Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-α and ceramide

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Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-α and ceramide. Am. J. Physiol. Cell Physiol. 278: C144–C153, 2000.—Brief “preconditioning” ischemia induces ischemic tolerance (IT) and protects the animal brain from subsequent otherwise lethal ischemia. A solution to this problem could be derived from an understanding of mechanisms by which the cell adapts to ischemic stress.

In animal models of brain ischemia, brief transient episodes of “preconditioning” ischemia induce tolerance and protect the brain from subsequent otherwise lethal ischemia (for review see Ref. 7). Development of the tolerant state takes time, usually 24–72 h (3). A cascade of signaling events initiated by sublethal stress proceeds during the latent period, resulting in a new, stress-resistant, biochemical makeup of brain cells. Identification of the signaling steps most proximal to the development of the tolerant state would allow compression of the latency period and make cells resistant to ischemic stress quickly.

Many deleterious as well as neuroprotective reactions in ischemic brain are mediated by the pleiotropic cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (16, 38). Recent studies suggest that the state of tolerance induced by ischemic preconditioning also might involve these cytokines. Thus intraperitoneal pretreatment of gerbils with IL-1 receptor antagonist abolished tolerance to global ischemia induced by ischemic preconditioning, and injection of IL-1α and IL-1β mimicked the effect of preconditioning (33). Similarly, intravenous pretreatment of spontaneously hypertensive rats with lipopolysaccharide (an agent that is known to induce production of TNF-α) led to development of ischemic tolerance, and combined administration with TNF-α-binding protein prevented it (42).

Furthermore, intracisternal pretreatment of mice with TNF-α (32) protected animals from ischemic injury in a permanent middle cerebral artery occlusion model. These observations suggest that cellular resistance to ischemic stress in brain originates at least partially from engagement of the TNF-α receptor and its downstream messengers.

The sphingolipid ceramide has been implicated as a second messenger in many of the multiple signaling pathways initiated on binding of TNF-α to its p55 receptor (13, 22). Although most of these studies have focused on the role of ceramide in induction of apoptosis and cell cycle control (34, 39), there is also evidence that ceramide can cause cytoprotection (15). We hypothesized that ceramide could play a role in induction of ischemic tolerance. This hypothesis was tested in an in vitro model of tolerance. We present evidence for the first time that preconditioning of cortical neurons with mild hypoxia results in TNF-α-mediated upregulation of ceramide.
of intracellular ceramide levels that is necessary and sufficient for induction of the tolerant state.

**MATERIALS AND METHODS**

Cortical neuronal cultures. Cortical neuronal cultures were established from 2-day-old Sprague-Dawley rats. Cerebral cortices without meninges were placed into a dissection medium [0.3% (wt/vol) glucose, 0.75% (wt/vol) sucrose, and 28 mM HEPES in Hanks' balanced salt solution, pH 7.3, osmolality 320 mosm/kg], cut into small pieces, treated with 0.25% trypsin for 20 min at 37°C, and then resuspended in DMEM-high-glucose (4,500 mg/l), 2 mM glutamine, 1% antibiotic/antimycotic (all from Gibco Life Technology), 10% fetal bovine serum (Summit Biotechnology), and 40 µg/ml DNase (Boehringer Mannheim) and triturated 20 times in culture medium. The cell suspension was centrifuged at low speed (1,000 rpm) to eliminate cell debris, resuspended in culture medium [Neurobasal-A with 2% B27 supplement, 1 mM L-glutamine (all from Gibco Life Technology), and 0.2% horse serum (Sigma Chemical)] at 5.5–5.8 × 10^5/ml (1 brain yielded a 24-ml cell suspension), and plated in 500 µl/well in 24-well plates (Costar) precoated with 2.5 µg/cm² of poly-L-lysine. The glucose concentration in Neurobasal-A was 4,500 mg/l. Nonneuronal cells were eliminated by changing the medium 20 min after plating and by adding 15 µg/ml 5'-fluoro-2'-deoxyuridine (Sigma Chemical) to the culture medium. Immunostaining of neurons with antibody against neuron-specific enolase (Chemicon International, Temecula, CA) and astrocytes with glial fibrillary acidic protein-specific antibody (Boehringer Mannheim) demonstrated that astrocyte contamination was <5%.

