypoxia (<0.4% O\(_2\), 5% CO\(_2\), and 95% N\(_2\)) for 2 h at 37°C in a commercially available culture incubator (Nuair, Tokyo, Japan). After hypoxia, the culture medium was exchanged for fresh D/F medium containing 5% HS and 5% FBS, and the neurons were further incubated for indicated periods in a 5% CO\(_2\) atmosphere (reperfusion).

**WST-8 Reduction Assay.** Cell viability was assessed using a WST-8 reduction assay kit (Dojin Laboratories, Tokyo, Japan) according to the manufacturer’s instructions. WST-8 was added to the culture and incubated for 3 h at 37°C before colorimetric measurement. The percentages of the WST-8 reduction activity were represented as the ratios of activities at different time points after reperfusion to that immediately after reperfusion.

**Double Staining with Propidium Iodide and an Anti-Active Caspase-3 Antibody.** Apoptotic or necrotic cell death was characterized using propidium-iodide (PI; Sigma-Aldrich) and anti-active caspase-3 antibody double staining. At the indicated times after seeding, cells were stained with 10 \( \mu \)g/ml PI before fixation with 4% PFA and then labeled with an anti-IR \( \beta \) chain antibody.

**Immunocytochemistry for Insulin Receptors.** Cortical cells on eight-well Lab-Tek chamber slides were fixed with 4% PFA in PBS for 30 min at 25°C, followed by permeabilization with 50% methanol and 100% methanol for 5 min each at 25°C. The cells were then rinsed twice with PBS and preincubated for 1 h at 25°C in blocking buffer (2% BSA and 0.1% Triton X-100 in PBS). The cells were incubated overnight at 4°C in blocking buffer containing a mouse or rabbit anti-IR \( \beta \) chain antibody (1:100; Santa Cruz Biotechnology, Inc.), rinsed with PBS, and then incubated for 2 h at 25°C with Texas Red-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit IgG (1:200; Santa Cruz Biotechnology, Inc.). To measure the total number of nuclei, neurons were stained with 10 \( \mu \)g/ml Hoechst 33342, a marker for nuclei, for 15 min at 25°C. Immunolabeled cells were incubated with Permafluor (Thermo Shandon) and imaged using an AxioVision 3.0 (Carl Zeiss) attached to a BX50 fluorescence microscope (Olympus). The percentages of IR-positive neurons were expressed as the ratios of the total number of nuclei stained with Hoechst 33342 for 100 to 500 nuclei in each of five separate fields. For detection of necrosis, neurons were stained with 10 \( \mu \)g/ml PI before fixation with 4% PFA and then labeled with an anti-IR \( \beta \) chain antibody.

**Characterization of Neurons by Immunocytochemical Analysis.** As a marker for cholinergic neurons, we used a goat anti-choline acetyltransferase (ChAT) antibody (1:50; Chemicon International, Tokyo, Japan). A rabbit anti-glutamic acid decarboxylase isofrom 67 (GAD67) antibody (1:500; Chemicon International) was used as a marker for GABAergic neurons, and a rabbit anti-vesicular transporter 2 (VGLUT2) antibody (1:200; Synaptic Systems, Goettingen, Germany) was used as a marker for glutamatergic neurons. Cortical cells on eight-well Lab-Tek chamber slides were fixed with 4% PFA in PBS for 30 min at 25°C. For the anti-ChAT antibody, cells were permeabilized using 0.3% Triton X in PBS for 5 min and then preincubated for 1 h at 25°C in blocking buffer (3% BSA and 0.1% Triton X-100 in PBS). For the anti-GAD67 and anti-VGLUT2 antibodies, cells were permeabilized using 50% methanol and 100% methanol for 5 min each at 25°C, rinsed twice with PBS, and preincubated for 1 h at 25°C in blocking buffer (3% BSA in PBS). Cells were incubated with the primary antibodies in blocking buffer overnight at 4°C, rinsed with PBS, and incubated for 2 h at 25°C with FITC-conjugated anti-goat IgG (1:200; Rockland, Gilbertsville, PA) or FITC-conjugated anti-rabbit IgG (1:200; Santa Cruz Biotechnology, Inc.). To count the total number of nuclei, neurons were stained with 10 \( \mu \)g/ml Hoechst 33342 for 15 min at 25°C. Immunolabeled cells were mounted with Permafluor (Thermo Shandon) and imaged using an AxioVision 3.0 (Carl Zeiss) attached to a BX50 fluorescence microscope (Olympus). The percentages of neurons positive for each marker were expressed as the ratios of the total number of nuclei stained with Hoechst 33342 for 100 to 500 nuclei in each of five separate fields. For detection of necrosis, neurons were stained with 10 \( \mu \)g/ml PI before fixation with 4% PFA and then labeled with neuron-specific markers. When neurons were double stained with an anti-IR \( \beta \) chain antibody and anti-ChAT, -GAD67, or -VGLUT2 antibody, we used a mixture of the primary antibodies.

