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Combination of Xenon and Isoflurane Produces a Synergistic Protective Effect against Oxygen–Glucose Deprivation Injury in a Neuronal–Glial Co-culture Model

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SUSTAINED exposure to glutamate causes neuronal death by overactivation of its receptors, particularly those of the *N*-methyl-D-aspartate (NMDA) subtype.[1,2] This process, denoted by the term *excitotoxicity*, is believed to play an important role in ongoing neuronal injury and death in acute insults, such as ischemic stroke and head trauma.[3,4] Consequently, the neuroprotective effects of NMDA receptor antagonists, including xenon, have been investigated in a variety of both *in vitro* and *in vivo* models of neuronal injury[5–9] of the type that may occur perioperatively.[10] Notwithstanding their putative beneficial effects, the clinical use of NMDA antagonists has been hindered[11] by the observation that several drugs of this class of compound produce neurotoxicity, characterized by distinctive behavioral and morphologic effects. Although xenon does not seem to have this side effect,[8] it may be desirable to use lower concentrations because of the prohibitive cost of this gas and to permit adequate oxygenation. Therefore, we sought to investigate whether neuroprotective efficacy can be observed when this NMDA receptor antagonist is combined with another putative neuroprotective agent that acts by modulating the γ-aminobutyric acid (GABA) receptor. The neuronal damage from ischemia may also be a result of loss of inhibitory influences. In the brain, GABA acts as the major inhibitory neurotransmitter; an agonist of the A subtype of the GABA receptor ($GABA_A$) has been shown to be neuroprotective in a transient forebrain ischemia model.[12] Isoflurane potentiates activation of the $GABA_A$ receptors,[13] and its neuroprotective effect has been demonstrated previously.[14–18] Because these anesthetics exert their neuroprotectant properties through different mechanisms, we hypothesized that in combination, their efficacy would be enhanced. To test our hypothesis, we studied the neuroprotective effect of the combination of xenon and isoflurane *versus* oxygen–glucose deprivation (OGD) injury in a neuronal–glial co-culture model.

Materials and Methods

The methodology for preparing mixed cortical cell cultures containing both neuronal and glial cell elements from mice has been reported elsewhere.[5] Before OGD exposure, the cell cocultures were carefully washed twice with HEPES buffer, after which prewarmed (37°C), deoxygenated balanced salt solution containing (in mM): 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 1.8 $CaCl_2$, and 26 $NaHCO_3$, pH 7.4, was added into each well, and the plates were placed in a temperature-controlled anaerobic chamber for 75 min at 37°C. Pilot studies showed that the maximal neuronal injury could be achieved by OGD for between 60 and 100 min. OGD was terminated by washing with Eagle's minimal essential medium enhanced with 25 mM glucose and 38 mM $NaHCO_3$, and the culture plates were returned to normoxic conditions containing a minimum of 20% O_2 for 6 h at 37°C. The balance of the gas mixture depended on the experimental group. Culture media for the OGD and the 6 h post-OGD periods were prepared by bubbling pure gases (O_2, N_2, CO_2, xenon, or isoflurane) through fine sintered-glass bubblers in Drechsel bottles filled with either balanced salt solution or Eagle's minimal essential medium for 20 min, a period that was found to be sufficient to allow equilibration as assessed by gas chromatography. For each set of experimental conditions, flow rates of gases were adjusted so that the gas concentrations in the solutions were equivalent to the corresponding gas composition in a purpose-built airtight, temperature-controlled cell-culture chamber equipped with inlet and outlet valves and an internal electric fan (as described previously[9]). Total gas flow was 100 ml/min, and the chamber was flushed for 40 min before a closed system was established. With the above-mentioned protocol, the final gas concentrations (determined by use of gas chromatography) were found to be stable, and there was no measurable leakage over 24 h. For the delivery of isoflurane, a Cyprane isoflurane vaporizer (AE Service and Supplies Ltd., Keighley, United

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Kingdom) was used; because this vaporizer is not calibrated to operate at these low flow rates, we monitored isoflurane concentration (Datex-Engstrom AS/3; Instrumentarium Corp, Helsinki, Finland) continuously to establish that the appropriate concentration was being delivered. Neuronal injury was assessed by lactate dehydrogenase (LDH) released into the medium, as previously described.[9] The data were fitted to a logistic equation of the form:

$$E(c) = \frac{\alpha^n}{\alpha^n + c^n},$$

where *E(c)* is the LDH release expressed as a fraction of the control at a xenon or isoflurane concentration *c*, α is the IC_{50}, and *n* is a slope factor. The results are expressed as mean ± SEM. The IC_{50} value is defined as the concentration of xenon or isoflurane that reduced the LDH release to 50% of the maximal value in control OGD exposures (*i.e.*, in the absence of either anesthetic).

