Xenon and Sevoflurane Protect against Brain Injury in a Neonatal Asphyxia Model


Background: Perinatal hypoxia–ischemia causes significant morbidity and mortality. Xenon and sevoflurane may be used as inhalational analgesics for labor. Therefore, the authors investigated the potential application of these agents independently and in combination to attenuate perinatal injury.

Methods: Oxygen–glucose deprivation injury was induced in pure neuronal or neuronal–glial cocultures 24 h after preconditioning with xenon and/or sevoflurane. Cell death was assessed by lactate dehydrogenase release or staining with annexin V–pro-pidium iodide. The mediating role of phosphoinositide-3-kinase signaling in putative protection was assessed using wortmannin, its cognate antagonist. In separate in vivo experiments, perinatal asphyxia was induced 4 hours after preconditioning with analgesic doses alone and in combination; infant size was assessed 7 days later, and neuromotor function was evaluated at 30 days in separate cohorts. The role of phosphorylated cyclic adenosine monophosphate response element binding protein in the preconditioning was assessed by immunoblotting.

Results: Both anesthetics preconditioned against oxygen–glucose deprivation in vitro alone and in combination. The combination increased cellular viability via phosphoinositide-3-kinase signaling. In in vivo studies, xenon (75%) and sevoflurane (1.5%) alone as well as in combination (20% xenon and 0.75% sevoflurane) reduced infarct size in a model of neonatal asphyxia. Preconditioning with xenon and the combination of xenon and sevoflurane resulted in long-term functional neuroprotection associated with enhanced phosphorylated cyclic adenosine monophosphate response element binding protein signaling.

Conclusions: Preconditioning with xenon and sevoflurane provided long-lasting neuroprotection in a perinatal hypoxic–ischemic model and may represent a viable method to preempt neuronal injury after an unpredictable asphyxial event in the perinatal period.

PERINATAL hypoxic–ischemic brain damage remains a major cause of acute mortality and chronic neurologic morbidity in infants and children. An estimated 2–4 per 1,000 full-term infants experience an asphyxial injury either antenatally or perinatally, and of these, approximately 15–20% die.1 Of the survivors, 25% exhibit permanent disability such as cerebral palsy, epilepsy, and learning difficulties.2 Given the magnitude of the problem, it is crucial that strategies are developed to minimize the long-term neurologic sequelae of neonatal hypoxia–ischemia. Because many of these devastating events occur in the perinatal period, we propose that if a laboring parturient could receive a pharmacologic agent that could protect her unborn child from subsequent perinatal neuronal injury (i.e., precondition against the later injury), this may represent a method of reducing the adverse sequelae of perinatal asphyxia.

Ischemic preconditioning of the heart3 was demonstrated before that of the brain was discovered,4 and hence the biologic phenomenon has been best described in cardiac tissue. When exposed to sublethal ischemic episode, the heart or brain initiates endogenous neuroprotective mechanisms to induce tolerance, so that it is protected against subsequent ischemic injuries. This phenomenon has been demonstrated in various in vivo and in vitro models.5–7 However, the iatrogenic induction of ischemia is both unfeasible and unethical, and therefore, research into pharmacologic preconditioning, including the use of anesthetic agents,8 to mimic ischemic preconditioning protection offers a mechanism to circumvent these problems. Therefore, preconditioning of the brain with pharmacologic agents against ischemic injury might be a strategy to reduce morbidity and mortality from perinatal injury. This is especially of importance because current peripartum fetal monitoring is poor at detecting the compromised fetus, thus delaying the institution of neuroprotective strategies to protect the at-risk neonate.9