**Immunocytochemistry for PKCs.** Cortical cells on eight-well Lab-Tek chamber slides were fixed with 4% PFA in PBS for 30 min at 25°C, followed by permeabilization using 50% methanol and 100% methanol for 5 min each at 25°C. The cells were then rinsed twice with PBS and preincubated for 1 h at 25°C in blocking buffer (2% BSA with 0.1% Tween 20 in PBS). The cells were next incubated with...
goat anti-phosphorylated PKC-α antibody, rabbit anti-PKC-β1 antibody, rabbit anti-PKC-βII antibody, rabbit anti-PKC-γ antibody, goat anti-phosphorylated PKC-ε antibody (1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-PKC-δ antibody (1:100; Invitrogen, Tokyo, Japan), or rabbit anti-phosphorylated PKC-ζ antibody (1:100; Cell Signaling Technology Inc.) in blocking buffer overnight at 4°C, rinsed with PBS, and then incubated for 2 h at 25°C with FITC-conjugated anti-rabbit IgG (1:200; Santa Cruz Biotechnology, Inc.) or FITC-conjugated anti-goat IgG (1:200; Rockland). Immunolabeled cells were mounted with PermaFluor (Thermo Shandon) and imaged using an AxioVision 3.0 (Carl Zeiss) attached to a BX50 fluorescence microscope (Olympus). For detection of necrosis, neurons were stained with 10 μg/ml PI and 10 μg/ml Hoechst 33342 before fixation with 4% PFA and then labeled with the PKC antibodies.

**Oligonucleotide Treatment.** To determine the importance of IRs or PKC-γ in the mechanism of necrosis inhibition, cultures were grown in the presence of antisense oligonucleotides (AS-ODNs) for rat IR or PKC-γ. The AS-ODNs were diluted in water to a concentration of 1 mM and added to the cultures at a final volume of 1:50 of the culture medium every 12 h after seeding for 3 days. In parallel, some cultures were treated with the corresponding missense ODNs (MS-ODNs) containing the same bases as the AS-ODNs but in a random order. Neither ODN resembled any other sequence in the GenBank database. Using Western blot or immunocytochemical analysis, we demonstrated that treatment of cortical neurons in culture with these AS-ODNs, but not the MS-ODNs, reduced the levels of the targeted proteins. The probes had the following sequences: IR AS-ODN, 5’-TCCCGGAGCCCATAGCAGTA-3’ (Obici et al., 2002); IR MS-ODN, 5’-ATCCACAGGACCCGAGGCCTG-3’; PKC-γ AS-ODN, 5’-AGGACCGAGCCCGCAT-3’ (Fleming et al., 1998); and PKC-γ MS-ODN, 5’-ACGACGGCGAACACCGCT-3’. All ODNs were purchased from QIAGEN (Tokyo, Japan).