Hypoxic pretreatment. Hypoxic pretreatment was performed on day 4 in vitro after withdrawal of 200 µl (of 400 µl/well) of culture medium. Neuronal cultures were placed in modular incubator chambers (Billups Rothenberg, Del Mar, CA) and flushed with a gas mixture of 5% CO₂-95% N₂ for 20 min at room temperature (when the cells were grown in 60-mm dishes, flushing time was 15 min). The chambers were sealed and incubated at 37°C for 20 min. O₂ concentration in the culture medium was monitored with an O₂ meter (Microelectrodes, Bedford, NH) and reached 8% at the end of incubation. Severe hypoxic treatment. Cells were subjected to severe hypoxic treatment on day 5 in vitro. Culture medium was completely removed from naive and preconditioned cells and substituted with 200 µl/well of Neurobasal-A medium plus 1 mM L-glutamine (no B27 supplement and horse serum). The plates were flushed with 5% CO₂-95% N₂ in hypoxic chambers until O₂ concentrations dropped to 2% (~40 min). Chambers were agitated every 5 min to ensure maximal gas exchange in the culture medium. The chambers were sealed and incubated for 2.5 h at 37°C (O₂ concentration in the medium was 5% at the end of incubation). For reoxygenation, 200 µl/well of normoxic culture medium containing double concentrations of B27 supplement and horse serum were added to the cells, cells were placed in a regular tissue culture incubator, and cell viability was measured at indicated times. Control cells were subjected to the same washing and feeding procedures with normoxic medium. For glucose deprivation studies, cells were incubated in 200 µl/well of DMEM containing no glucose, and 200 µl/well of DMEM containing double concentrations of glucose were added to the cultures on reoxygenation.

Pretreatment of neuronal cultures with TNF-α and C-2 ceramide and blocking reagents. TNF-α was added to neuronal cultures on day 4 at 25 ng/ml in Neurobasal-A plus 1 mM L-glutamine and 2% B27 supplement and 0.2% horse serum for 24 h (it was washed out just before the severe hypoxic treatment). N-acetylcarnosine (C-2 ceramide) was added to the cultures at the beginning of severe hypoxia (day 5 in vitro) at 10 µM and remained in the medium during the entire reoxygenation period. Anti-TNF-α neutralizing antibody (8 µg/ml at ND50 = 3-6 µg/ml; R & D Systems) or fumonisin B₁ at 50 µM (Alexis Biochemicals, San Diego, CA) was added to neuronal cultures at the time of preconditioning. Both reagents were washed out just before incubation of cells in severe hypoxia.

Quantitation of neuronal injury. Quantitation of neuronal injury was performed by means of an ethidium homodimer fluorescence exclusion test. Ethidium homodimer and other DNA-binding fluorescent dyes are commonly used for cytotoxicity tests. These dyes are polar and, therefore, cell impermeant, unless the integrity of the cell membrane is compromised by a cytotoxic reagent. Ethidium homodimer has been specifically designed for live/dead assays, and it is superior to other stains because of its higher DNA affinity, very low membrane permeability, and very low background fluorescence. It has been used in cytotoxicity assays since 1995; however, its application for neuronal cultures has been limited, although it has been documented in several studies (26, 31). One of the reasons is that neuronal cultures do not form monolayers, and the number of cells per culture or per certain area of the culture varies. This variability becomes even greater when cells are subjected to stress, such as hypoxia, because many cells disintegrate or detach from the dish. Thus it was necessary to measure not only the number of cells that fail to exclude the dye but also the number of cells that remained in the culture after treatment. Our modification of the assay was to add a mild detergent at the end of the assay to make all the cells in the culture permeable to the dye, which allowed us to estimate the total number of cells remaining in the culture wells after each experimental condition.

