

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

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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-propidium 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; infarct 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 vitro* and *in vivo* 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

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superior to that of nitrous oxide (50%)-oxygen¹⁸; however, it is unknown whether sevoflurane can precondition against perinatal asphyxial injury like xenon can.¹⁴ The inert gas xenon is an *N*-methyl-D-aspartate receptor antagonist, whereas sevoflurane acts mainly *via* γ -aminobutyric acid type A receptors.^{19,20} 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-Glial Coculture

The neuronal-glial cells were cultured according to our established protocol.^{11,21} 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), 2 mM glutamine (Sigma, Poole, 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 1.25×10^5 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 (12.5%) and sevoflurane (0.67%), 5% CO₂, and 20% O₂ (with the balance of one atmosphere of gas represented by nitrogen) for 2 h. Cells were then returned to a culture incubator filled with air and 5% CO₂.

Twenty-four hours later, cell injury was induced by oxygen-glucose deprivation. Culture medium was replaced by deoxygenated balanced salt solution in the absence of glucose and maintained in an anoxic chamber at 37°C for a period of 75 min, which causes submaximal neuronal injury as reported previously.²¹ The injury was terminated by washing with Eagle minimal essential medium. The medium was harvested 16 h later, and the amount of lactate dehydrogenase (LDH) released into

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 1.2×10^5 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 ($\times 1$) and glutamine (25 μ M). On day 5 after neuronal plating, 100 μ l/10 ml cytosine arabinoside (10 μ M; 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 HEPES buffer and then detached with 0.2% trypsin-EDTA. Thereafter, cells were stained with annexin V-fluorescein isothiocyanate conjugate (0.4 μ g/ml) and subsequently with propidium iodide (0.8 μ g/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.²² 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- μ m 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 \pm 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 $55 \pm 12\%$ and to $49 \pm 12\%$ 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 $64 \pm 6\%$ ($P < 0.05$) and $37 \pm 5\%$ ($P < 0.01$) 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 $59 \pm 5\%$ 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 effects *via* an antiapoptotic or antinecrotic mechanism. Control cultures (without intervention) had a majority of viable cells ($76 \pm 1\%$). Oxygen-glucose deprivation provoked $70 \pm 2\%$, $75 \pm 2\%$, and $81 \pm 2\%$ 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-