**Western Blot Analysis.** After ODN treatment for 3 days after seeding, cells were immediately lysed in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol), boiled and reduced with 10 mM dithiothreitol. Samples and molecular weight standards (Invitrogen, Tokyo, Japan) were electrophoresed in 10% SDS-PAGE acrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Tokyo, Japan). Membranes were blocked for 2 h at 25°C with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20, incubated with a rabbit anti-IR β chain antibody (1:200; Santa Cruz Biotechnology, Inc.) or rabbit anti-PKC-γ antibody (1:500) overnight at 4°C, and then with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; Zymed Laboratories, South San Francisco, CA) for 2 h at 25°C. All visualization of immunoreactive bands was performed using Light Capture (AE-6960/C/SD, Atto, Tokyo, Japan) with an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, Super Signaling FC; Atto, Tokyo, Japan). The detection of necrosis was performed using Light Capture (AE-6960/C/S, Atto, Tokyo, Japan) with a nuclear reactivity bands was performed using Light Capture (AE-6960/C/S, Atto, Tokyo, Japan). The detection of necrosis was performed using Light Capture (AE-6960/C/S, Atto, Tokyo, Japan). The detection of necrosis was performed using Light Capture (AE-6960/C/S, Atto, Tokyo, Japan).

**Statistical Analysis.** Data were analyzed using Student’s t test after multiple comparisons by analysis of variance. The criterion of significance was set at P < 0.05. All results are expressed as the mean ± S.E.M.

**Results**

**Hypoxia-Reperfusion Stress-Induced Neuronal Necrosis.** Cortical cells of embryonic day 17 rats were seeded at a density of 0.5 × 10^5 cells/cm^2 in D/F medium containing 5% HS and 5% FBS. To remove non-neuronal cells, 0.3 μM Ara-C was added to the culture medium on the following day, and the cells were cultured for a further 2 days. On the third day of culture, we observed that almost all cells were neurons, with extended neurites, under a phase-contrast microscope. Furthermore, these neurons were positive for microtubule-associated protein 2 as evaluated by immunocytochemical analysis (data not shown). When these neurons were exposed to hypoxia-reperfusion stress, the survival activity measured by the WST-8 reduction assay rapidly decreased. Survival activity at 3 h after reperfusion was as low as 25% of that immediately after reperfusion (Fig. 1A). Necrosis, as evaluated by PI staining, was found in approximately 50% of the total neurons as early as 3 h after reperfusion, and the level then reached a plateau (Fig. 1, B and C). PI-positive neurons were without nuclear fragmentation or condensation when stained with Hoechst 33342 (data not shown). On the other hand, apoptosis, as evaluated by staining with an anti-active caspase-3 antibody, was found in 20% of the total neurons at 3 h after reperfusion, and the level slightly increased to 25% at 6 h and later (Fig. 1, B and C). Thus, it is evident that most of the cortical neurons subjected to ischemic hypoxia-reperfusion stress died by necrosis. Since there was only limited apoptosis, delayed cell death, the cause of the apoptosis is likely to be attributable to other mechanisms, including the Ara-C pretreatment, rather than to the ischemic hypoxia-reperfusion stress.

In the transmission electron microscopic (TEM) analysis, the control neurons without hypoxia-reperfusion stress showed intact features in their plasma membrane, nuclei, and mitochondria at 3 days after the start of the serum-containing culture (Fig. 2, a and b). However, neurons after hypoxia-reperfusion stress had typical necrotic features such as a disrupted plasma membrane, grossly swollen mitochondria with no clear cristae structures, and decreased electron density in the cytoplasm at 3 h after reperfusion (Fig. 2, c and d), as reported previously for serum-free culture (Fujita and Ueda, 2003a,b). In this TEM analysis, approximately 40 to
50% of the total neurons had typical necrotic morphology, whereas none of the neurons had the typical apoptotic morphology characterized by nuclear fragmentation or condensation at this time point. Thus, the cell death characterized by PI staining at 3 h after reperfusion was confirmed to be necrosis by the TEM analysis.