This is illustrated by a representative experiment presented in Fig. 1A. There were only a few dead cells in control cultures (Fig. 1A) compared with hypoxia-treated wells (Fig. 1C). To estimate how many cells remained in the well, all the cells were made permeable to the dye by addition of the detergent saponin. This procedure revealed a significant cell loss in hypoxia-treated cultures (Fig. 1D) compared with control (Fig. 1B). To quantify fluorescence changes, neurons were plated in 24-well plates and subjected to the treatments mentioned above. At the end of each experiment, culture medium was withdrawn and cells were incubated with 6 µM ethidium homodimer (Molecular Probes, Eugene, OR) in Hanks' buffer at 300 µl/well for 30 min at 37°C. Cell fluorescence was measured with a CytoFluor 4000 fluorescent plate reader (PerSeptive Biosystems, Framingham, MA) at excitation/emission wavelengths of 530/620 nm. Background fluorescence was measured on each plate and subtracted. The percentage of dead cells was calculated by means of the following formula:

\[
\text{% dead cells} = \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \right) \times 100
\]

where F is fluorescence of unknown sample, Fₘᵢₙₐ is fluorescence of untreated healthy control cultures, and Fₘᵢₙₐ is fluorescence of the control cultures treated with 0.03% saponin for 1 h at 37°C. To estimate the percentage of cells that
had already detached from the dish, saponin was added to all the wells at the end of incubation with ethidium homodimer, and fluorescence of each sample (Fsaponin) was related to that of control untreated samples (Fmax) with use of the following formula

\[
\text{% lost cells} = \frac{\text{F}_{\text{max}} - \text{F}_{\text{saponin}}}{\text{F}_{\text{max}}} \times 100
\]

To take into account uneven distribution of cells on the surface of any well, the fluorescent plate reader was programmed to read five different areas of each well, and the mean signal was calculated. To overcome variability in cell plating density, each experimental value was obtained as an average of measurements performed in eight wells.

Intracellular ceramide levels. Intracellular ceramide levels were measured in neurons that were grown in 60-mm culture dishes and subjected to preconditioning hypoxia or to TNF-\(\alpha\) pretreatment. At 0, 16, 20, 24, 28, and 32 h after reoxygenation, cells were washed twice with cold PBS, scraped off, and pelleted. Cell pellets were subjected to lipid extraction, and intracellular ceramide was quantitated by means of reverse-phase HPLC according to Santana et al. (37), as described in detail elsewhere (13). Ceramide values were normalized per lipid phosphate, as described previously (13).

Measurements of TNF-\(\alpha\) concentrations. Neuronal cultures in 24-well plates were covered with 0.4 ml of culture medium and subjected to hypoxic pretreatment. Aliquots of culture medium were withdrawn at 4, 8, and 24 h after preconditioning, and TNF-\(\alpha\) levels were measured in these samples with an ELISA kit for rat TNF-\(\alpha\) (Endogen, Woburn, MA) according to the manufacturer’s instructions.

Visualization and quantitation of apoptotic cells. Visualization and quantitation of apoptotic cells were performed with the In Situ Cell Death Detection Kit (POD, Boehringer Mannheim). Staining of control and C-2 ceramide-treated neuronal cultures was performed according to the manufacturer’s protocol. The samples were analyzed with a Zeiss Axiovert 10 light microscope (magnification \(\times 40\)). Digitized images of 20 microscopic fields per experimental condition were generated using a DAGE MTI DEI-750 attached camera. The same microscope and camera settings were used for all samples. The number of apoptotic cells within each image was determined by means of a Scion Image computer program and expressed as a sum in pixels of all positively stained areas. The size of one apoptotic nucleus was 80–145 pixels \([113 \pm 22.5 (SD), n = 10]\). Areas <45 pixels were considered “debris” and were excluded from the measurements.

Immunostaining for TNF-\(\alpha\) receptor. Neurons were subjected to preconditioning hypoxia and 24 h later were fixed with Bouin’s solution and immunostained with goat polyclonal antibody directed against TNF-\(\alpha\) type 1 receptor/p55 (Santa Cruz) at 1:100 dilution. Digitized images of the samples were generated as described above for evaluation of apoptosis. TNF-\(\alpha\) type 1 receptor/p55 expression was quantified by means of Scion Image and expressed as a mean intensity of staining. A background intensity was measured for each image and subtracted from mean intensity; 12–14 images were analyzed per experimental condition.

Statistical analysis. Statistical analysis was carried out by two-factor ANOVA and by paired t-test by use of Excel software.

