Heme Oxygenase-1 Mediates Neuroprotection Conferred by Argon in Combination with Hypothermia in Neonatal Hypoxia–Ischemia Brain Injury

Hailin Zhao, Ph.D., Sian Mitchell, M.B.B.S., Stefania Koumpa, M.B.B.S., Yushi Tracy Cui, B.Sc., Qingquan Lian, M.D., Ph.D., Henrik Hagberg, M.D., Ph.D., Mark R. Johnson, M.D., Ph.D., Masao Takata, M.D., Ph.D., F.R.C.A., Daqing Ma, M.D., Ph.D., F.R.C.A.

ABSTRACT

Background: Hypoxic–ischemic encephalopathy is a major cause of mortality and disability in the newborn. The authors investigated the protective effects of argon combined with hypothermia on neonatal rat hypoxic–ischemic brain injury.

Methods: In in vitro studies, rat cortical neuronal cell cultures were challenged by oxygen and glucose deprivation for 90 min and exposed to 70% Ar or N₂ with 5% CO₂ balanced with O₂ at 33°C for 2 h. Neuronal phospho-Akt, heme oxygenase-1 and phospho-glycogen synthase kinase-3β expression, and cell death were assessed. In in vivo studies, neonatal rats were subjected to unilateral common carotid artery ligation followed by hypoxia (8% O₂ balanced with N₂ and CO₂) for 90 min. They were exposed to 70% Ar or N₂ balanced with oxygen at 33°C, 35°C, and 37°C for 2 h. Brain injury was assessed at 24 h or 4 weeks after treatment.

Results: In in vitro studies, argon–hypothermia treatment increased phospho-Akt and heme oxygenase-1 expression and significantly reduced the phospho-glycogen synthase kinase-3β Tyr-216 expression, cytochrome C release, and cell death in oxygen–glucose deprivation–exposed cortical neurons. In in vivo studies, argon–hypothermia treatment decreased hypoxia/ischemia-induced brain infarct size (n = 10) and both caspase-3 and nuclear factor-kB activation in the cortex and hippocampus. It also reduced hippocampal astrocyte activation and proliferation. Inhibition of phosphoinositide-3-kinase (PI3K)/Akt pathway through LY294002 attenuated cerebral protection conferred by argon–hypothermia treatment (n = 8).

Conclusion: Argon combined with hypothermia provides neuroprotection against cerebral hypoxia–ischemia damage in neonatal rats, which could serve as a new therapeutic strategy against hypoxic–ischemic encephalopathy. (Anesthesiology 2016; 125:180-92)

Hypoxic–ischemic encephalopathy (HIE) is a major cause of mortality and disability in the newborn and is associated with cerebral palsy, epilepsy, mental retardation, and learning difficulties. HIE is associated with hugely negative emotion and financial costs to the family of the affected infant and is a burden to society in general. Given the severity and lifelong nature of the adverse effects of perinatal HIE, there is a pressing need to develop novel neuroprotective strategies.

Currently, therapeutic hypothermia, which provides modest neuroprotection in perinatal HIE, has been widely adopted in clinical practice. Hypothermia exerts inhibitory effects at many levels within the pathological cascade of HIE that leads to delayed neuronal death. Furthermore, hypothermia has shown to delay the onset of secondary energy failure and nearly double the duration of the latent phase, the period when additional neuroprotective agents could be given, in experimental models. However, despite hypothermia treatment, the rate of death and disability remains high, with approximately half of cooled infants...
dying or exhibiting neurodevelopmental disability. 4, 5 There is, therefore, a pressing need to discover better and more effective treatment strategies to prevent or ameliorate neonatal brain damage after perinatal hypoxia–ischemia.

The noble gas xenon has shown great promise as a neuroprotectant when administered alone 6 or in combination with therapeutic hypothermia in rat 7, 8 and piglet 9 models of neonatal HIE. The combination of xenon and hypothermia has either a synergistic 7 or an additive 10 neuroprotective effect. However, xenon is present in very low concentrations in air and its extraction is very costly, perhaps prohibiting its widespread use. Argon, on the other hand, is the most common noble gas in the atmosphere and is emerging as a viable alternative. Unlike xenon, argon is not an anesthetic gas and lacks anesthetic/sedative properties; hence, it may be more safely administered to neonatal patients with hypoxia–ischemia brain injury. 11 The aim of this study is to investigate whether argon in combination with hypothermia is neuroprotective in our in vitro and in vivo models of HIE and to explore the underlying molecular mechanisms.