**Insulin-Induced Necrosis Inhibition.** When insulin was added to the culture medium at the indicated concentrations throughout hypoxia and reperfusion, the survival activity measured by the WST-8 reduction assay increased in a concentration-dependent manner in a range of 0.1 to 100 μg/ml 3 h after reperfusion (Fig. 3A). The addition of 100 μg/ml insulin just after hypoxia also significantly increased survival activity (data not shown). When necrosis was characterized by PI-positive staining, the percentage of necrotic neurons was significantly decreased by 100 μg/ml insulin 3 h after reperfusion (55.1%; Fig. 3B). However, because there was no difference in the necrosis-inhibiting effect between 10 and 100 μg/ml in WST-8 reduction assay, we decided to use 100 μg/ml insulin for PI staining experiments, expecting complete action.

**Lack of Neuronal Phenotype Specificity.** As shown in Fig. 3B, insulin protected only 50% of neurons from necrosis. It seems that this effect of insulin is limited to some neuron populations; thus, we decided to characterize the neurons and determined the effect of insulin in each group of neurons. Neuronal phenotypes can be identified by specific immunostaining with antibodies against ChAT, GAD67, and VGLUT2, molecular markers for cholinergic, GABAergic, and glutamatergic neurons, respectively (Eckenstein and Baughman, 1984; Kaufman et al., 1986; Ni et al., 1994). As shown in Fig. 4, A and B, most neurons that were positive for each antibody also showed positive staining with the anti-IR β chain antibody 3 days after the start of culture. When the necrosis was evaluated by PI staining 3 h after reperfusion, 88% of the IR-positive neurons had died by necrosis after ischemic hypoxia-reperfusion stress, whereas the addition of 100 μg/ml insulin reduced the number of necrotic neurons to 30%, as shown in Fig. 4C. On the other hand, 76 to 97% of neurons that were ChAT-, GAD67-, or VGLUT2-positive had died by necrosis 3 h after reperfusion (Fig. 4C). The addition of 100 μg/ml insulin reduced the necrosis to 30 to 60%, and there were no significant differences among the neuronal phenotypes.

**Insulin Receptor-Mediated Necrosis Inhibition.** Pretreatment with AS-ODN for IR abolished the necrosis (PI-positive) inhibition by 100 μg/ml insulin when observed 3 h after ischemic reperfusion, whereas the MS-ODN had no effect (Fig. 5A). Quantitative analysis revealed a significant effect of the AS-ODN for IR (Fig. 5B). Treatment with the AS-ODN, but not with the MS-ODN, significantly reduced the IR expression level in a Western blot analysis (Fig. 5B, inset). On the other hand, there was no significant increase or decrease in insulin receptor expression levels by 100 μg/ml insulin (Supplemental Data Fig. 1).

**Blockade by PKC Inhibitors.** Inhibition of necrosis by 100 μg/ml insulin was only attenuated by 1 μM HBDBDE, an inhibitor of PKC-α and PKC-γ (Ma et al., 2002), as shown in Fig. 6, whereas no toxic effects were observed with HBDBDE alone under conditions of hypoxia-reperfusion stress (Fig. 6A). On the other hand, there were no significant attenuations by 1 μM Gö6976, an inhibitor of PKC-α and PKC-β (Ma et al., 2002), or 1 μM rottlerin, an inhibitor of novel types of PKCs, including the PKC-δ isoform (Murugappan et al., 2004). These results suggest that PKC-γ is more likely to be involved in insulin-induced necrosis inhibition. On the other hand, no attenuation was observed with either 1 μM phosphatidyl inositol-3-OH-kinase (PI3-K) inhibitor wortmannin or 1 μM mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (Fig. 6B), as reported previously for serum-free culture (Hamabe et al., 2003).

**Selective Translocation of PKC-γ by Insulin.** That translocation or phosphorylation of PKC can be a functional event when the enzyme is activated by various cellular signals, such as calcium ions and diacylglycerol, is well known.

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**Fig. 2.** TEM analysis of neurons under hypoxia-reperfusion stress. Low-magnification (LM) and high-magnification (HM) photographs are shown (top and bottom, respectively). a and b, neuron in the serum-containing culture 3 days after the start of the culture. c and d, neuron in the hypoxia-reperfusion stress culture 3 h after reperfusion. Arrows, arrows, and white arrows represent intact, swollen mitochondria, and membrane disruption, respectively. N, nucleus. Scale bars, 1 μm (LM) and 200 nm (HM).