We and others have demonstrated that the noble anesthetic gas xenon has a neuroprotective potential in both in vivo and in vitro models of acute neuronal injury.10–13 Xenon also protected against brain damage induced by hypoxic–ischemic injury when administered in a preconditioning setting.14 In contrast, nitrous oxide, another N-methyl-D-aspartate receptor antagonist, which is widely used for labor analgesia, is unable to precondition against subsequent perinatal neurologic injury or myocardial ischemia.14,15 Volatile anesthetics, such as sevoflurane, have also been shown to protect myocardium from ischemic injury in a preconditioning setting.16,17 Furthermore, sevoflurane (0.8% in oxygen) has recently been shown to provide labor analgesia that is
superior to that of nitrous oxide (50%–oxygen); however, it is unknown whether sevoflurane can precondition against perinatal asphyxial injury alike xenon can. The inert gas xenon is an N-methyl-D-aspartate receptor antagonist, whereas sevoflurane acts mainly via γ-aminobutyric acid type A receptors. Because these preconditioning agents exert their effects through different mechanisms, we hypothesized that in combination, their efficacy would be enhanced. Furthermore, a potential combination should reduce the costs of xenon administration. The current study sought to investigate whether xenon and sevoflurane could act both independently and in combination to prevent neuronal damage in vitro and in an in vivo model of neonatal asphyxia.

Materials and Methods

The study protocol was approved by the Home Office (London, United Kingdom), and all efforts were made to minimize the animals’ suffering and the number of animals used.

Neuronal–Gliial Coculture

The neuronal–glial cells were cultured according to our established protocol. Briefly, the glial cells derived from cerebral neocortices were obtained from early postnatal (1- to 2-day-old) pups of Balb/c mice. They were cultured in a medium consisting of Eagle minimum essential medium augmented with 20 mM glucose, 26 mM NaHCO₃, 10% fetal bovine serum, 10% heat-inactivated horse serum (Gibco, Paisley, United Kingdom), and 10 ng/ml murine epidermal growth factor (GibcoBRL). One week later, neuronal cells obtained from cerebral neocortices of fetal BALB/c mice at 14–16 days of gestation were plated at a density of 1.25 × 10⁶ cells/cm² on the confluent monolayer of glial cells. Neuronal cells reached confluence 10 days after plating.

Cells were kept in purpose-built airtight, temperature-controlled, cell-culture chambers. Preconditioning conditions were achieved with the desired concentration of xenon (25–75% atm) or sevoflurane (0.67–3.3%) or a combination of xenon (25–75% atm) and sevoflurane (0.67–3.3%) or in combination to prevent neuronal damage in vitro and in an in vivo model of neonatal asphyxia.

The medium was analyzed using a standardized colorimetric enzyme kit (Sigma). Neuronal protection provided by the exposure to xenon and/or sevoflurane was expressed as the percentage of the LDH released without intervention.

Pure Neuronal Culture

Neuronal cells derived from cerebral neocortices were harvested from 16-day-old embryonic mice by cesarean delivery for pregnant Balb/c mice as described in the Neuronal–Glial Coculture section. The cells were plated at a density of 1.2 × 10⁵ cells/cm² on 24-well plates precoated with poly-L-lysine (Cater, Cambridge, MA), and the cultures were maintained at 37°C in a humidified 5% CO₂ and air environment and fed with neurobasal media supplemented with B27 (×1) and glutamine (25 µM). On day 5 after neuronal plating, 100 µl/10 ml cytosine arabinoside (10 µM; Sigma) was added to the cell cultures to halt nonneuronal cell division. Neuronal cell cultures were ready to use on day 7.

Cells were preconditioned with 12.5% xenon and 0.67% sevoflurane alone or in combination with the same protocol described above (neuronal–glial coculture section). To study the effects of the phosphoinositide-3-kinase (PI3K) pathway on preconditioning, 100 nM wortmannin (a dose chosen from pilot studies to block the PI3K pathway without cytotoxicity) was added to the culture medium just before the combined preconditioning of xenon and sevoflurane. Immediately after preconditioning, the culture medium was replaced by a wortmannin-free medium.