RESULTS

Description of the model of hypoxic-ischemic tolerance in vitro. Analysis of morphological changes of cortical neurons subjected to 2.5 h of hypoxic treatment demonstrated significant cell death (Fig. 2B) compared with control untreated cultures (Fig. 2A). Cultures preconditioned with mild hypoxia were more resistant to hypoxic treatment, with fewer dead cells (Fig. 2C). The effect of hypoxic treatment on neuron viability was quantified by means of the ethidium homodimer assay (see MATERIALS AND METHODS). Measurements of dead cell number demonstrated that hypoxia-induced injury of neuronal cells had already begun during hypoxic treatment and then progressed after reoxygenation. At the end of 2.5 h of hypoxic incubation, \(~13\%\) of the cells...
were dead ($P = 0.004, n = 4$; Fig. 3A). The number of dead cells doubled during 8 h of reoxygenation ($P = 0.0001, n = 6$). Progression of hypoxia-initiated cell death continued for up to 24 h of observation. No significant cell death was observed in sham-washed control cultures maintained in normoxia during the entire period of observation (Fig. 3A). Hypoxic preconditioning of neuronal cultures 24 h before the hypoxic insult inhibited cell death during the period of incubation in the hypoxic environment by 50% ($P = 0.044, n = 4$) and during progression of neuronal injury after reoxygenation by 52 and 39% at 8 h ($P = 0.0003, n = 7$) and 24 h ($P = 0.0013, n = 7$) after reoxygenation, respectively (Fig. 3A). Measurements of ethidium fluorescence in hypoxia-preconditioned cultures 24 h after preconditioning (immediately before the main insult) revealed no cell death and no cell loss as a result of preconditioning (data not shown).

Glucose deprivation without hypoxia did not affect cell viability (Fig. 3B), but when superimposed on hypoxic treatment ($O_2$-glucose deprivation), it resulted in a higher death toll (33.4 vs. 23.6% dead cells, $P = 0.03, n = 4$). Hypoxic preconditioning, however, was as effective in protection against $O_2$-glucose deprivation as it was against hypoxia alone (52% at 8 h after reoxygenation and addition of glucose, $P = 0.045, n = 4$; Fig. 3B).

On the basis of these observations, we have chosen to study molecular mechanisms of hypoxic preconditioning in the model of hypoxic injury of neurons, rather...
than in the more complicated model of O₂-glucose deprivation.

Role of TNF-α in hypoxic preconditioning of neurons. Experiments in animal models have demonstrated that intracisternal administration of TNF-α had a protective effect in the middle cerebral artery occlusion model of brain ischemia (32). On the basis of these observations, we sought to investigate whether TNF-α can mimic the protective effect of preconditioning in neuronal cells in our model. TNF-α was added to neuronal cultures at 25 ng/ml 24 h before hypoxic treatment. Immediately before placement of the cells into hypoxic chambers, the culture medium with TNF-α was completely exchanged for the culture medium containing no TNF-α, and cells were subjected to 2.5 h of hypoxia. The results of this experiment are presented in Fig. 4A. The number of dead cells at 0, 8, and 24 h after cell reoxygenation demonstrated that pretreatment with TNF-α protected neurons from a hypoxic insult to the same degree as did hypoxic preconditioning: 40% (P = 0.004, n = 3), 53% (P = 0.002, n = 5), and 44% (P = 0.002, n = 5), respectively.

Because the timetable for the TNF-α pretreatment and the amplitude of its protective effect were shown to be very similar to that for hypoxic preconditioning, we hypothesized that hypoxic preconditioning is at least partially mediated by TNF-α. This hypothesis has been tested in two ways: 1) we investigated whether preconditioned neurons released TNF-α in the culture medium, and 2) we wanted to know whether inhibition of TNF-α activity during preconditioning would compromise the tolerant state. By means of the TNF-α ELISA, increase of TNF-α concentrations could be detected by 4 h after preconditioning (Fig. 4B, inset). At 8 h after preconditioning, 6.15 ± 2.0 (SE) pg of TNF-α were released by 2.5 × 10⁵ preconditioned neurons (plated in 1 well), in contrast to 1.0 ± 1.2 pg TNF-α released by the same number of control, untreated neurons (n = 3, P < 0.045). Also, cultured neurons during the entire period of preconditioning (24 h) were treated with sheep polyclonal antibody, which neutralizes the biological activity of rat TNF-α. The antibody was washed out with the culture medium as the severe hypoxic treatment began. As shown in Fig. 4B, hypoxic preconditioning decreased the percentage of dead cells in severe hypoxia-treated cultures from 20.2 to 7.4% (P = 0.01, n = 4) and from 27.2 to 14.7% (P = 0.002, n = 4) when measured at 8 and 24 h after reoxygenation, respectively. When preconditioning was performed in the presence of anti-TNF-α antibody, however, it had no protective effect, resulting in 17.7% (P = 0.026 vs. tolerant cells, n = 4) and 23.8% (P = 0.017, n = 4) dead cells, respectively (Fig. 4B). In contrast, sheep IgG with no specificity against TNF-α did not block the effect of preconditioning on neuronal survival (data not shown).