Materials and Methods

Primary Cortical Neuronal Cell Culture
The cortical neuronal cultures were derived from gestational day 16 fetal Sprague-Dawley rats. The neuronal cells were seeded into poly-l-lysine precoated plates and fed with neurobasal medium (Gibco, Thermo Fischer Scientific, USA) with the addition of B27 supplement and glutamine (25 μM).

Oxygen–Glucose Deprivation and Gas Exposure
Oxygen–glucose deprivation (OGD) was induced. 7 Culture medium was replaced by deoxygenated balanced salt solution and maintained in a purpose-built cell-culture chamber at 37°C for 90 min. Cells were then recovered in the neuronal culture medium in the purpose-built chamber, which was randomly filled with 75% Ar or N₂ (Air Products, United Kingdom) and 5% CO₂ balanced with O₂ at 33°C for 2 h. They were further recovered in a normal cell culture incubator for 24 h at 37°C.

Determination of Apoptosis and Necrosis In Vitro
Neuronal cells were stained with annexin V–propidium iodide (PI) apoptosis kit (e-Bioscience, United Kingdom) according to the manufacturer’s guidelines. A count of 10,000 cells per sample was analyzed with flow cytometry (FACSCalibur; Becton Dickinson, USA) to determine the percentage population of apoptotic (annexin V positive, PI negative), necrotic (annexin V and PI positive), and live cells (unstained).

HO-1 siRNA Transfection and PI-3K/Akt Inhibition
Neuronal cells were transfected with heme oxygenase (HO)-1 siRNA (S01522122, Qiagen, United Kingdom) using lipofectamine (Invitrogen, United Kingdom) at 20 nM, while scrambled siRNA served as negative control. Cells were incubated with siRNA for 6 h at 37°C in humidified air containing 5% CO₂, after which it was removed and replaced with experimental medium followed by OGD treatment. For PI-3K/Akt inhibition, cultured neurons received 100 mM LY294002. 12

Rat Hypoxic–Ischemic Brain Injury
Seven-day-old Sprague-Dawley rat pups and their mother were purchased (Harlan, United Kingdom) and housed in the animal facilities in Chelsea-Westminster Hospital campus, Imperial College London, London, United Kingdom. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Right common carotid artery ligation was performed under surgical anesthesia. 13 After 1 h of recovery, the pups were exposed to hypoxia (8% O₂ balanced with N₂) for 90 min in purpose-built multichambers. They were exposed to 70% Ar or N₂ balanced with 30% O₂ for 2 h through our established protocol. 14 Hypothermia (33°C or 35°C) or normothermia (37°C) was achieved and sustained by the temperature-controlled water bath. Rat pups were randomly allocated to experimental conditions, and their number used per group was based on the similar experimental settings established previously. 7 All the animal experiments conform to the United Kingdom Animal Research: Reporting In Vivo Experiments guidelines. 15 Efforts were made to minimize the number used and suffering of animals throughout.

Drug Administration In Vivo
LY294002 (Calbiochem [Germany], 0.2 mol/l in 5 μl of phosphate-buffered saline) or the phosphate-buffered saline vehicle was injected intracerebroventriculally before gas treatment, as described previously. 16, 17