Twenty-four hours after oxygen–glucose deprivation, cells were stained for flow cytometry analysis. The cells were washed with HEPBS buffer and then detached with 0.2% trypsin–EDTA. Thereafter, cells were stained with annexin V–fluorescein isothiocyanate conjugate (0.4 µg/ml) and subsequently with propidium iodide (0.8 µg/ml; Sigma-Aldrich, Poole, United Kingdom). A minimum of 10,000 cells per sample was analyzed with flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, CA) to determine the population of apoptotic cells (annexin V positive and PI negative), necrotic or dead cells (propidium iodide positive), and viable cells (both annexin V and propidium iodide negative). Thus, annexin V–positive and propidium iodide–negative cells are apoptotic. Cells staining positive for both annexin V and propidium iodide may represent either necrotic cells or cells entering the final phases of apoptosis, thus approaching cell death.

Animal Model of Hypoxic–Ischemic Injury

The model of neonatal asphyxia was adapted from a previous report. Briefly, 7-day-old postnatal rats (n = 6/group) were preconditioned by a 120-min exposure to 20% or 75% xenon, 0.75% or 1.5% sevoflurane, or 20% xenon plus 0.75% sevoflurane in 25% oxygen balanced.
with nitrogen. Four hours after exposure, the animals underwent right common carotid artery ligation during surgical anesthesia followed by hypoxia as previously described.22 The exposure chambers were partially submerged in a water bath, and the desired brain temperature (37°C), measured with a telemetry temperature monitoring system (VitalView; Mini-Mitter, Bond, OR), was targeted using an appropriate water temperature in the bath. In each set of experiments, a temperature probe was implanted (~2 mm from the bregma and 2 mm away from the sagittal sinus; the tip of the probe was advanced to the subcortex and fixed on the skull with glue) in a sentinel rat pup that was not further assessed for brain injury.

Seven days after the injury, the pups were killed by an overdose of pentobarbitone (100 mg/kg, intraperitoneal) and perfused transcardially with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and placed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight. The blocks of brain were dehydrated, embedded in wax, and sectioned into 5-μm slices. After staining with the cresyl violet (Nissl), six slices were selected from each pup to match predefined brain regions relative to the bregma in adults (+2, +1, 0, −2, −4, and −5 mm; Rat Brain Atlas, Academic Press, San Diego, CA). Once identified, each slice was photographed, and the infarct size (mm²; the area of contralateral intact hemisphere − the area of ipsilateral healthy tissue) was calculated using data analysis software (ImageJ version 1.31, NIH Image software; Bethesda, MD) by one author who was blinded to the treatment that the pups had received. The area under curve was calculated from the plot constructed from the infarct area against the distance relative to the bregma for statistical analysis.

Other cohorts were allowed to live up to 30 days after hypoxia-ischemia, and then they underwent coordination testing. Coordination was tested by placing rats on a rotarod rotating at 30 rpm, and the latency to falling off the rod was assessed (maximal latency was 300 s). For each of the functional assays, the rat pup was tested three times, with a 10-min interval between each assessment, and the mean of the three assessments was used for each rat in the analysis.

Western Blot Analysis
The ipsilateral hemispheres harvested 4 h after gas exposure were used for Western blotting with our established protocol.14 The investigated antigens were reacted with rabbit monoclonal antibody directed against phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) or cyclic adenosine monophosphate response element binding protein (CREB) (1:1,000; New England Biolab, Hitchin, United Kingdom) overnight at 4°C. Horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G (1:2,000; New England Biolab) was used to detect the primary antibodies. The bands were visualized with the enhanced chemiluminescence system (ECL; Amersham Biosciences, Little Chalfont, United Kingdom), and intensities were quantified by densitometry. Results are normalized with CREB and expressed as fractions of control.

Statistical Analysis
Data were expressed as mean ± SEM. Statistical analysis was performed with analysis of variance followed by the Student–Newman–Keuls test for parametric data. Long-term neurologic outcome after preconditioning was assessed using nonparametric analysis of variance with Dunn post hoc tests. P < 0.05 was considered to be of statistical significance. Data analyses were performed using INSTAT (London, United Kingdom).

