Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms

Evelyne Gozal,1,2 L. R. Sachleben, Jr.,1 M. J. Rane,3 C. Vega,1 and D. Gozal1,2

1Kosair Children’s Hospital Research Institute, Department of Pediatrics, University of Louisville; 2Department of Pharmacology and Toxicology, University of Louisville; and 3Kidney Disease Program, Department of Medicine, University of Louisville, Louisville, Kentucky

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Gozal, Evelyne, L. R. Sachleben, Jr., M. J. Rane, C. Vega, and D. Gozal. Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. Am J Physiol Cell Physiol 288: C535–C542, 2005. First published November 10, 2004; doi:10.1152/ajpcell.00270.2004.—Episodic hypoxia, a characteristic feature of obstructive sleep apnea, induces cellular changes and apoptosis in brain regions associated with neurocognitive function. To investigate whether mild, intermittent hypoxia would induce more extensive neuronal damage than would a similar degree of sustained hypoxia, rat pheochromocytoma PC-12 neuronal cells were subjected to either sustained (5% O2) or intermittent (alternating 5% O2 35 min, 21% O2 25 min) hypoxia for 2 or 4 days. Quantitative assessment of apoptosis showed that while mild sustained hypoxia did not significantly increase cell apoptosis at 2 days (1.31 ± 0.29-fold, n = 8; P = NS), a significant increase in apoptosis occurred after 4 days (2.25 ± 0.4-fold, n = 8; P < 0.002), without increased caspase activation. Furthermore, caspase inhibition with the general caspase inhibitor N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) did not modify sustained hypoxia-induced apoptosis. In contrast, mild, intermittent hypoxia induced significant increases in apoptosis at 2 days (3.72 ± 1.43-fold, n = 8; P < 0.03) and at 4 days (4.57 ± 0.82-fold, n = 8; P < 0.001) that was associated with enhanced caspase activity and attenuated by Z-VAD-FMK pretreatment. We conclude that intermittent hypoxia induces an earlier and more extensive apoptotic response than sustained hypoxia and that this response is at least partially dependent on caspase-mediated pathways. In contrast, caspases do not seem to play a role in sustained hypoxia-induced apoptosis. These findings suggest that different signaling pathways are involved in sustained and intermittent hypoxia-induced cell injury and may contribute to the understanding of differential brain susceptibility to sustained and intermittent hypoxia.

Episodic hypoxia; neuronal cell death; caspase; hypoxic adaptation

Episodic hypoxia is a characteristic feature of a highly prevalent human disease, sleep apnea, and induces cellular changes, architectural disorganization, and apoptosis in brain regions associated with neurocognitive functions (14). In contrast, long-term exposure to chronic, sustained hypoxia (SH) is associated with adaptive signaling pathways, ultimately leading to improved energy conservation and more efficient cellular use of O2 (7, 20, 37). The neurobehavioral and pathological consequences of intermittent hypoxia (IH) suggest that this type of hypoxic stimulus fails to induce the adaptive response required for neuronal cell survival in the setting of hypoxia. Recent efforts to classify cell death as either apoptosis or necrosis have led to a wider definition of apoptosis. Programmed cell death is a process mediated by a signaling cascade, while necrosis involves the recruitment of separate cell death-related mechanisms and is triggered when cells are challenged beyond repair by a physical or chemical challenge (19, 22, 23). The widely accepted pathway of apoptotic signaling involves a caspase activation cascade and mitochondrial membrane permeabilization, ultimately leading to the typical features of apoptosis, i.e., nuclear condensation, DNA fragmentation, and targeted cell degradation (23, 29, 32, 50). Caspases are a family of cysteine proteases existing in normal cells as inactive enzymes, activated by one or more sequential proteolytic events, and leading to the degradation or functional inactivation may not confer complete cytoprotection in various experimental systems, and cell death may still occur with characteristic apoptotic features (48, 52, 56).