Immunohistochemistry
For in vitro fluorescence staining, cells were fixed in paraformaldehyde, incubated in 10% donkey serum for 1 h, and then incubated overnight with rabbit anti-phospho-Akt (p-Akt; 1:200, Cell signaling, USA), or rabbit or mouse anti–HO-1 (1:200, Abcam, United Kingdom), or rabbit anti-phospho-glycogen synthase kinase (GSK)–3β Tyr-216 (1:200, Abcam), or rabbit anticytochrome C (1:200, Cell Signaling), or mouse anti–α-tubulin (1:200, Sigma-Aldrich, United Kingdom), followed by secondary antibody for 1 h. The mitochondria were stained with MitoRed (Sigma-Aldrich). For in vivo fluorescence staining, the pups were sacrificed and transcardially perfused with 4% paraformaldehyde. After dehydration, brain was cryosectioned into 25-μm slices. Coronal sections between approximately –2.5 and –3.7 mm from bregma (relative to the adult rat brain) were incubated with 3% donkey serum (Millipore, USA) and were then incubated overnight with rabbit anti–Bcl-2 (1:200, Abcam), rabbit or mouse anti–HO-1 (1:200, Abcam), rabbit anti–α-tubulin (1:200, Sigma-Aldrich), and goat anti–phospho-Akt (p-Akt; 1:200, Cell Signaling, USA) or rabbit anti–cytochrome C (1:200, Cell Signaling).
antiphospho–GSK-3β Tyr-216 (1:200, Abcam), rabbit anti-cleaved caspase-3 (1:200, Cell Signaling), rabbit anti-nuclear factor (NF)-κB p65 (1:200, Abcam), or rabbit anti–glial fibrillary acidic protein (GFAP) (1:200, Dako, Denmark) primary antibody, followed by fluorescein-conjugated secondary antibodies (Millipore, United Kingdom). For dual fluorescence labeling, cells or brain sections were incubated with the two primary antibodies overnight, followed by the secondary antibodies.

The slides were counterstained with nuclear dye 4',6-diamidino-2-phenylindole (DAPI) and mounted with Vectorshield mounting medium (Vector Laboratories, USA). Ten fields at ×20 view were first photographed using an AxioCam digital camera (Zeiss, United Kingdom) mounted on an Olympus BX60 microscope (Olympus, United Kingdom). Staining was quantified using ImageJ software (US National Institutes of Health, USA). Fluorescence intensity was observed by one author who was blinded to the treatment and then calculated as percentages of the mean value of the naive controls.

**Western Blotting**

The tissue lysates from brain samples were centrifuged, and the protein extracts (40 μg per sample) underwent electrophoresis and then were transferred to a polyvinylidene difluoride membrane. The membrane was treated with blocking milk solution and probed with rabbit anti–Bcl2 (1:1,000, Abcam), rabbit anti–HO-1 (1:1,000, Abcam), and rabbit anti–GSK-3β phospho Tyr-216 (1:1,000, Abcam), followed by horseradish peroxidase-conjugated secondary antibody. The loading control was α-tubulin (1:10,000, Sigma-Aldrich). The blots were visualized with enhanced chemiluminescence system (Santa Cruz Biotechnology, USA) and analyzed with Genesnap (Syngene, United Kingdom). Protein band intensity was normalized with α-tubulin and expressed as ratio of the naive control.

**Assessment of Brain Infarction through Caspase 3 or Cresyl Violet Staining**

Double-labeled fluorescence staining was performed on 25-μm vibratome brain sections as described previously.18 The brain sections at the level of striatum (approximately Bregma-0.35 mm) were labeled with the rabbit anti-cleaved caspase-3 (1:200, Cell Signaling). The vibratome sections were also stained with nuclear staining PI and examined using the Olympus BX60 microscope (Olympus).

For histology, 5-μm paraffin sections were stained with 0.5% cresyl violet. The coronal sections (5 mm) from rats were selected from each pup to match predefined brain regions relative to the bregma (+2, +1, 0, −1, −2, and 5 mm) relative to adult brain. Each slice was photographed, and the size (arbitrary unit) of the healthy matter of both hemispheres was calculated with data analysis software (ImageJ version 1.31; National Institutes of Health image software) by one author blinded to the treatment. The infarct size was calculated with a formula of (left hemisphere – right hemisphere)/left hemisphere (%). Then, the data were used to plot curves, and the area under curve was calculated to indicate the infarction volume (arbitrary units).14

**Statistical Analysis**

All numerical data were expressed as mean ± SD. To study the treatment effects on protein expression with time, two-way ANOVA and a post hoc Tukey test was performed; otherwise, one-way analysis of variance followed by post hoc Student–Newman–Keuls test was performed for statistical comparisons (GraphPad Prism 5.0 software, GraphPad Software, Inc., USA). A two-tailed P < 0.05 was considered to be statistically significant.

**Results**

**Argon Exposure Up-regulated Heme Oxygenase-1 in the Cultured Cortical Neurons and Neonatal Rat Brain**

To investigate whether HO-1 was up-regulated in vitro and in vivo after argon exposure, cultured rat neuronal cells or 7-day-old neonatal rats were given 70% Ar for 2 h (fig. 1). Four hours after gas exposure, up-regulation of HO-1 was observed in cultured neuronal cells (fig. 1, A and B). The immunostaining of HO-1 in the cortex and CA1 and CA3 regions of the hippocampus is shown in figure 1C.