Results

Xenon and Sevoflurane Alone and in Combination In Vitro
Preconditioning with xenon for 2 h produces a concentration-dependent reduction in LDH release after oxygen–glucose deprivation. LDH release was significantly reduced by xenon concentrations of 50% and 75% to 55 ± 12% and to 49 ± 12% of control, respectively (P < 0.05). Xenon at 12.5% and 25% displayed a trend of decreasing LDH release with increasing concentrations; however, the results were not statistically significant (P > 0.05; fig. 1A). Sevoflurane preconditioning did not produce a significant reduction of LDH release up to 1.9%, but at sevoflurane concentrations of 2.7% and 3.3%, LDH release was decreased to 64 ± 6% (P < 0.05) and 37 ± 5% (P < 0.01) of control, respectively (fig. 1B). Individually, sevoflurane at 0.67% and xenon at 12.5% did not cause statistically significant reduction in LDH release. However, when the two gases are used (at these ineffective doses) in combination, LDH release was significantly reduced to 59 ± 5% of control (P < 0.01; fig. 1C).

Assessment of Necrotic, Viable, and Apoptotic Cell Populations
Flow cytometry analysis was used to determine whether the gas combination exerts its effect via an antiapoptotic or antinecrotic mechanism. Control cultures (without intervention) had a majority of viable cells (76 ± 1%). Oxygen–glucose deprivation provoked 70 ± 2%, 75 ± 2%, and 81 ± 2% of necrotic cell death in the sham, 12.5% xenon, and 0.67% sevoflurane exposure conditions, respectively. However, exposure to the combination of 12.5% xenon and 0.67% sevoflurane increased the number of viable cells by 59% and reduced necrotic cell death by 41% when compared with oxygen–glucose deprivation without gas exposure (P < 0.01; fig. 2A). These data suggest that xenon and sevoflurane, when used in combination, exert their precondi-
tioning action through an antinecrotic mechanism. It is notable that there was a significant increase in the number of apoptotic neurons (35 ± 4% [P < 0.01] vs. 21 ± 1% in the sham group). To establish whether the preconditioning afforded by the xenon and sevoflurane combination is dependent on the PI3K pathway, wortmannin, a PI3K inhibitor, was added during the gas exposures. Wortmannin almost abolished the preconditioning effect of the combination of xenon and sevoflurane without exerting any effect on cell death when given alone (fig. 2B), suggesting that PI3K may be involved in the neuroprotective effect provided by the combination of xenon and sevoflurane.

Morphology and Long-term Function In Vivo
To determine whether the interaction between xenon and sevoflurane in vitro can also be observed in a clinically relevant model in vivo, we used the Rice-Vanucci model of neonatal hypoxic-ischemic injury. Under our experimental conditions, cerebral focal infarction of pup brain is highly reproducible, as presented in figures 3A and B.14,21 Xenon at 20% and sevoflurane at 0.75% did not provide any protection when compared with controls. There was no statistically significant decrease in the area under the curve of infarction when control (73.2 ± 10 arbitrary units), 20% xenon (71.6 ± 8.8), and 0.75% sevoflurane (64.3 ± 8.9) were compared (P > 0.05; fig. 3C). However, 75% xenon and 1.5% sevoflurane significantly reduced the area under the curve to

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The combination of 20% xenon and 0.75% sevoflurane also significantly reduced the area under the curve to 28 ± 16 (P < 0.05), although neither was effective alone (fig. 3C). Long-term neurologic functional assessment of coordination corroborated our histologic findings in the animals treated with 75% xenon and the combination of 20% xenon and 0.75% sevoflurane (fig. 4).

Phosphorylated Cyclic Adenosine Monophosphate Response Element Binding Protein

Preconditioning stimuli exert their effects by triggering signaling cascades that ultimately converge onto nuclear transcription factors, e.g., CREB.23,24 Therefore, we examined whether xenon alone or in combination with sevoflurane triggers phosphorylation of CREB. A more than threefold increase in pCREB was noted with either 75% xenon alone or 20% xenon in combination with 0.75% sevoflurane (fig. 5).