Hypoxic or hypoxia-reoxygenation injuries induce both necrosis and apoptosis in neuronal cells (1, 4, 31, 47, 49). Hypoxia-induced pathophysiology, as well as the extent of apoptotic injury, is dependent on the type and severity of O2 deprivation (1, 31, 34). Investigators at our laboratory previously showed that exposure to mild IH resulted in partially irreversible memory and learning impairments in both animal and human studies (13–16, 39–41). In addition, such neurobehavioral consequences of IH are preceded by marked increases in apoptosis in the rat cortex and the CA1 region of the hippocampus, peaking at 48-h exposure (14). These findings are compatible with the concept of a slowly evolving excitotoxicity that may occur as a consequence of impaired cellular energy metabolism and free radical production and that may lead to neuronal apoptosis (1, 3, 31, 39, 42). However, SH of similar magnitude appears not to be associated with neuronal apoptosis to such an extent or with such extensive alterations in neurocognitive function (43, 45), suggesting that the mode of hypoxic stimulus presentation has major implications for neuronal cell outcome. Thus whether both SH and IH trigger a common signaling pathway leading to neuronal apoptosis is currently unclear. Determining the differences in signaling pathways triggered by SH and IH could therefore contribute to the understanding of the fundamental differences in the pathophysiology of these two hypoxic stimuli.

Address for reprint requests and other correspondence: E. Gozal, Kosair Children’s Hospital Research Institute, 570 South Preston St., Suite 321, Louisville, KY 40202 (E-mail: evelyne.gozal@louisville.edu.).

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The rat pheochromocytoma (PC)-12 cell line is a dopaminergic, neoplastic cell line that exhibits unique sensitivity to changes in O_2 availability and is frequently used as a cellular model to study neuronal vulnerability to hypoxia (9, 24, 45, 46). Similarly to type I carotid body cells, PC-12 cells respond to O_2 changes with membrane depolarization, increased intracellular Ca^{2+}, and release of neurotransmitters, followed by changes in the expression of specific genes that are known to play a role in the cellular response to hypoxia (9, 45, 46). We therefore took advantage of this well-established cellular model to further explore the effect of mild SH and IH on neuronal cell death and the potential involvement of caspase-3 activation in hypoxia-mediated cell death after either mild SH or IH challenges.

**MATERIALS AND METHODS**

**Cell culture and hypoxic exposures.** Rat PC-12 cells (American Type Culture Collection, Manassas, VA) were cultured either on collagen-coated six-well plates in RPMI 1640 medium (2 ml/well) supplemented with 10% horse serum and 5% fetal bovine serum or in glass slide chambers in 0.5 ml of medium per chamber. After reaching 60% confluency, cells were pretreated for 1 h with vehicle or with 20 μM N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK; Calbiochem, San Diego, CA) and then were exposed to normoxia (RA; 21% O_2, 5% CO_2, and balance N_2), SH (5% O_2, 5% CO_2, and balanced N_2), or IH (5% O_2, 5% CO_2, and balance N_2 for 35 min alternating with 25-min RA), using a custom-designed, computer-controlled incubation chamber attached to an external O_2-CO_2 computer-driven controller (Biospherix, Redfield, NY) for 2 and 4 days. Chamber O_2, N_2, and CO_2 levels were continuously monitored and adjusted according to the desired programmed profile. In addition, O_2 content in the medium was measured with a fiberoptic O_2 sensor (Ocean Optics, Dunedin, FL) placed 1 mm above the cell layer to ensure the specific experimental profile implementation. O_2 levels in the air phase as well as in the medium were continuously monitored and recorded using appropriate AD-controller interface and software (Biospherix).

**Western blotting.** Cells exposed to RA or 2 and 4 days of SH or IH were lysed in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS, 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 mg/ml aprotinin, and 20 mg/ml leupeptin). Proteins were quantified by performing a modified Lowry protein assay (DC-Bio-Rad, Hercules, CA). Equal amounts of proteins were separated on a Tris-glycine 4–12% gradient precast gel (Invitrogen, San Diego, CA), transferred to a nitrocellulose membrane, and immunoblotted with a hemoglobin (HO-1) monoclonal antibody (1:2,500 dilution; Stressgen, Victoria, BC, Canada). Proteins were revealed using enhanced chemiluminescence (ECL Plus; Amersham, Piscataway, NJ). Equal loading and uniform transfer of proteins in the different lanes were verified using Ponceau S staining of the transferred nitrocellulose membrane.