Argon exposure significantly increased HO-1 expression in the cortex at all three time points with the highest increase at 4 h (fig. 1D), in the CA1 region at 4 and 24 h with the highest increase at 4 h (fig. 1E), and in the CA3 region at 4 h, compared with that in the nitrogen controls (fig. 1F).

**Argon and Hypothermia Up-regulated p-Akt and HO-1 in the Cortical Neuronal Cell Culture**

First, the effect of argon exposure on the expression of p-Akt and HO-1 was investigated in the neuronal cell culture through immunofluorescence technique (fig. 2). HO-1 was expressed at basal levels in the naive control group but was slightly increased during hypothermia treatment (fig. 2A). Argon significantly augmented the up-regulation of HO-1 (fig. 2, A and C). p-Akt was detected at low level in the naive control group; however, expression of p-Akt was moderately enhanced in neurons after being treated with hypothermia and was also greatly enhanced by argon–hypothermia treatment (fig. 2, A and B). To assess the neuroprotective effects of argon–hypothermia treatment, the neuronal cultures were challenged with OGD for 90 min, followed by nitrogen–or argon–hypothermia treatment for 2 h. Four hours after treatment, activation of GSK-3β was found in the neurons, indicating a possible role in apoptosis. In the argon–hypothermia-treated group, p-GSK-3β Tyr-216 was barely expressed in neurons (fig. 2, D and E). In addition, α-tubulin staining indicated that
Argon–hypothermia treatment improved cellular morphology and preserved neuronal dendrites well under OGD challenge (fig. 2D).

**CRITICAL CARE MEDICINE**

**Fig. 1.** Enhanced expression of heme oxygenase (HO)-1 in cultured cortical neuronal cells, brain cortex, and hippocampus after argon (Ar) exposure. Rat neuronal cell culture was exposed to Ar gas (70% Ar and 5% CO₂ balanced with O₂) or N₂ gas (70% N₂ and 5% CO₂ balanced with O₂) for 2 h and then to air cell incubator at 37°C for 24 h. (A) Dual immunolabeling of α-tubulin (green fluorescence) and HO-1 (red fluorescence); (B) fluorescence intensity of HO-1 at 4 h after gas exposure. Seven-day-old neonatal rats were exposed to Ar gas (70% Ar balanced with 30% O₂) or N₂ gas (70% N₂ balanced with 30% O₂) for 2 h and then to room air for 24 h. HO-1 expression (green fluorescence) was assessed at 0, 4, and 24 h after gas exposure. (C) The example images of the naive control (NC), N₂, and Ar-treated cortex, hippocampus CA1 and CA3 at 4 h after gas exposure. Fluorescence intensity (% of NC) of HO-1 after gas exposure in (D) cortex, (E) hippocampus CA1 region, and (F) CA3 region. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Data are represented as mean ± SD; n = 8. *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bar: 50 μm.

Argon–hypothermia treatment improved cellular morphology and preserved neuronal dendrites well under OGD challenge (fig. 2D).

**Argon-Hypothermia Reduced Ischemic Neuronal Injury Induced by OGD In Vitro**

Four hours after treatment, dual labeling of mitochondria and cytochrome C demonstrated the cytochrome c release from mitochondria under OGD-induced injury (fig. 3). Argon–hypothermia treatment significantly restrained the cytochrome c within the mitochondria, indicated by colocalization of mitochondria and cytochrome C (fig. 3, A and B). Twenty-four hours later, neuronal death was assessed with PI/annexin V staining (fig. 3, C and D). Hypothermia alone confers a certain level of protection (P = 0.02, OGD vs. OGD + hypothermia group), and argon–hypothermia increased the percentage of live neurons after OGD (69 ± 7.9% vs. 38.5 ± 6.2%, Ar vs. N₂, P < 0.01). These data indicate that argon combined with hypothermia promotes neuronal resistance against hypoxic–ischemic injury induced by OGD.