**Fig. 3.** Insulin-induced inhibition of necrosis under hypoxia-reperfusion stress. A, concentration-dependent increase in survival activity by insulin. The WST-8 reduction assay was performed 3 h after reperfusion. B, quantitative analyses of the percentages of neuronal necrosis stained with PI 3 h after reperfusion. Details of the PI staining are given under Materials and Methods. The “control” value is that of the culture without insulin (none). The results are expressed as the means ± S.E.M. from three independent experiments. *, P < 0.05 versus the control culture without insulin (none).
PKC-γ Activation in Insulin Signaling Prevents Necrosis

Fig. 4. Lack of neuronal phenotype-specific inhibition of necrosis by insulin. Cultured cortical neurons were stained with several antibodies against IR and ChAT, GAD67, or VGLUT2. A, Representative photographs of double staining for IR and ChAT, GAD67, or VGLUT2. A red signal (Texas Red) indicates IR expression, whereas a green signal (FITC) indicates ChAT, GAD67, or VGLUT2 expression. All neurons also were stained with Hoechst 33342. B, quantitative analyses of the percentages of IR- and ChAT-, GAD67-, or VGLUT2-expressing neurons. C, quantitative analyses of the percentages of neuronal necrosis within neurons positively stained with each neuronal marker. Details of the immunocytochemistry are given under Materials and Methods. The results are expressed as the means ± S.E.M. from three independent experiments.

Discussion

The major finding of this study is that in hypoxia-reperfusion stress, IR stimulation rescues neurons from necrosis through PKC-γ activation. In the well-known pathological view of ischemia (Dirnagl et al., 1999), it is considered that inhibition of necrosis, an acute phase of ischemic cell death, is important in therapeutic strategies for ischemic injury. Although we have already reported that insulin inhibits neuronal necrosis (Hamabe et al., 2003), the experiments were under serum-free stress, which does not completely mimic physiological conditions. To clarify the nature of insulin-induced necrosis inhibition under culture conditions closer to ischemia, we established a neuronal in vitro ischemic model. It is interesting to note that our culture model differs from other previously reported models. In a common in vitro ischemic model used in other reports, neurons that are seeded at 2 to 5 × 10^5 cells/cm^2 and exposed to 1 to 2 h of oxygen-glucose deprivation die by apoptosis 24 h after reperfusion (Gonzalez-Zulueta et al., 2000; Kapinya et al., 2002). In some cases, a small population of cells, less than 10%, die by necrosis (Malagelada et al., 2004). In contrast, we report here that approximately 50% of neurons died by necrosis within 3 h after reperfusion when seeded at 0.5 to 10^5 cells/cm^2 and exposed to serum and oxygen deprivation for 2 h by using deaerated glucose-containing BSS (hypoxia) followed by a return to the complete culture conditions (reperfusion).

In addition, compared with our earlier report using serum-free starvation stress, there are several advances with this hypoxia-reperfusion stress. First, before the induction of necrosis, cells were grown for 3 days under complete culture conditions. Then, we pretreated neurons with the AS-ODN for 3 days before exposing them to hypoxia-reperfusion stress (Fig. 5). By such experiments, we could prove that IRs and PKC-γ mediate the insulin-induced inhibition of necrosis.

Fig. 5. Blockade of insulin-induced inhibition of necrosis by treatment with an AS-ODN for IR. A, representative photographs of PI staining 3 h after reperfusion. B, top, AS-ODN, but not MS-ODN, treatment-induced reduction of the IR expression level in Western blot analysis. Representative data are shown, and all results were reproducible in another set of experiments. Bottom, effects of the AS-ODN and MS-ODN for IR on insulin-induced inhibition of necrosis. Details of the PI staining are given under Materials and Methods. The control value is the number of PI-positive neurons in the vehicle (water)-treated culture without insulin. The results are expressed as the means ± S.E.M. from three independent experiments. *P < 0.05 versus the control culture with insulin; †, P < 0.05 versus the MS-ODN-treated culture with insulin.