Preconditioning does not change surface expression of TNF-α type 1 receptor (p55). Neurons are not only capable of TNF-α synthesis in response to ischemic stress (16, 25), but they also express TNF-α receptors and amplify this response through paracrine and autocrine mechanisms (41). Studies of lipopolysaccharide- and TNF-induced tolerance in macrophages and monocytes (18) suggest that unresponsiveness of preconditioned cells to the second stimulation with these agents could result from downregulation of the respective receptors caused by the preconditioning treatment. Because our data demonstrated that hypoxic preconditioning was mediated by TNF-α and because TNF-α is also known to mediate cytotoxic effects during ischemia, we wanted to rule out the possibility that the small amount of TNF-α that is released during preconditioning could downregulate expression of TNF-α type 1 receptor by shedding or by endocytosis, and this would make preconditioned cells more resistant to cytotoxic
amounts of TNF-α, which are released during the severe hypoxia.

To investigate this possibility, neuronal cultures were immunostained with a monoclonal antibody directed against TNF-α type 1 receptor (p55), which is known to mediate signaling for the majority of TNF-α-induced responses. Two representative images of control and preconditioned neurons are shown in Fig. 6, A and B, respectively. The majority of cells in both cultures demonstrated positive staining. As demonstrated by binary images of the same microscopic fields (Fig. 5, C and D), TNF-α type 1 receptor is predominantly expressed on the plasma membrane of intact and preconditioned neurons, rather than in cytoplasm, which argues against endocytosis of the receptor. Comparative analysis with the Scion Image program of the mean staining intensities of control and preconditioned neurons revealed no changes in TNF-α type 1 receptor expression: 14.5 ± 0.9 (n = 12) and 15.1 ± 1.7 (n = 14), respectively.

Ceramide is a messenger of ischemic tolerance. The results of the experiments presented above strongly suggested that TNF-α release in neurons preconditioned with mild hypoxia initiated a signaling cascade responsible for cellular resistance to subsequent hypoxic insult. The role of a sphingolipid, ceramide, as a mediator of TNF-α effects has been demonstrated in many cellular models. It has been shown that cell-permeable ceramide analogs, when added to the neuronal cultures, mimic TNF-α effects such as apoptosis (6) or cytoprotection (15, 20). Accordingly, we sought to investigate whether ceramide was a mediator of tolerance.

This hypothesis was tested in three ways: 1) by mimicking preconditioning effects of hypoxia and TNF-α with the exogenous ceramide analog N-acetylsphingosine (C-2 ceramide), 2) by measuring intracellular ceramide levels in neurons preconditioned with TNF-α and hypoxia, and 3) by inhibiting development of tolerance by blocking ceramide biosynthesis in preconditioned neurons.

Cell-permeable C-2 ceramide (10 µM) was added to neuronal cultures immediately before the hypoxic insult. Cells were subjected to 2.5 h of hypoxia, and the number of dead cells was measured by ethidium homodimer exclusion test at 8 and 24 h after reoxygenation. The presence of C-2 ceramide in the culture medium decreased the cell death rate from 30.1 to 13.5% (P = 0.001, n = 4) and from 34.1 to 16.1% (P = 0.009, n = 4) at 8 and 24 h after reoxygenation (Fig. 6A). The efficacy of ceramide-induced protection (55 and 54%, respectively) was similar to that of hypoxic preconditioning (52 and 39%, respectively) and TNF-α preconditioning (53 and 44%, respectively).