**Inhibition of Akt and HO-1 Abolished Argon–Hypothermia–mediated Protection in Cultured Rat Cortical Neurons**

To further investigate whether p-Akt/HO-1 essentially contribute to the neuroprotective effects of argon–hypothermia treatment, the neurons were treated with either PI-3K-Akt inhibitor LY294002 or HO-1 siRNA and then subjected to the OGD challenge (fig. 4). Argon–hypothermia-treated neurons exhibited a relative intact neuronal morphology, as demonstrated by better preserved cytoplasm and neuronal dendrites (fig. 4, A and C). Furthermore, either the PI-3K–Akt inhibitor LY294002 or HO-1 siRNA blocked the protective effects of argon–hypothermia on neurons after OGD treatment. The LY294002- or HO-1 siRNA-treated neurons had shrunken cell soma and fragmented neurites, in contrast to the intact cell morphology in the argon only group. In addition, blocking either PI-3K/Akt pathway or
HO-1 expression induced significant cell death at 24 h after treatment (fig. 4, B, D, F, and H). Taken together, all these indicate that p-Akt and HO-1 mediate the neuroprotective effects of argon–hypothermia against OGD-induced injury.

**Argon Combined with Hypothermia Induced the Up-regulation of HO-1 and Bcl-2 and the Suppression of GSK-3β Activation in the Cortex and Hippocampus**

These observations suggest that HO-1 plays the central role in argon-mediated protection. Consistent with in vitro data, Western blot analysis of in vivo samples showed that argon–hypothermia significantly increased HO-1 (3.4 ± 0.4 vs. 2.3 ± 0.5, Ar vs. N2; *P < 0.05) and Bcl-2 (2.9 ± 0.25 vs. 2.02 ± 0.5, Ar vs. N2; *P < 0.05) and reduced p-GSK-3β Tyr 216 (0.9 ± 0.2 vs. 2.5 ± 0.4, Ar vs. N2; *P < 0.05) at 4 h posttreatment in the cortex of neonatal rats with hypoxic–ischemic injury (fig. 5, A to C). Furthermore, the dual immunolabelings of Bcl-2 and HO-1 and HO-1 and p-GSK-3β in the CA1 and CA3 regions of the hippocampus are shown in figure 5, D and E. Colocalization of HO-1 and Bcl-2 was observed, and HO-1 expression negatively correlated with the activation of GSK-3β after argon–hypothermia treatment (fig. 5, D to G).

**Argon Combined with Hypothermia Decreased Cell Death and Tissue Inflammation in the Cortex and Hippocampus after Hypoxia–Ischemia Challenge**

To assess the level of brain injury after hypoxic–ischemic insult, the brain section was stained with caspase-3 (fig. 6). Argon–hypothermia treatment caused a notable reduction of caspase-3–positive areas (fig. 6A). Cleaved caspase-3 expression in the cortex and the CA1 and CA3 regions of the hippocampus was assessed (fig. 6, B and C). Hypothermia alone
significantly reduced the expression of cleaved caspase-3 in these areas compared with the normothermic controls. Argon combined with hypothermia caused further significant reductions in caspase-3 expression in the cortex and hippocampus, compared to hypothermia alone (fig. 6, B and C). NF-κB activation is an essential component of the inflammatory response in the brain. The expression and nuclear translocation of NF-κB in the cortex and the CA1 and CA3 regions of the hippocampus after the injury were readily detected (fig. 6, D and E). Hypothermia alone did not significantly reduce the expression of NF-κB compared to the injury controls. Argon combined with hypothermia resulted in significant reductions in NF-κB p-65 expression and translocation when compared with control, hypothermia alone, and normothermia injury controls (fig. 6, D and E). Astrocyte activation and proliferation (reactive gliosis) is a hallmark of neuroinflammation during hypoxia–ischemia-induced neuronal injury processes. Fluorescence intensity of GFAP in the region between the pyramidal cell layer and the alveus in the CA1 and CA3 regions of the hippocampus was significantly elevated (fig. 6, F and G) in the normothermia injury group. Hypothermia alone did not significantly reduce GFAP expression compared with the injury controls. Argon combined with hypothermia significantly reduced GFAP expression in the CA1 and CA3 regions of the hippocampus when compared with the injury controls (fig. 6G).