**Assessment of cell death.** After 2 and 4 days of SH, IH, or RA, cells were collected and lysed, and apoptosis was assessed using a Cell Death ELISA Plus kit (Roche Molecular Biology, Indianapolis, IN) according to manufacturer’s protocol. This assay quantifies nucleosomal DNA fragments by using mouse monoclonal antibodies against DNA and histone, providing a quantitative measure of apoptosis. Proteins were assayed using a modified Lowry protein assay (DC-Bio-Rad) and results were normalized per milligram of protein and expressed as the increase in apoptosis relative to the respective controls. Results are presented as means ± SE of relative increases compared with RA. In addition, PC-12 cells were grown in chamber slides, exposed to the various hypoxic profiles described above, and stained with bisbenzimide H33258 Hoechst nuclear stain (Sigma, St. Louis, MO). Nuclear morphology was then observed under a fluorescence microscope (Olympus IX50), revealing chromatin condensation, one of the hallmark features of apoptosis.

**In situ activity staining.** Cells grown in chamber slides were exposed to SH, IH, or RA for 2 or 4 days. To visualize general caspase activation, the Caspase-3 FITC-VAD-FMK in situ marker (Promega, Madison, WI) was used according to the manufacturer’s instructions and was added to the cells to obtain a final concentration of 10 μM, followed by incubation at 37°C for 20 min. The Caspase-3 FITC-VAD-FMK in situ marker solution was removed, and cells were washed with PBS, fixed in 10% formalin for 30 min, and washed three times for 5 min each time with PBS. Active caspase staining was then observed under a fluorescence microscope (Olympus IX50).

**Caspase-3/7 activity assay.** Caspase-3/7 activity was assessed quantitatively by performing the Apo-ONE homogeneous caspase-3/7 assay (Promega) according to the manufacturer’s instructions. Cells in duplicate wells were assayed after exposure to RA, SH, or IH for 2 or 4 days. Caspase activity was assessed using fluorescence reading of cleaved rhodamine 110 bis-([N-CBz-i-aspartyl]-l-glutamyl-l-valyl-l-aspartic acid amide) with a SPECTRAFluor Plus microplate reader at 485-nm excitation and 535-nm emission wavelengths. In this assay, fluorescence of the cleaved substrate is proportional to the amount of caspase-3/7 activity present. Measurements were expressed in relative fluorescence units (RFU) ± SE for each condition.

**RESULTS**

**Induction of apoptosis by mild sustained and intermittent hypoxia.** Controlled exposure to IH in an in vitro cellular model is complicated by the requirement of reliably controlling and monitoring oscillations of O_2 levels in the cell culture medium. In this study, we used custom-designed chambers placed inside a cell culture incubator and attached to an external O_2-CO_2 computer-driven controller such that the prescribed hypoxia profiles were accurately delivered and controlled in the culture medium by monitoring dissolved O_2 tension in one of the culture dishes inside the chambers, using non-O_2-consuming fiberoptic O_2 sensors placed 1 mm above the cell layer. The IH profile recorded in the gas phase (dashed line), as well as in medium (solid line), is shown in Fig. 1A, demonstrating the time lag between air and medium hypoxia. The IH profile was selected to allow O_2 levels to decrease in the medium, reach an equilibrium at 5% O_2, and maintain hypoxic conditions in the medium to stimulate hypoxia-inducible gene expression. To confirm that such hypoxic exposures were sufficient to induce hypoxic gene response in both IH and SH, we assessed the expression of HO-1, a well-established hypoxia-inducible gene (25). HO-1 protein expression increased in cells exposed to 2- and 4-day SH and IH, suggesting that the hypoxia profile applied in our study adequately stimulated the cells to induce hypoxia-inducible gene expression (Fig. 1B).