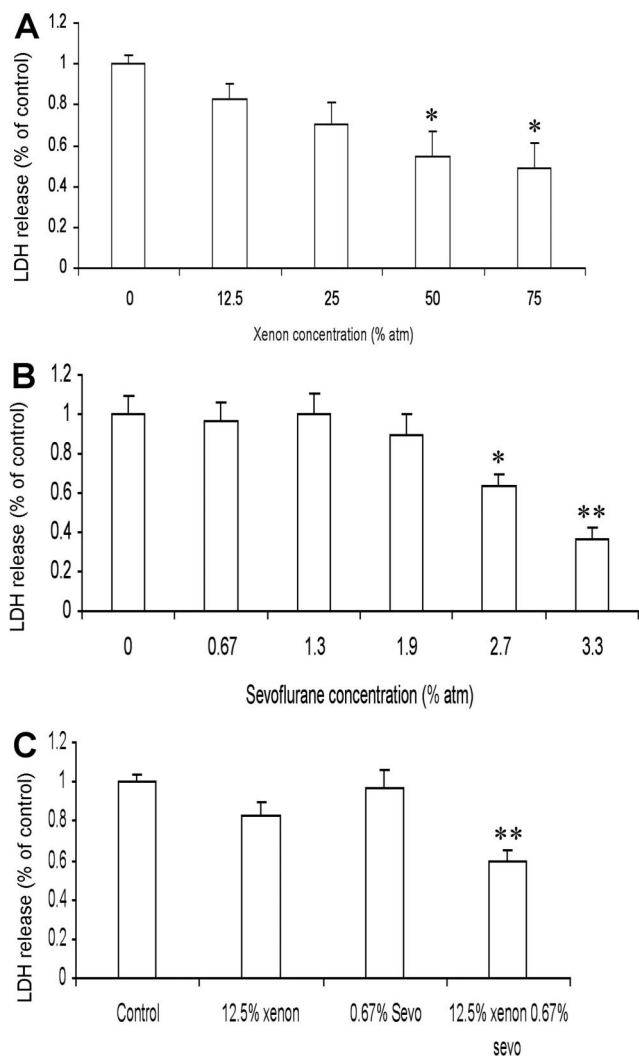


Fig. 1. Xenon and/or sevoflurane preconditioning reduce lactate dehydrogenase (LDH) release in neuronal–glial cocultures. Twenty-four hours after 2 h of gas exposure, LDH release from cultured neurons provoked by 75 min of oxygen–glucose deprivation was measured at 16 h after the oxygen–glucose deprivation period. LDH release, expressed as a fraction of the maximum LDH release in the absence of agents (mean \pm SEM, n = 5–6). (A) Concentration–response of neuroprotection afforded by xenon preconditioning. (B) Concentration–response of neuroprotection afforded by sevoflurane preconditioning. (C) Effect of xenon and sevoflurane (sevo) alone and in combination on LDH release. * $P < 0.05$, ** $P < 0.01$ versus control.

tioning action through an antinecrotic mechanism. It is notable that there was a significant increase in the number of apoptotic neurons ($35 \pm 4\%$ [$P < 0.01$] vs. $21 \pm 1\%$ 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.

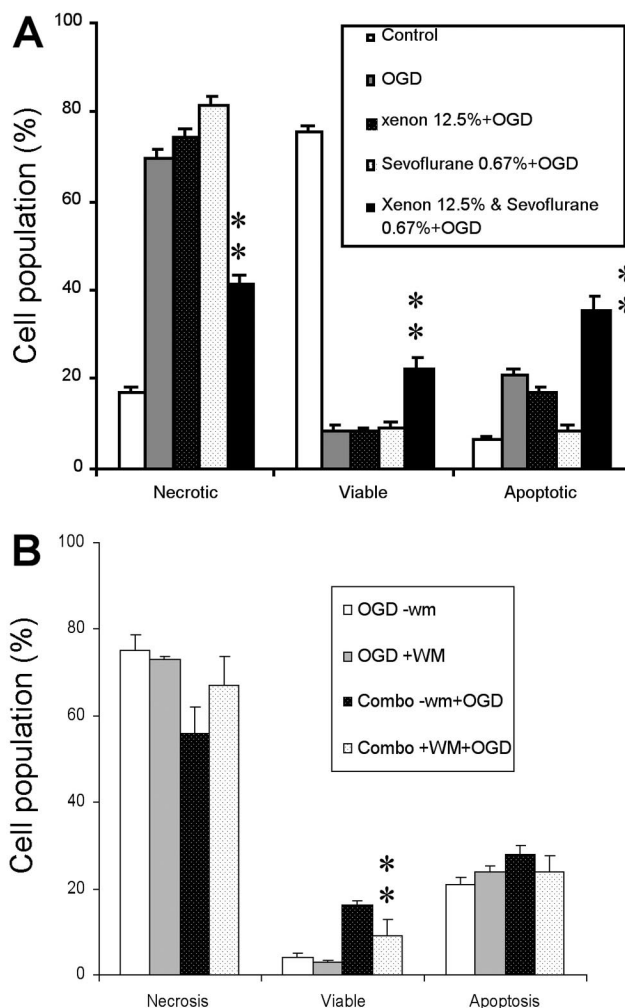


Fig. 2. Xenon and sevoflurane in combination attenuate cell death *in vitro*. Pure neuronal cells were preconditioned with 12.5% xenon and 0.67% sevoflurane alone and in combination for 2 h and then 24 h after they were provoked by 75 min of oxygen–glucose deprivation (OGD). Subsequently, cells were stained with annexin V (for apoptosis) and propidium iodide (for necrosis) for flow cytometry analysis. Different cell populations with various gas exposure in the absence (A) or presence (B) of wortmannin (WM). Mean \pm SEM (n = 6). ** $P < 0.01$ versus OGD.