Discussion

Xenon and sevoflurane precondition to reduce the amount of neuronal cell death provoked by oxygen-glucose deprivation injury in vitro. Furthermore, the combination of xenon and sevoflurane increases cellular viability through an antinecrotic mechanism in a PI3K-dependent manner. In vivo xenon preconditioning and the combination of xenon and sevoflurane provided long-lasting neuroprotection assessed by histologic and neurologic outcomes. These outcomes correlated with an increased phosphorylation of the transcription factor CREB, which controls the expression of various cellular survival cascades.

If we can extrapolate to the clinical situation, the combination of xenon and sevoflurane may be able to...
improve long-term neurologic outcome from perinatal injury, in addition to providing analgesia for laboring parturients. In this regard, it is important that nitrous oxide, which is widely used for labor analgesia, is unable to precondition against neuronal injury.14 In this study, we have shown that, unlike xenon and the combination of xenon and sevoflurane (fig. 4), sevoflurane (1.5%) is unable to induce long-term functional neurologic protection in this paradigm. This may likely be because of the small size of samples and its large variation for statistical significance to be reached. However, consistent with these preconditioning data, some evidence suggests that isoﬂurane induces phosphorylation. Interestingly, postconditioning with the volatile anesthetic isoflurane induces phosphorylation of Akt; this effect was blocked by wortmannin,33 implying that PI3K is also important in volatile anesthetic preconditioning. We have now found that xenon is also capable of activating Akt (unpublished Western blot data, Dr. Ma, May 1, 2008). As activated Akt phosphorylates CREB (i.e., pCREB), leading to transcription of survival genes, we suggest that the combination activates PI3K–pAKT–pCREB pathway.34 Therefore, the long-term neuroprotection afforded by xenon and xenon–sevoflurane is consistent with an increase in pCREB and a dependency on PI3K signaling (fig. 5).

Protein kinase C is thought to be activated possibly by reactive oxygen spices, which are generated by preconditioning.35 Experimental studies have implied that PKC activation is an important step in preconditioning by ischemia, xenon, and sevoflurane.18–20 An isoform of PKC, PKC-δ, was found to be translocated to the mitochondria in response to both xenon and sevoflurane preconditioning in the heart37,38 and by ischemia in neurons.36 Activation of PKC acts to stabilize open states of mitochondrial K_{ATP} channels, which leads to cytoprotection by decreasing cytosolic and mitochondrial overload of Ca^{2+}.39 Chelerythrine, a speciﬁc inhibitor of 

The minimum alveolar concentration (MAC) is a measure of potency of an anesthetic. The MAC of xenon is estimated to be 63% in humans26 and 161% in rats.27 The MAC of sevoflurane is reported to be 1.71–2.05% in humans28 and 2.68% in rats.29 Therefore, the in vivo combinations used (20% xenon and 0.75% sevoflurane) represent 0.3 and 0.4 MAC in humans and 0.12 and 0.28 MAC in rats. Currently, 0.5 MAC nitrous oxide (50%) is used for labor analgesia. Therefore, we estimate that the combination is able to provide effective preconditioning at clinically acceptable doses (0.4–0.7 MAC).

Both xenon and sevoflurane are capable of crossing the blood–brain barrier, are pleasant for inhalation, and have low blood/gas solubility, resulting in rapid pharmacokinetics and delivery to the site of action.30 Xenon lacks the neurotoxic and the pharmacokinetic problems of other N-methyl-D-aspartate receptor antagonists while providing subanesthetic analgesic and neuroprotective effects.30,31 Combination with sevoflurane will reduce the required dose and therefore the cost of xenon inhalation, but it is still likely that the use of a semiclosed breathing and scavenging system will require further technological development to make this strategy economically viable.