To examine differences in neuronal vulnerability, apoptosis was assessed in PC-12 cells exposed to either SH or IH. To avoid severe traumatic cell injury, a relatively moderate (5% O_2) level of hypoxia was selected. Exposure of PC-12 cells to
SH did not appear to significantly induce apoptosis after 2 days as assessed using Hoechst nuclear staining, but increased cell death was detected in the 4-day SH condition on the basis of the appearance of characteristic morphological apoptotic features such as nuclear condensation (Fig. 2). Increased chromatin condensation also was identified in the 2- and 4-day IH conditions.

One of the earliest detectable morphological alterations of apoptosis is the condensation of nuclear chromatin into more densely packed material along the nuclear membrane, resulting in a brighter signal when assessed with Hoechst nuclear staining. To confirm the qualitative assessment of apoptotic morphology obtained using Hoechst staining, we performed a quantitative determination of nucleosomal DNA resulting from apoptotic DNA fragmentation using a nucleosomal DNA ELISA. Indeed, there was no significant DNA fragmentation after 2-day SH (1.31 ± 0.29-fold increase, n = 8; P = NS) (Fig. 3), indicating that such a mild level of SH is not detrimental to cell survival. However, increased DNA fragmentation was detected in the 4-day SH-treated cells (2.25 ± 0.40-fold increase, n = 8; P < 0.002) (Fig. 3). In contrast, exposure to IH at a similar level of hypoxia induced significant increases in apoptosis at 2 days (3.72 ± 1.43-fold increase, n = 8; P < 0.03) (Fig. 3) and increased further after 4 days of IH (4.57 ± 0.82-fold increase, n = 8; P < 0.001) (Fig. 3), data that are in agreement with the qualitative data obtained using Hoechst staining (Fig. 2). In addition, the extent of apoptosis induced by 2 days of IH was comparable to that induced by 4 days SH (Fig. 3).

Involvement of caspases in hypoxia-induced apoptosis. In situ active caspase staining showed no significant caspase
activation after 2 or 4 days of SH compared with RA (Fig. 4).

In contrast, IH-exposed PC-12 cells stained positively for active caspase after either 2 or 4 days of hypoxia (Fig. 4), suggesting that in contrast to SH-induced apoptosis, IH-induced apoptosis involves caspase-dependent pathways in PC-12 cells. In agreement with these findings, inhibition of caspase activation with a general caspase inhibitor, Z-VAD-FMK, did not inhibit SH-induced apoptosis (SH: 2.2 ± 0.4 vs. SH + Z-VAD-FMK: 1.82 ± 0.2; n = 5) (Fig. 5), while caspase inhibition significantly decreased IH-induced apoptosis (IH: 4.6 ± 0.8 vs. IH + Z-VAD-FMK: 2.5 ± 0.4; n = 5) (Fig. 6). Furthermore, RA-, SH-, and IH-exposed PC-12 cells were subjected to caspase-3/7 activity assays to corroborate the findings from in situ active caspase staining. Caspase-3/7 activity findings confirmed that SH did not significantly alter caspase-3/7 activity compared with RA after 2 days (SH: 576.1 ± 78.3 RFU vs. RA: 379.5 ± 97.9 RFU) or after 4 days.
In contrast, IH treatment significantly increased caspase-3/7 activity 2.6-fold compared with RA at 2 days (IH: 740.6 ± 163.8 RFU vs. RA: 379.5 ± 97.9 RFU, n = 3; P < 0.04) and 4.8-fold at 4 days (IH: 1,636.5 ± 435.8 vs. RA: 337.9 ± 45.8, n = 3; P < 0.002) (Fig. 7).

Taken together, these findings suggest that PC-12 cells are more susceptible to IH than to SH and that even mild hypoxia such as that used in the present experiments can induce apoptotic features and cell death. Furthermore, we could not detect caspase activation with such a mild SH stimulus, while caspase-mediated cell death was present in PC-12 cells exposed to IH.