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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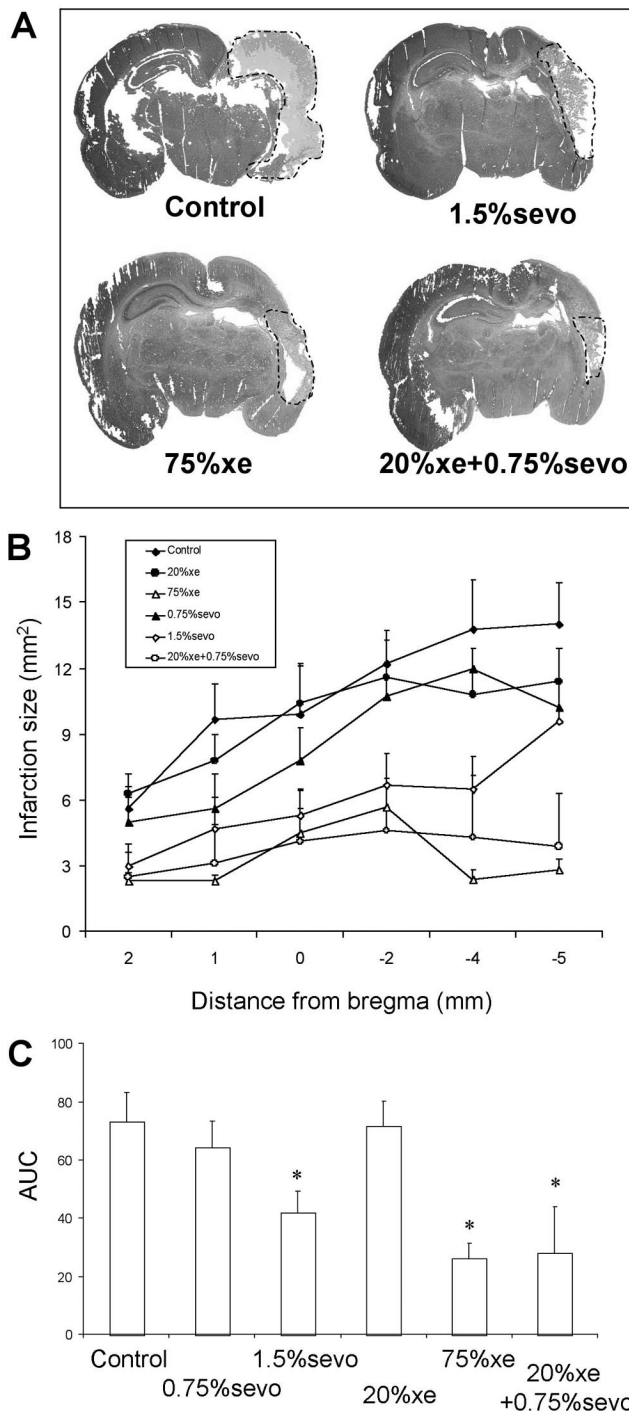


Fig. 3. Effect of preconditioning on focal infarct size. After exposure to xenon (xe) and sevoflurane (sevo) alone or in combination or air, 7-day-old rat pups underwent hypoxic-ischemic injury 4 h later. Infarct size was assessed 7 days later. (A) Example microphotographs from animals treated with sevoflurane and xenon alone and in combination, compared with control. (B) Mean infarct size at six adjacent slices relative to the bregma (+2, +1, 0, -2, -4, and -5 mm). (C) Mean area under the curve (AUC) derived from A. Mean \pm SEM (n = 6). * $P < 0.05$.

26.4 \pm 5.1 and 42 \pm 7.5, respectively ($P < 0.05$ vs. control). The combination of 20% xenon and 0.75% sevoflurane also significantly reduced the area under the

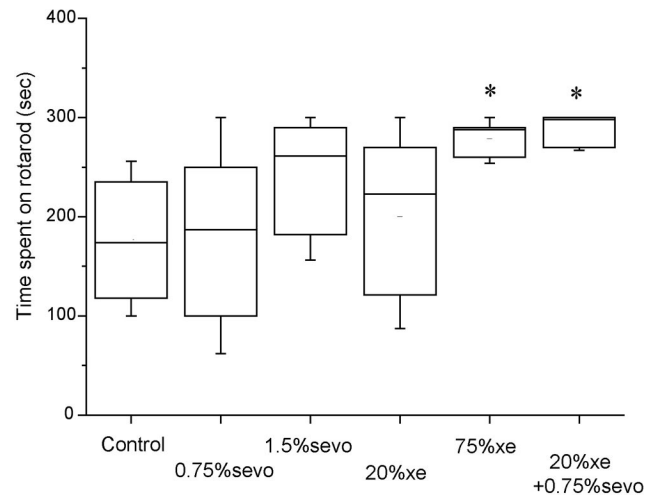


Fig. 4. Long-term functional neurologic outcome after preconditioning. Seven-day-old Sprague-Dawley pups were exposed for 2 h to xenon (xe) and sevoflurane (sevo) alone or in combination or air; 4 h later, pups underwent hypoxic-ischemic injury for 90 min. Time spent on the rotarod was assessed 30 days after injury and is presented in a box-and-whisker plot (maximum, 75% interquartile range, median, 25% interquartile range, and minimum). * $P < 0.05$ versus control (n = 6).