Preconditioning stimuli induce new genes and proteins, via intracellular signals and mediators, such as protein kinases and transcription factors. PI3K is a signal transduction molecule upstream of protein kinase C (PKC) and is activated in ischemic and anesthetic preconditioning in the heart.51,52 Our results suggest that PI3K is essential for the preconditioning provided by the combination of these anesthetics. It is thought that activated PI3K induces the activation of Akt proteins by phosphorylation. Interestingly, postconditioning with the volatile anesthetic isoflurane induces phosphorylation of Akt; this effect was blocked by wortmannin,33 implying that PI3K is also important in volatile anesthetic postconditioning. We have now found that xenon is also capable of activating Akt (unpublished Western blot data, Dr. Ma, May 1, 2008). As activated Akt phosphorylates CREB (i.e., pCREB), leading to transcription of survival genes, we suggest that the combination activates the PI3K–pAKT–pCREB pathway.34 Therefore, the long-term neuroprotection afforded by xenon and xenon–sevoflurane is consistent with an increase in pCREB and a dependency on PI3K signaling (fig. 5).
PKC, was found to nullify the opening of mitochondrial K_{ATP} channel opening and block preconditioning with sevoflurane or xenon.

Our flow cytometry data imply that the two gases, when given in combination, reduce necrotic death but increase apoptotic death. The factors determining whether a cell dies from apoptosis or necrosis include the type, timing, duration, and intensity of the stimulus and the vulnerability of the cell. For example, a wave of intense excitotoxic injury may promote necrotic cell death, which then triggers apoptotic injury in further cells. Furthermore, some cells may be damaged rapidly, lyse, and be identified as necrotic, whereas other cells sustaining a terminal injury may be damaged in a way that is initially less severe but results in apoptosis. The morphologic characteristics of cells undergoing apoptosis or necrosis were thought to be different; however, more recently, there have been reports of similarities in expression of specific cellular markers in both apoptotic and necrotic bodies in pathologic entities such as stroke, suggesting overlapping mechanism. This continuum of apoptosis and necrosis may make detection of true apoptotic or true necrotic cells more difficult and is likely interrelated in the neonate. The shift toward an apoptotic mode of cell death may be of clinical importance for two reasons: First, necrotic cell death occurs quickly and is hard to inhibit; and second, because apoptosis occurs more slowly, the shift may make the injury more amenable to further therapy, such as hypothermia, in the postnatal phase.

In summary, we have shown that the combination of xenon and sevoflurane can precondition against ischemic injury in vitro and in vivo and these effects are mediated by PI3K and pCREB signaling. If translatable to the clinical situation, we hope this combination may offer pharmacologic preconditioning with long-term effects to ameliorate the consequences of perinatal hypoxic-ischemic injury. It is also notable that sevoflurane, when given in combination, reduce necrotic death but increase apoptotic death. The factors determining whether a cell dies from apoptosis or necrosis include the type, timing, duration, and intensity of the stimulus and the vulnerability of the cell. For example, a wave of intense excitotoxic injury may promote necrotic cell death, which then triggers apoptotic injury in further cells. Furthermore, some cells may be damaged rapidly, lyse, and be identified as necrotic, whereas other cells sustaining a terminal injury may be damaged in a way that is initially less severe but results in apoptosis. The morphologic characteristics of cells undergoing apoptosis or necrosis were thought to be different; however, more recently, there have been reports of similarities in expression of specific cellular markers in both apoptotic and necrotic bodies in pathologic entities such as stroke, suggesting overlapping mechanism. This continuum of apoptosis and necrosis may make detection of true apoptotic or true necrotic cells more difficult and is likely interrelated in the neonate. The shift toward an apoptotic mode of cell death may be of clinical importance for two reasons: First, necrotic cell death occurs quickly and is hard to inhibit; and second, because apoptosis occurs more slowly, the shift may make the injury more amenable to further therapy, such as hypothermia, in the postnatal phase.

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