Because ceramide has been implicated in signaling pathways leading to apoptosis and because the ethidium homodimer exclusion test, which was used to measure neuronal death, did not detect apoptotic cells, which preserve an intact plasma membrane, there was a possibility that C-2 ceramide, while protecting against hypoxic injury, induces apoptosis in cultured neurons. To rule out this possibility, neuronal cultures, control and treated with 10 µM C-2 ceramide for 24 h, were analyzed for the presence of apoptotic nuclei by TdT-mediated dUTP nick end labeling. Quantitation of the number of apoptotic cells within each image as a sum of positively stained areas in pixels revealed no statistically significant differences between control and ceramide-treated cultures: 11,785 ± 1,479 vs. 12,333 ± 2,199 (SD) pixels (n = 19). Total area occupied by all the cells in one image was equal to 58,655 ± 7,786 pixels.

Fig. 5. Effect of hypoxic preconditioning on expression of TNF-α type 1 receptor (p55) in neurons. Neurons were preconditioned with 20 min of hypoxia and 24 h later fixed and immunostained with anti-TNF-α type 1 receptor antibodies. Positively stained cells were visualized with a Zeiss Axiovert 10 light microscope (magnification ×40). Representative digitized images of control (A and C) and preconditioned (B and D) cultures are shown. Most cells exhibit positive staining, and no differences between control and preconditioned cells were seen. Binary images (C and D) of same cultures confirm membrane localization of TNF-α receptor.
(n = 10), suggesting that there were ~20 and 21% apoptotic cells in control and ceramide-treated cultures, respectively.

The fact that C-2 ceramide exerted its inhibitory effect when added immediately before the hypoxic treatment suggested that its levels should be elevated near the time of hypoxic insult, that is, ~24 h after the start of the preconditioning treatment. We have observed such a late ceramide response to TNF-α-induced preconditioning in astrocytes and brain endothelial cells (14). To test this possibility, ceramide concentrations in neuronal cells were measured at 0, 16, 20, 24, 28, and 32 h after hypoxic preconditioning and after TNF-α pretreatment. Increased intracellular ceramide levels 120–140% of baseline (0.7 ± 0.1 pmol/nmol lipid phosphate) were observed by 16 h (P = 0.018, n = 3) and reached 180–200% of baseline at 24 h after preconditioning (P = 0.035, n = 3; Fig. 7A). In two of five experiments, ceramide accumulation peaked at 20 h. A similar ceramide time course was observed in TNF-α-pretreated neurons (Fig. 7B). It is noteworthy that both preconditioning exposures, hypoxia and TNF-α, failed to induce ceramide accumulation in the presence of fumonisin B1, a mycotoxin produced by Fusarium moniliforme, which inhibits ceramide synthase (sphingosine-N-acyltransferase), in many cell types including neurons (30). Actually, ceramide levels in the presence of fumonisin B1 were below the baseline (Fig. 7).

The inhibitory effect of fumonisin B1 on ceramide levels in preconditioned cells was paralleled by its ability to attenuate tolerance. Hypoxic preconditioning without fumonisin B1 rescued 45.8% (P = 0.0013, n = 4) and 51.8% (P = 0.023, n = 4) of cells subjected to 2.5 h of hypoxic insult, which would otherwise die at 8 and 24 h, respectively, after reoxygenation. The percentage of dead cells in the cultures preconditioned in the presence of fumonisin B1 was not significantly different from that in the naive cultures: 17.9 vs. 17.4% at 8 h and 20.8 vs. 25.4% at 24 h after reoxygenation (P = 0.01 and 0.026 for 8 and 24 h, respectively, vs. tolerant cells, n = 4; Fig. 6B). These studies indicate that ceramide de novo synthesis is required for induction of tolerance to severe hypoxia by hypoxic or TNF-α preconditioning.

Fig. 6. Role of ceramide in hypoxic preconditioning of neurons against hypoxia-induced neuronal injury. A: neuronal cultures were subjected to 2.5 h of hypoxia in presence of 10 µM C-2 ceramide. Number of dead cells was determined at 8 and 24 h after cell reoxygenation. Dead cells were also measured in C-2-treated and untreated cultures maintained in normoxic conditions. Each measurement was performed in 8 wells and averaged. Each bar represents mean ± SD of 4 experiments. B: neuronal cultures were preconditioned with 20 min of hypoxia in absence or presence of 50 µM fumonisin B1 and then subjected to 2.5 h of hypoxia. Each measurement was performed in 8 wells and averaged. Each bar represents mean ± SD of 3 experiments. *Significant difference from naive cells; **significant difference from preconditioned cells.