Argon Combined with Hypothermia Reduced the Infarction Size

The long-term protective profile of argon–hypothermia treatment was explored (fig. 7). Argon combined with hypothermia (33°C and 35°C) significantly reduced infarction volume, when compared with nitrogen control under normothermia (reduction of infarct size by 48, 52, and 65% when argon combined with 37°C, 35°C, and 33°C, respectively, compared with the nitrogen group) (fig. 7, A and B). The reduction in pathological changes by the argon–hypothermia treatment correlated well with the body weight (fig. 7C), while argon exposure increased the body weight, when compared with nitrogen control.
Inhibition of PI3K/Akt Pathway Attenuated Neuroprotection Conferred by Argon–Hypothermia Treatment

Treatment with LY294002 dramatically decreased HO-1 expression levels in argon–hypothermia group (fig. 8, A and B). Caspase-3 expression was evident in LY294002-treated animals (fig. 8, C and D). The significant reduction of infarct size afforded by argon–hypothermia was lost by this treatment on day 28 (fig. 8, E and F). These data indicated that inhibition of PI3-K/Akt significantly attenuated HO-1 up-regulation and neuronal protection.

Discussion

The absence of a safe and effective therapy for hypoxia–ischemia brain injury in newborns has prompted the investigation of the possible protective effects of noble gases, especially xenon. Argon, another noble gas, possesses similar protective properties. However, the neuroprotective potential of argon combined with hypothermia has not been explored to date. The current study demonstrates that argon, when combined with mild or moderate hypothermia (35° and 33°C), elicits robust and prolonged neuroprotection against ischemic brain injury in neonatal rats. PI-3K/Akt pathway activation, HO-1 up-regulation, and GSK-3β inhibition were demonstrated to be the possible molecular mechanisms underlying the beneficial effects of the combined treatment both in vivo and in vitro (fig. 9). Furthermore, inhibition of HO-1 and PI-3K/Akt pathway activation significantly attenuated argon–hypothermia-induced neuroprotection against OGD-induced injury in vivo or in vitro. These findings support our hypothesis that argon works synergistically with hypothermia to provide robust neuroprotection against a hypoxia–ischemia insult in neonatal rats.
an in vivo model of acute focal cerebral ischemia in adult rats, exposure to 50% Ar significantly reduced infarct volumes and neurological deficits after the occlusion.23 Although noble gases are chemically inert, they are capable of forming induced dipole, which is attracted to the charge that induced it, or instantaneous dipole, which produces and binds to an induced dipole in a second molecule.24 Thus, they might produce biological effects by stabilizing receptors or enzymes in active or inactive forms via interactions with amino acids at the binding sites.25

The PI3K/Akt pathway elicits a survival signal against apoptotic insults26 and has been proposed to be involved in the well-documented neuroprotective effect of insulin-like growth factor-1 in the immature brain.27 Recently, it has been demonstrated to be the up-stream pathway of HO-1,28 which acts against cellular stress, such as oxidative stress. HO-1 is an enzyme induced by oxidative stress; it catabolizes free heme into labile iron, carbon monoxide, and biliverdin.29 HO-1 provides cytoprotection mainly through the catabolism of heme and several end products: HO-1 reduces oxidative stress by breaking down the prooxidant heme; production of carbon monoxide leads to the degradation of proapoptotic p38α mitogen-activated protein kinase and activation of antiapoptotic p38β mitogen-activated protein kinase,30 which may induce the up-regulation of Bcl-231; production of iron limits transcription of proinflammatory genes by inhibiting phosphorylation of NF-κB p6532; biliverdin may also serve as an antioxidant.29 In vitro studies using neuronal cultures have shown that HO-1 protects neurons against oxidative injury.33 In this study, a significant up-regulation of this cytoprotective protein by argon was found in both the cortex and hippocampus. Therefore, we postulate that argon–hypothermia increases HO-1 expression mainly in these interneurons, providing cytoprotection to them although it is plausible that multiple molecular pathways could also be involved in the protective mechanisms.