**DISCUSSION**

Consequences of IH or SH on neuronal cell viability are relevant to clinical conditions such as cardiopulmonary disease, stroke, and sleep apnea. While stroke and other ischemic diseases involve episodes of severe ischemia-hypoxia, most pathological conditions are associated with milder and often intermittent hypoxic events. While a short exposure to severe SH or to a few cycles of IH may induce preconditioning and increase tolerance to hypoxia (37, 55), previous studies uncovered increased neuronal vulnerability to long-term IH characterized by impaired adaptive response to hypoxic injury. IH, a hallmark of obstructive sleep apnea, has severe cardiovascular and neurocognitive consequences (11–14, 21, 39 – 41). In contrast, chronic SH does not alter blood pressure (21) and may be associated with milder neural deficits than those associated with IH (44).

Investigators at our laboratory (14) previously established that, unlike SH, prolonged IH exposure results in neuronal apoptosis in vulnerable brain regions such as the CA1 hippocampal region. Thus, while neuronal cells appear to mount adaptive responses to SH, they seem unable to do so effectively when exposed to IH. We now present evidence suggesting that hypoxia-induced cell death in the PC-12 cell line, a neuronal cell line model for the study of hypoxic sensitivity, is dependent on stimulus presentation (IH vs. SH) and involves differential recruitment of caspase signaling pathways.

The study of hypoxia at the cellular level has been complicated by technical limitations inherent in accurate O2-level control in a cell culture environment. To achieve the suitable level of IH in the cell culture medium, the O2 diffusion rate into the liquid medium is a major limiting factor that requires a longer cycle duration than the IH cycles used for animal exposure. Strategies that enable accurate delivery of targeted oxygenation profiles were recently reported for adherent cells (2), and in the present study we used a newly developed incubator chamber system that allows accurate control and monitoring of O2 levels in cell culture medium at 1 mm above the cell layer. Measuring PO2 closer to the cell layer would disturb the cells and interfere with cell viability; however, we cannot exclude the possibility that the PO2 on the cells may be lower because of cellular O2 consumption. Our findings show that both mild SH and mild IH can induce apoptotic charac-...
Ca²⁺ activation. Calpain is a Ca²⁺-dependent proteolytic enzyme and results in apoptosis-like cell death with either some or all of the morphological features of this process (48, 53, 56). Furthermore, under pathological conditions, apoptosis and necrosis often coexist (27, 28, 31, 53) or may occur sequentially, with necrosis usually occurring after apoptosis as a result of energy depletion (28). Cellular ATP levels were not assessed in the present study; however, researchers in numerous studies have reported ATP depletion in severe hypoxic conditions (20, 31). In anoxia or hypoxia, neurons are protected from energy depletion by reducing non-vital cellular energy consumption and releasing autocrine-paracrine growth factors (17, 20, 33, 54). Primary embryonic rat cortical neurons exposed to severe hypoxia displayed a 10% reduction in intracellular ATP and an 85% reduction in protein synthesis, while glycolytic rates did not change significantly (33). In addition, improved electrophysiological functions in parapyramidal neurons, compared with dentate gyrus neurons exposed to severe hypoxia, correlated with mild hypoxia-induced ATP depletion (37). Thus, in PC-12 cells, reducing cellular energy use may provide an initial protective mechanism and delay the apoptotic response to SH, thereby resulting in greater cell viability after 2-day exposure compared with IH.