curve to 28 \pm 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

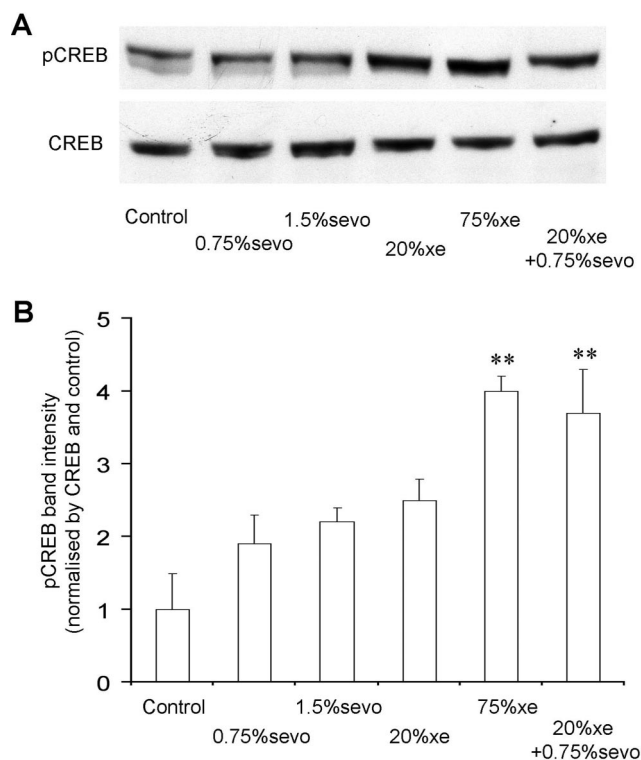


Fig. 5. Phosphorylated cyclic adenosine monophosphate response element binding protein (pCREB) immunoreactivity in pups' cerebral cortex 4 h after exposure to xenon (xe) and sevoflurane (sevo) alone or in combination or air. (A) Representative band of pCREB and cyclic adenosine monophosphate response element binding protein (CREB). (B) Mean data of band intensity from three independent experiments. ** $P < 0.05$ versus control.

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 has a transient neuroprotective effect against hypoxic-ischemic injury in adult rats,²⁵ underlying the importance of studying long-term functional endpoints in neuroprotection studies. It is possible that higher doses of sevoflurane may have had an enhanced effect, but they also would have limited clinical translation, because maternal sedation is a significant complication of sevoflurane at concentrations in excess of 0.8%.¹⁸ In aggregate, a preconditioning dose of sevoflurane alone during labor does not seem to be a clinically acceptable option.

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.^{10,11} 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.^{31,32} 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 pCREB and a dependency on PI3K signaling (fig. 5).

Protein kinase C is thought to be activated possibly by reactive oxygen species, which are generated by preconditioning.³⁵ Experimental studies have implied that PKC activation is an important step in preconditioning by ischemia, xenon, and sevoflurane.^{36–38} An isoform of PKC, PKC- ϵ , was found to be translocated to the mitochondria in response to both xenon and sevoflurane preconditioning in the heart^{37,39} 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

PKC, was found to nullify the opening of mitochondrial K_{ATP} channel opening⁴⁰ and block preconditioning with sevoflurane or xenon.^{37,38}

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.⁴¹ 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.⁴² 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 (1.5%) reduced infarct size but did not induce long-term functional protection. Whether secondary anesthetic injury may have contributed to the lack of neurologic protection is a concern, because isoflurane has been reported to induce apoptosis when administered during neurodevelopment with subsequent neurocognitive impairment in adulthood.⁴³ The safety profile of sevoflurane should be urgently investigated in this regard.