Fig. 5. Expression of heme oxygenase (HO)-1, Bcl-2, and glycogen synthase kinase (GSK)-3β in brain cortex and hippocampus after combined treatment of argon and hypothermia. Seven-day-old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% O₂ balanced with N₂ for 90 min and then exposed to argon (Ar) gas (70% Ar balanced with 30% O₂) or N₂ gas (70% N₂ balanced with 30% O₂) under hypothermia (33°C) for 2 h and then room air for 24 h. Expression of (A) HO-1, (B) Bcl-2, and (C) phospho-glycogen synthase kinase (p-GSK-3β) Tyr 216 in the cortex was assessed by Western blot, at 4 h after gas exposure. (D) Dual labeling of HO-1 (green fluorescence) and Bcl-2 (red fluorescence) in the hippocampus; (E) dual labeling of HO-1 (green fluorescence) and p-GSK-3β Tyr 216 (red fluorescence) in rat hippocampus; fluorescence intensity (% of naïve control [NC]) of (F) Bcl-2 and (G) p-GSK-3β Tyr 216 in CA1 and CA3 regions of rat hippocampus. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Data are represented as mean ± SD (n = 8); *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bar: 50 μm. HI = hypoxic-ischemic insult; Hy = hypothermia.
Argon and Hypothermia Confer Neuroprotection

Zhao et al. 

Glycogen synthase kinase 3β is a proline-directed serine/threonine kinase in mammals. Dysregulation of GSK has been linked to many diseases such as cancer and neurodegenerative disease. The β-subunit of GSK-3β is activated by phosphorylation of the tyrosine 216 residue in the kinase domain and inactivated by phosphorylation of the amino terminal serine 9 residue. Activation of GSK-3β has been associated with cell death through the intrinsic pathway. In this study, argon significantly decreased GSK-3β activation after OGD-induced injury in vitro and hypoxia–ischemia-induced injury in vivo.

Fig. 6. Effect of argon (Ar) combined with hypothermia treatment on expression of cell death, tissue inflammation, and astrocyte activation in the cortex or hippocampus after hypoxia–ischemia. Seven-day-old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% O₂ balanced with N₂ for 90 min and then exposed to Ar gas (70% Ar balanced with 30% O₂) or N₂ gas (70% N₂ balanced with 30% O₂) under hypothermia (33°C) for 2 h and then room air for 24 h. (A) Coronal sections of the brain 16 h after hypoxia–ischemia are shown. The caspase-3+ areas indicated initiation of caspase-3 activation which is shown with green fluorescence and whose intact region was counterstained with nuclear-staining propidium iodide (red). Expression of (B) caspase-3 (green fluorescence) and (C) Nuclear factor (NF)-κB (red fluorescence) was assessed by immunofluorescence at 4 h after gas exposure. Fluorescence intensity (% of naive control [NC]) of (D) caspase-3 and (F) NF-κB. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). (F) Glial fibrillary acidic protein (GFAP; green fluorescence) in the hippocampus (hippo) CA1 and CA3 of rat brain of NC, injury treated only, N₂ or Ar combined with hypothermia treated at 24 h; scale bar: 50 μm. (G) Fluorescence intensity (% of NC) of GFAP. Data are represented as mean ± SD (n = 8); *P < 0.05, **P < 0.01, and ***P < 0.001; scale bar: 50 μm. HI = hypoxic–ischemic insult; Hy = hypothermia.
Reduction of GSK-3β correlated strongly with caspase-3 activation; this observation is consistent with the previous study by Petit-Paitel et al., which demonstrated that the phosphorylation of tyr216 was involved in mitochondria-dependent neuronal cell death and that inactivation of GSK-3β has been proposed to be important for the neuroprotection afforded by insulin-like growth factor-1 and hexarelin.

During acute neuronal injury, cytochrome c causes the activation and release of apoptotic protease-activating factor-1 into apoptosome, which activates caspase-9 and subsequently caspase-3. An up-regulation of Bcl-2 suppresses this pathway and therefore protects the neurons against apoptosis. In the current study, 70% Ar significantly increased Bcl-2 expression in the cortex and the CA1 and CA3 regions of the hippocampus at 4 h after the gas exposure. Hypoxic-ischemic injury caused marked activation of caspase-3 in the cortex and hippocampus; this was decreased by hypothermia alone and still further by the combination of hypothermia and argon.

Nuclear factor-κB activation is the hallmark of neuroinflammation, which is closely associated with neuronal cell death. Upon removal of inhibitor protein IκB by IκB kinase, the NF-κB (p50/p65) heterodimer translocates into the nucleus and drives the expression of many inflammatory mediators, e.g., tumor necrosis factor-α, cyclooxygenase-2, inducible nitric oxide synthase, and intercellular adhesion molecule-1. Suppression of NF-κB activation has been shown to reduce neuronal damage in a rat model of global cerebral ischemia. In our study, NF-κB was highly up-regulated and nuclear translocation was evident in association with cortical and hippocampal injury. NF-κB activation was suppressed by the combination of argon and hypothermia.