Fig. 7. Intracellular ceramide levels in neurons preconditioned with hypoxia or TNF-α. Neuronal cultures were grown in 60-mm tissue culture plates and preconditioned with mild hypoxia for 20 min (A) or pretreated with 25 ng/ml TNF-α (B) in absence or presence of 100 µM fumonisin B1. Ceramide levels in preconditioned neurons were measured by means of reverse-phase HPLC at indicated times after preconditioning or TNF-α addition. Results are presented as percent increase over concentrations in unstimulated cells (0.7 ± 0.1 pmol/nmol lipid phosphate). Each data point represents mean ± SD of 3–4 experiments.
DISCUSSION

A molecular mechanism(s) that is switched on at the end of the lag period after ischemic preconditioning and confers cell resistance to ischemic stress should meet the following requirements. First, it should affect various types of brain cells as well as their multiple responses to ischemia. A mechanism affecting expression of multiple genes would suit this requirement. Second, the release or synthesis of such a “tolerizing” factor(s) should be delayed after onset of preconditioning and reach optimal levels when tolerance is demonstrated in the model system.

We have presented evidence that ischemic tolerance is mediated by de novo synthesis of ceramide triggered by TNF-α. Ceramide kinetics in preconditioned cells, its key role in TNF-α receptor signaling, and its pleiotropic effects on cells (including neuroprotection) conform to the above requirements well. This new function of ceramide is supported by the following observations.

First, exogenous TNF-α and exogenous C-2 ceramide were able to substitute for hypoxic pretreatment in making cortical neurons resistant to a subsequent hypoxic insult. Under these conditions, TNF-α and C-2 ceramide protected neurons to the same degree as did hypoxic preconditioning.

The role of TNF-α and its downstream messenger ceramide as mediators of hypoxic preconditioning is further supported by the fact that hypoxic preconditioning resulted in no tolerance if it was performed in the presence of TNF-α-neutralizing antibody (but not in the presence of nonspecific antibody) or in the presence of the inhibitor of ceramide de novo synthesis, fumonisin B₁. Double the ND₅₀ of antibody was used in the experiments. According to the manufacturer, this dose was able to neutralize 0.025 ng/ml of TNF-α. There were ~2.5 x 10⁶ cells/well, which means that, during preconditioning, 100 pg of TNF-α could be released per 10⁶ neurons. TNF-α ELISA demonstrated that the amount of TNF-α in culture medium increased by 6 pg/well, which contained ~2.5 x 10⁵ neurons, suggesting that ≥30 pg/10⁶ cells were released. These calculations do not include cell membrane- and plastic surface-bound TNF-α. In fact, the amount of TNF-α in the medium peaked at 8 h after preconditioning and decreased by 24 h, probably because of TNF-α binding to these surfaces. Our observation that preconditioned neurons expressed high levels of TNF-α p55 receptor further suggests engagement of TNF-α signaling pathways in induction of tolerance. Because the majority of TNF-α effects have been attributed to p55 receptor (1, 35), expression of p75 receptor has not been tested, although one cannot exclude its role in preconditioning.

The inhibitory effect of fumonisin B₁ on neuronal ceramide synthase (sphingosine-N-acyltransferase) has been previously demonstrated (30). The ability of fumonisin B₁ to block hypoxic preconditioning suggests that de novo synthesis of ceramide contributes to induction of tolerance.

Further evidence for the ceramide being a TNF-α messenger in induction of ischemic tolerance is derived from the measurements of ceramide levels in cells preconditioned with hypoxia or pretreated with TNF-α. Both treatments resulted in a delayed increase of intracellular ceramide levels, which coincided with development of resistance to severe hypoxic insult. These data are consistent with our previous observations which demonstrated that astrocytes and brain endothelial cells preconditioned with TNF-α also exhibited delayed ceramide responses, which coincided with a tolerant state (14). The ability of fumonisin B₁ to abolish ceramide synthesis parallels its effect on ischemic tolerance and strongly argues for the role of ceramide as a mediator of tolerance.