References

- Vannucci RC, Perlman JM: Interventions for perinatal hypoxic-ischemic encephalopathy. *Pediatrics* 1997; 100:1004-14
- Low JA: The relationship of asphyxia in the mature fetus to long-term neurologic function. *Clin Obstet Gynaecol* 1993; 36:82-90
- Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124-36
- Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamata T: "Ischemic tolerance" phenomenon found in the brain. *Brain Res* 1990; 528:21-4

- Ytrehus K, Liu Y, Downey JM: Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994; 266:1145-52
- Millar CGM, Baxter GF, Thiemeermann C: Protection of the myocardium by ischaemic preconditioning: Mechanism and therapeutic implications. *Pharmacol Ther* 1996; 69:143-51
- Gonzalez-Zulueta M, Feldman AB, Klesse LJ, Kalb RG, Dillman JF, Parada LF, Dawson TM, Dawson VL: Requirement for nitric oxide activation of p21ras/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc Natl Acad Sci U S A* 2000; 97:436-41
- Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK, Dirnagl U: Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. *Stroke* 2002; 33:1889-98
- Sanders RD, Manning HJ, Ma D, Maze M: Perinatal neuroprotection. *Curr Anaesth Crit Care* 2007; 18:215-24
- Ma D, Wilhelm S, Maze M, Franks NP: Neuroprotective and neurotoxic properties of the "inert" gas, xenon. *Br J Anaesth* 2002; 89:739-46
- Wilhelm S, Ma D, Maze M, Franks NP: Effects of xenon on *in vitro* and *in vivo* models of neuronal injury. *ANESTHESIOLOGY* 2002; 96:1485-91
- Petzelt C, Blom P, Schmehl W, Muller J, Kox WJ: Prevention of neurotoxicity in hypoxic cortical neurons by the noble gas xenon. *Life Sci* 2003; 72:1909-18
- Ma D, Yang H, Lynch J, Franks NP, Maze M, Grocott HP: Xenon attenuates cardiopulmonary bypass-induced neurologic and neurocognitive dysfunction in the rat. *ANESTHESIOLOGY* 2003; 98:690-8
- Ma D, Hossain M, Pettet GK, Luo Y, Lim T, Akimov S, Sanders RD, Franks NP, Maze M: Xenon preconditioning reduces brain damage from neonatal asphyxia in rats. *J Cereb Blood Flow Metab* 2006; 26:199-208
- Weber NC, Toma O, Awan S, Frassdorf J, Preckel B, Schlack W: Effects of nitrous oxide on the rat heart *in vivo*: Another inhalational anesthetic that preconditions the heart? *ANESTHESIOLOGY* 2005; 103:1174-82
- Piriou V, Chiari P, Lhuillier F, Bastien O, Loufoua J, Raisky O, David JS, Ovize M, Lehot JJ: Pharmacological preconditioning: A comparison of desflurane, sevoflurane, isoflurane, and halothane in rabbit myocardium. *Br J Anaesth* 2002; 89:486-91
- Garcia C, Julier K, Bestmann L, Zollinger A, von Segesser LK, Pasch T, Spahn DR, Zaugg M: Preconditioning with sevoflurane decreases PECAM-1 expression and improves one year cardiovascular outcome in coronary artery bypass graft surgery. *Br J Anaesth* 2005; 94:159-65
- Yeo ST, Holdcroft A, Yentis SM, Stewart A: Analgesia with sevoflurane during labour, I: Determination of the optimum concentration. *Br J Anaesth* 2007; 98:105-9
- Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR: How does xenon produce anaesthesia? *Nature* 1998; 396:324
- Krasowski MD, Harrison NL: The actions of ether, alcohol and alkane general anaesthetics on GABA_A and glycine receptors and the effects of TM2 and TM3 mutations. *Br J Pharmacol* 2000; 129:731-43
- Ma D, Hossain M, Rajakumaraswamy N, Arshad M, Sanders RD, Franks NP, Maze M: Dexmedetomidine produces its neuroprotective effect *via* the alpha 2A-adrenoceptor subtype. *Eur J Pharmacol* 2004; 502:87-97
- Rice JE III, Vannucci RC, Brierley JB: The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* 1981; 9:131-41
- Meller R, Minami M, Cameron JA, Impey S, Chen D, Lan JQ, Henshall DC, Simon RP: CREB-mediated Bcl-2 protein expression after ischemic preconditioning. *J Cereb Blood Flow Metab* 2005; 25:234-46
- Lee HT, Chang YC, Wang LY, Wang ST, Huang CC, Ho CJ: cAMP response element-binding protein activation in ligation preconditioning in neonatal brain. *Ann Neurol* 2004; 56:611-23
- Kawaguchi M, Kimbro JR, Drummond JC, Cole DJ, Kelly PJ, Patel PM: Isoflurane delays but does not prevent cerebral infarction in rats subjected to focal ischemia. *ANESTHESIOLOGY* 2000; 92:1335-42
- Nakata Y, Goto T, Ishiguro Y: Minimum alveolar concentration (MAC) of xenon with sevoflurane in humans. *ANESTHESIOLOGY* 2001; 94:611-4
- Koblin DD, Fang Z, Eger EI, Laster MJ, Gong D, Ionescu P, Halsey MJ, Trudell JR: Minimum alveolar concentrations of noble gases, nitrogen, and sulfur hexafluoride in rats: Helium and neon as nonimmobilizers (nonanesthetics). *Anesth Analg* 1998; 87:419-24
- Smith I, Nathanson M, White PF: Sevoflurane: A long awaited volatile anaesthetic. *Br J Anaesth* 1996; 76:435-45
- Kashimoto S, Furuya A, Nonaka A, Oguchi T, Koshimizu M, Kumazawa T: The minimum alveolar concentration of sevoflurane in rats. *Eur J Anaesthesiol* 1997; 14:359-61
- Sanders RD, Franks NP, Maze M: Xenon: No stranger to anaesthesia. *Br J Anaesth* 2003; 91:709-17
- Tong H, Chen W, London RE, Murphy E, Steenbergen C: Preconditioning enhanced glucose uptake is mediated by p38 MAP kinase not by phosphatidylinositol 3-kinase. *J Biol Chem* 2000; 275:11981-6
- Tong H, Chen W, Steenbergen C, Murphy E: Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ Res* 2000; 87:309-15
- Chiari PC, Bienengraeber MW, Pagel PS, Krolkowski JG, Kerten JR, Wardler DC: Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: Evidence for anaesthetic induced preconditioning in rabbits. *ANESTHESIOLOGY* 2005; 102:102-9