Activation of caspases has historically been viewed as occurring downstream from the commitment to die. ATP is required to conduct a caspase-3 activation and cell suicide program (28, 35). Prolonged SH could therefore induce cell death by ATP depletion that may also prevent caspase-3 activation and result in caspase-independent cell death with mixed features of apoptosis and necrosis (51). Indeed, in a model of permanent rat middle cerebral artery occlusion, neurons with apoptotic nuclear morphological features but no cleaved caspase-3 expression were identified in the ischemic zone, in addition to caspase-3-positive apoptotic cells (10). Furthermore, colchicine treatment of primary murine cerebellar granule neurons induced apoptosis with characteristic nuclear chromatin condensation in the presence or absence of the caspase inhibitor Z-VAD-FMK (51). These findings suggest that nuclear condensation can occur in the absence of caspase activity, consistent with our observation of caspase-independent apoptotic cell death in PC-12 cells after 4 days of SH. However, the mechanisms underlying SH induction of caspase-independent apoptosis in these cells remains to be identified. Other proteases, such as calpain alone or in conjunction with caspase activity, could be implicated. Yamakawa et al. (53) reported that PC-12 cells exposed to severe SH (1% O₂) for up to 72 h undergo apoptosis as well as necrosis in a Ca²⁺-dependent manner, involving both caspase-3 and calpain activation. Calpain is a Ca²⁺-dependent protease, and addition of EGTA, a Ca²⁺ chelator, nearly completely abolished hypoxia-induced PC-12 apoptosis. Furthermore, hypoxia induces depolarization and increases intracellular Ca²⁺ concentration in PC-12 cells (57), similar to the in vivo response of carotid body type I cells (5). Thus long-term SH may induce the release of Ca²⁺, the activation of calpain, and the depletion of ATP reserves, consequently preventing caspase-3 activation and potentially resulting in the apoptotic features identified after 4 days of SH.

Alternatively, SH could induce other caspase-independent pathway mediators such as apoptosis-inducing factor, which has been shown to induce the nuclear condensation and DNA fragmentation that are characteristic of apoptotic cell death, in the absence of caspase activation (8). Caspase activation has been reported after hypoxia-reoxygenation in cultured neurons from embryonic rat forebrain, persisting in late reoxygenation, neonatal rat cerebral slices, and cerebral microvascular endothelial cells, essentially during reoxygenation (4, 18, 26). Because intermittent reoxygenation during IH most likely allows cells to restore the pool of ATP, caspase activation could proceed in IH-exposed PC-12 cells, thereby resulting in caspase-dependent apoptosis. In addition, the severity of the insult may play a role in cells’ fate (1, 28, 31, 34), and our selection of a relatively mild level of hypoxia (5% O₂) may explain the relatively low magnitude of cell death induced by SH, while the reoxygenation in IH-exposed cells may induce apoptosis via caspase proteolytic activity, free radical generation, or irreversible mitochondrial damage (31, 43). We have shown in the present study that a relatively mild hypoxic stimulus, whether intermittent or sustained, can induce HO-1 expression, indicating that both IH and SH can induce this hypoxia-inducible gene. The extent of the apoptotic response to IH is to the least surprising, considering that the duration of hypoxia in IH is essentially half that endured by SH-exposed cells. Thus the magnitude of IH-induced cell death at 4 days is further increased compared with that which occurs after 2 days of SH, with an equivalent amount of time spent in hypoxia. These findings therefore suggest that the intermittent characteristics of IH and the recurring pattern of reoxygenation may affect PC-12 cell survival to a greater extent than the severity or duration of hypoxia. These observations are consistent with our previous in vivo findings of marked neuronal cell loss in a rat model of obstructive sleep apnea (14, 16, 30, 39, 41, 42).

In summary, although the most common signaling pathway for apoptosis involves caspase activation, it is unlikely that this pathway, notwithstanding its diversity, could be uniquely responsible for neuronal cell death in hypoxia. Moreover, the energetic requirement for caspase activation is likely to induce the activation of alternative, more energy-efficient, caspase-independent pathways when energy depletion occurs, such as during prolonged SH. This strategy may allow PC-12 cells to survive and mount an appropriate adaptive response to hypoxia. In contrast, IH, which essentially involves recurrent reoxygenation episodes, leads to increased generation of 'O₂ and induces oxidant stress (39). Cellular energy availability during IH allows for caspase activation and eventual irreversible mitochondrial damage, preventing neuronal adaptation to hypoxic injury. Further understanding of the mechanisms of the increased PC-12 susceptibility to IH uncovered in the present study will allow for the formulation of novel therapeutic strategies that aim to minimize or to prevent the adverse neuropathological consequences of conditions such as obstructive sleep apnea syndrome.

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