Different culture conditions and the older age of neuronal cells could explain the different temporal parameters required to achieve tolerance in our model compared with the model designed by Brueer and coworkers (5). Nevertheless, our study confirms their findings that a minimal lag period of ~24 h is required for cells to become tolerant after hypoxic preconditioning. Employment of fluorescent techniques for rapid quantitation of dead cells has allowed us to detect neuronal damage and protection immediately after hypoxic treatment even before reoxygenation, which has been impossible with traditional lactate dehydrogenase measurements because of the latency of the enzyme leakage (21). We demonstrate that hypoxic pretreatment attenuates neuronal injury that occurs during the hypoxic insult and neuronal injury that develops during reoxygenation. The fact that preconditioning did not result in cell loss or cell death rules out the possibility that selection of “stronger” cells during hypoxic pretreatment explains the data.

Ceramide effects in neuronal cells range from protective to apoptotic. Cell-permeable ceramide analogs induced apoptosis in embryonic chick hemispheric neurons (43) and in mesencephalic neurons (6). In different models, the same ceramide derivatives added exogenously exhibited a protective effect. Thus exogenous ceramide induced protection of hippocampal neurons against glutamate, FeSO₄, and amyloid β-peptide toxicity. This protective effect of ceramide was blocked by inhibitors of RNA and protein synthesis (15). Controversial results on the role of ceramide in apoptosis signaling in neurons have been obtained when endogenous ceramide levels were manipulated. Increase of intracellular ceramide levels by blocking ceramide catabolism in sensory neurons (36) and inhibition of intracellular ceramide levels with the inhibitor of its de novo synthesis (12) were found to promote apoptosis. In one study, both effects of ceramide (protective and proapoptotic) have been demonstrated in the same cells (spinal motoneurons), depending on ceramide concentrations (19). We previously reported that the C-2 ceramide effect on cell viability depends on its dose and demonstrated that 10 μM ceramide was not harmful to astrocytes and brain microvascular endothelial cells (14). According to measurements of apoptosis presented here, ~20% of cells in neuronal cultures were apoptotic, most probably due to culture conditions, to low numbers of astrocytes in these cultures, and to...
constitutive apoptosis, which occurs in developing brain. Addition of 10 µM C-2 ceramide did not cause an increase in the percentage of apoptotic cells.

Little is known about the molecular mechanisms mediating TNF-α and ceramide-induced cytotoxicity. Protection of sympathetic neurons against nerve growth factor (NGF) deprivation by exogenous ceramide has been associated with NGF binding to its low-affinity receptor (20). Ceramide was also shown to decrease the levels of microtubule-associated protein τ and to increase the calpain-derived spectrin breakdown product by modifying the activity of calpain 1 proteinase in PC-12 cells. It was suggested that the latter effect of ceramide might increase neuronal regeneration and remodeling. We have noticed that pretreatment with C-2 ceramide not only rescued hypoxia-injured cultures but also made control cultures look “healthier,” which might be related to described effects of C-2 ceramide on calpain. Much evidence has been accumulated that implicates activation of nuclear factor-κB (NF-κB) in mechanisms controlling apoptosis/survival in neuronal cells through stabilization of intracellular Ca²⁺ concentration, increase in density of the outward K⁺ currents, and induction of antioxidants (2, 11, 28). Which of these mechanisms is involved in ceramide-mediated hypoxic preconditioning in neuronal cells is not clear. Recent studies of myocardial preconditioning (29, 45) suggest a participation of NF-κB in adaptation to ischemia. In support of this hypothesis, loss of NF-κB activity was observed during brain ischemia and inhibition of NF-κB sensitized brain cells to cytotoxic effects of TNF-α in vitro (4).

More experiments are needed to elucidate the signaling events downstream from ceramide that make neurons tolerant to ischemic injury. The recently identified ceramide-activated protein kinase (24) and a serine-threonine phosphatase (9) could be suggested as attractive initial targets for these studies. Sphingosine or sphingosine 1-phosphate could be suggested as attractive initial targets for these events. Sphingosine or sphingosine 1-phosphate could be suggested as attractive initial targets for these reactions downstream from ceramide that make neurons tolerant to ischemic injury. The recently identified ceramide-activated protein kinase (24) and a serine-threonine phosphatase (9) could be suggested as attractive initial targets for these studies. Sphingosine or sphingosine 1-phosphate could be suggested as attractive initial targets for these events.

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REFERENCES