34. Liang MH, Wendland JR, Chuang DM: Lithium inhibits Smad3/4 transactivation *via* increased CREB activity induced by enhanced PKA and AKT signaling. *Mol Cell Neurosci* 2008; 37:440-53
35. Baines CP, Goto M, Downey JM: Oxygen radicals release during ischemic preconditioning contribute to cardioprotection in rabbit myocardium. *J Moll Cell Cardiol* 1997; 29:207-16
36. Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Perez Pinzon MA: ϵ PKC is required for the induction of tolerance by ischemic and NMDA mediated preconditioning in the organotypic hippocampal slice. *J Neurosci* 2003; 23:384-91
37. Weber NC, Toma O, Wolter JI, Obal D, Mullenheim J, Preckel A, Schlack W: The noble gas xenon produces pharmacological preconditioning in the rat heart *in vivo via* induction of PKC- ϵ and p38 MAPK. *Br J Pharmacol* 2005; 144:123-32
38. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF: Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. *ANESTHESIOLOGY* 2003; 99: 421-8
39. Julier K, da Silva R, Garcia C, Bestmann L, Frascarolo P, Zollinger A, Chassot P, Schmid ER, Turina MI, von Segesser LK, Pasch T, Spahn D, Zaugg M: Preconditioning by sevoflurane decreases biochemical markers for myocardial and renal dysfunction in coronary artery bypass graft surgery: A double-blinded, placebo-controlled, multicenter study. *ANESTHESIOLOGY* 2003; 98:1315-27
40. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC: Volatile anaesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K_{ATP} channels *via* multiple signalling pathways. *ANESTHESIOLOGY* 2002; 97:4-14
41. Syntichaki P, Tavernarakis N: The biochemistry of neuronal necrosis: Rogue biology? *Nat Rev Neurosci* 2003; 4:672-84
42. Ma D, Williamson P, Januszewski A, Nogaro MC, Hossain M, Ong LP, Shu Y, Franks NP, Maze M: Xenon mitigates isoflurane-induced neuronal apoptosis in the developing rodent brain. *ANESTHESIOLOGY* 2007; 106:746-53
43. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003; 23:876-82