(Shirai and Saito, 2002). By immunocytochemical analysis, the addition of 100 μg/ml insulin only altered the staining pattern of the PKC-γ signal 1 h after ischemic reperfusion (Fig. 7A, g and h). In a high-magnification photograph, the PKC-γ signal was found in the cytoplasm in the control neuron, whereas it was translocated to the perinuclear region in the insulin-treated neuron (Fig. 7B, g' and h'). When we double stained with anti-PKC-γ antibody and Hoechst 33342, it was clear that PKC-γ translocated to the perinuclear region 1 h after 100 μg/ml insulin stimulation (Fig. 7C). Quantitative analysis indicated that 62% of insulin-stimulated neurons show perinuclear localization of PKC-γ. However, there was no apparent translocation (Fig. 7A, c–f, i, and j) or increase in phosphorylation levels (Fig. 7A, a, b, and k–n) of other PKC isoforms.

PKC-γ Is Involved in the Insulin-Induced Necrosis Inhibition. Three hours after ischemic reperfusion, vehicle-treated neurons without insulin were double stained with an anti-PKC-γ antibody (green) and PI (red), as shown in Fig. 8B, a. When 100 μg/ml insulin was added, PKC-γ was redistributed to the cytoplasm 3 h after reperfusion (Fig. 8B, b). These neurons were no longer PI-positive. On the other hand, treatment with the AS-ODN for PKC-γ, but not the MS-ODN, significantly reduced the PKC-γ expression level in a Western blot analysis (Fig. 8A). When neurons were pre-treated with the AS-ODN, the signal disappeared, and significant PI staining appeared, whereas treatment with the MS-ODN had no effect (Fig. 8B, c and d). Quantitative analysis revealed that treatment with the AS-ODN, but not the MS-ODN, reversed the 100 μg/ml insulin-induced inhibition of necrosis 3 h after reperfusion, as evaluated by PI staining (Fig. 8C). However, AS-ODN or MS-ODN alone-treated neurons did not show cell death under normal or hypoxia-reperfusion conditions (Fig. 8C).
Second, we found that necrosis was not observed just after hypoxia but was triggered by the reperfusion and that incubation with insulin during hypoxia could be a necrosis-preventive pretreatment, as shown by the subsequent results. Another advantage was that the concentration of insulin required for necrosis inhibition, 100 ng/ml, was 100-fold lower in the present ischemic reperfusion model than in the serum-free culture (Hamabe et al., 2003). In the current culture system, about 90% of neurons possess IRs (Fig. 4, A and B), and extensive coexpression of IRs with ChAT (a marker for cholinergic neurons), GAD67 (a marker for GABAergic neurons), and VGLUT2 (a marker for glutamatergic neurons) was observed (Fig. 4, A and B). Because insulin protected only 50% of neurons from necrosis (Fig. 3), we thought we needed to discuss whether neuronal phenotype specificity is involved in necrosis-inhibition by insulin. However, we could not detect any significant changes among several neuronal phenotypes, glutamatergic, GABAergic, and cholinergic neurons, all major neurons in the cortex. These findings suggest that all types of neurons may be direct targets for insulin and that they can be partially rescued from necrosis by insulin (Fig. 4C).

Insulin-induced inhibition of necrosis seems to be unique, since inhibitors of PI3-K or MEK had no effects (Fig. 6B). Such characteristics are in good contrast with apoptosis inhibition (Navarro et al., 1998; Barber et al., 2001) and may be related to our previous observation that brain-derived neurotrophic factor, a known trophic factor that requires activation of extracellular signal-regulated kinase or PI3-K for its survival activity, did not inhibit neuronal necrosis (Hamabe et al., 2003). Thus, it is suggested that the necrosis inhibition
may be insulin-specific and extracellular signal-regulated kinase- or PI3-K-independent.