Neuronal inflammation caused by cerebral ischemia induces astrocyte activation and proliferation (reactive astrogliosis) and an increased production of GFAP, a cytoskeletal intermediate filament protein specific to activated astrocytes. Activated astrocytes also release inflammatory mediators such as inducible nitric oxide synthase and cytotoxic molecules such as reactive oxygen species and cause glial scar formation, which impedes axon regeneration and remyelination. In our study, a large increase in GFAP expression was found in the hippocampus of normothermic injury group and this was reversed by the combination of...
Argon and hypothermia confer neuroprotection after hypoxic–ischemic injury. In our study, we treated neonatal rat pups with argon and hypothermia, reversing the potentially harmful overactivation of astrocytes.

Our study is not without limitations: first, only 70% of Ar for 2 h duration of treatment was investigated; its optimal concentration and exposure duration were not sufficiently explored, and this certainly warrants further investigation. Second, the effect of argon on the in vitro OGD-induced neuronal injury was primarily investigated and the effects on other cell types, e.g., glia, during and after insult were not assessed.

Our study has significant clinical implementations; HIE is a devastating condition, which current treatments do little to reverse.1 Our results show that treatment with the combination of argon and hypothermia results in short- and long-term neuroprotection in our in vitro and in vivo models of HIE. This could serve as basis for further research with argon and hypothermia, reversing the potentially harmful overactivation of astrocytes.

Fig. 8. Inhibition of phosphoinositide-3-kinase (PI-3K)/Akt abolished argon–hypothermia–mediated neuroprotection. Seven-day-old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% O2 balanced with N2 for 90 min. PI3K-Akt inhibitor LY294002 or vehicle was then administered intracerebroventriculally after hypoxic–ischemic injury before exposure to argon (Ar) gas (70% Ar balanced with 30% O2) or N2 gas (70% N2 balanced with 30% O2) under moderate hypothermia (33°C) for 2 h and then room air for 24 h. (A) Heme oxygenase (HO)-1 expression (green fluorescence) in the cortex and hippocampus at 4 h after gas treatment. Scale bar: 10 μm. (B) Immunofluorescence intensity of HO-1 at 4 h after gas treatment. (C) Caspase-3 expression (green fluorescence) in the cortex and hippocampus at 4 h after gas treatment. Scale bar: 50 μm. (D) Immunofluorescence intensity of caspase-3 at 4 h after gas treatment. (E) Representative brain micrograph, stained by cresyl violet, on 28 days after treatment. (F) Infarct volume on 28 days after treatment. Data are represented as mean ± SD (n = 8); *P < 0.05 and ***P < 0.001.

HI = hypoxic–ischemic insult; Hy = hypothermia; Ly = LY294002; NC = naive control; Ve = vehicle; Vh = vehicle.

Fig. 9. Putative molecular mechanisms of argon combined with hypothermia-mediated neuroprotection. Argon combined with hypothermia activated phosphoinositide-3-kinase (PI-3K)/Akt pathway, enhanced heme oxygenase (HO)-1 and Bcl-2 expression and reduced phospho-glycogen synthase kinase (p-GSK)-3β Tyr216 expression. This leads to reduced tissue damage and inflammation in neonatal rat after hypoxia–ischemia brain injury. NF-κB = nuclear factor-κB.
in combination with hypothermia as an effective strategy against hypoxia-ischemia brain injury in neonates.

Research Support
This work was supported by a grant (10IMP01) from SPARKS, London, United Kingdom.

Competing Interests
The authors declare no competing interests.

Correspondence
Address correspondence to Dr. Ma: Faculty of Medicine, Section of Anaesthetics, Pain Medicine and Intensive Care, Department of Surgery and Cancer, Imperial College London, Chelsea and Westminster Hospital, London SW10 9NH, United Kingdom. d.ma@imperial.ac.uk. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References
29. Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM, Cuadrado A: Regulation of heme oxygenase-1...
expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem 2004; 279:8919–29