In the current study, we observed that PKC-activating pathways play important roles in insulin-induced necrosis inhibition, supporting our previous findings that a broad-spectrum PKC inhibitor abolishes neuronal necrosis under conditions of serum-free stress (Hamabe et al., 2003). PKC is a central protein in neuronal metabolism, function, and survival (Dekker and Parker, 1994), and changes in PKC activity have been hypothesized to play either a protective or a detrimental role in the cell death, including ischemic damage (Hara et al., 1990; Busto et al., 1994; Durkin et al., 1997). However, we could not detect any significant effects of various PKC inhibitors treated alone on hypoxia-reperfusion-induced necrosis in additional experiments (Fig. 6A). Thus, it is unlikely that any PKC isoforms contribute to stress-induced necrosis.

The PKC family can be classified into three major groups, i.e., conventional PKC-α, -βI, -βII, and -γ; novel PKC-δ, -ε, -η, -θ, and -μ; and atypical PKC-ζ, -η, and -λ (Goodnight et al., 1995). Accumulating observations suggest that these PKC isoforms are each activated by different signals and play contrasting roles in cell survival; for example, PKC-α and PKC-ɛ have an antiapoptotic effect, whereas PKC-δ and PKC-θ have a proapoptotic effect (Gutcher et al., 2003).

To clarify the PKC isoform that plays an important role in necrosis inhibition, we performed pharmacological analysis using a panel of different PKC inhibitors (Ma et al., 2002; Murugappan et al., 2004). As shown in Fig. 6A, Go6976, an inhibitor of PKC-α and PKC-β, or rottlerin, an inhibitor of PKC-δ and PKC-θ, failed to block the insulin-induced inhibition of necrosis, as evaluated by PI staining. In contrast, necrosis was significantly blocked by HBDDE, an inhibitor of PKC-γ and PKC-α (Fig. 6A). Immunocytochemical examination revealed that only PKC-γ showed subcellular translocation from the cytoplasm to a compact perinuclear structure that resembles the Golgi apparatus (Fig. 7). This finding was consistent with the observation that PKC-γ AS-ODN treatment completely blocked insulin-induced necrosis inhibition (Fig. 8). As AS-ODN-treatment did not affect the necrosis level in hypoxia-reperfusion stress or normal conditioned culture (Fig. 8C), another redundant PKC family is unlikely involved in necrosis-inhibition by insulin.

It is well accepted that PKC translocation is a typical feature representing its activation. In most cases, activated PKC isoforms are translocated to plasma membranes, but in some cases they are translocated to several cytosolic organelles (Shirai and Saito, 2002). There are reports that activated PKC-γ is translocated to Golgi-like organelles (Goodnight et al., 1995) or a perinuclear region (Szalay et al., 2001) as well as to plasma membranes (Goodnight et al., 1995; Szalay et al., 2001), although the role of these translocations in cellular signaling remains to be determined. In the present study, insulin stimulation of cortical neurons also caused translocation of PKC-γ to a perinuclear region, although it remains to be determined whether it is a direct consequence of insulin receptor activation. However, as treatment of neurons with AS-ODN or a relatively specific inhibitor for PKC-γ abolished the necrosis inhibition by insulin, activated PKC-γ is clearly involved in necrosis inhibition. PKC-γ is expressed solely in brain and spinal cord, and its localization is restricted to neurons, whereas other PKC isoforms are expressed in many tissues in addition to brain (Nishizuka, 1988). There are conflicting results regarding the effects of PKC-γ on cell survival in a variety of models. In some ischemic models, translocation of PKC-γ to membranes contributes to ischemic cell death (Shamloo and Wieloch, 1999; Kurihara et al., 2004; Matsumoto et al., 2004), whereas PKC-γ-deficient mice are more susceptible to ischemia (Aronowski et al., 2000). Furthermore, Lin et al. (1997) reported that age-induced apoptosis of cerebellar granule cells was prevented by induction and up-regulation of the PKC-γ isoform. Thus, the physiological roles of the PKC-γ isoform, including that in the present insulin-induced necrosis inhibition, may vary under different biological conditions.

In summary, we report the first evidence of IR- and PKC-γ-mediated necrosis inhibition under hypoxia-reperfusion...
stress. In light of the significant roles of necrosis in ischemic injury, our results could offer potential targets for neuroprotection in ischemia.

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