To determine whether or not the combined effects of xenon and isoflurane were additive when administered as a mixture, we compared the observed IC_{50} value for the mixture with that predicted assuming additivity. In general, for additivity, the following condition holds:

$$\frac{a}{\alpha_a\left(\frac{1 - E(c_a)}{E(c_a)}\right)^{1/n_a}} + \frac{b}{\alpha_b\left(\frac{1 - E(c_b)}{E(c_b)}\right)^{1/n_b}} = 1,$$

where *a* and *b* are the concentrations of xenon and isoflurane, respectively, that are present in a mixture, and α_a and α_b are the IC_{50} concentrations of xenon and isoflurane when applied alone, with associated slope factors n_a and n_b. For the special case of 50% inhibition of LDH release (*i.e.*, E = 0.5), this equation reduces to:

$$\frac{a}{\alpha_a} + \frac{b}{\alpha_b} = 1.$$

Thus, having determined α_a and α_b from the concentration–response curves of each agent individually, the concentration of a (xenon) in the presence of a fixed concentration of b (isoflurane), or *vice versa*, to achieve a 50% reduction in LDH release from the control value can be predicted, assuming that additivity holds. This predicted value can then be compared with the observed value. Statistical significance was assessed by use of the Student *t* test with Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant.

Results

Neuronal damage induced by a 75-min duration of OGD was reduced significantly by the presence of increasing concentrations of either xenon (fig. 1A) or isoflurane (fig. 1B) as indicated by LDH release. The IC_{50} values (mean ± SEM) were 35.9 ± 2.2% atm for xenon and 2.72 ± 0.35% atm for isoflurane. We next determined the effects of a low concentration of one agent on the concentration–response curve for the other agent. In the presence of 0.6% isoflurane, the concentration–response curve for xenon was changed significantly (fig. 1A), and the IC_{50} value for xenon-mediated neuroprotection decreased to 18.9 ± 2.5% atm, which is significantly lower than 28 ± 2.0% atm ($P < 0.05$), the predicted IC_{50} value for xenon in the presence of 0.6% isoflurane, assuming that the effects of isoflurane and xenon are additive (see Materials and Methods). Similarly, in the presence of 14% xenon, the concentration–response curve for isoflurane was changed significantly (fig. 1B), with the IC_{50} value being decreased to 0.92 ± 0.13% ($P < 0.05$), which is significantly lower than 1.66 ± 0.22% atm, the predicted IC_{50} value for isoflurane, assuming additivity. Therefore, for both isoflurane added to xenon and xenon added to isoflurane, the data are consistent with a synergistic neuroprotective effect, which is represented isobolographically (fig. 2).

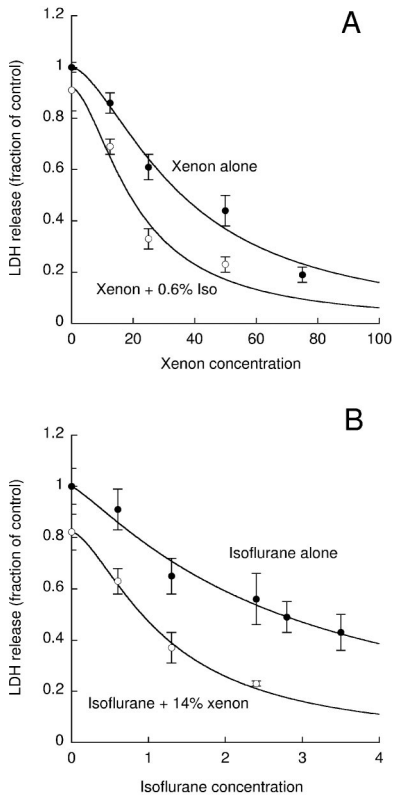

Fig. 1. Xenon and isoflurane alone and in combination inhibited lactate dehydrogenase (LDH) release, expressed as a fraction of the maximum LDH release in the absence of agents (mean ± SEM, *n* = 3 or 4) induced by 75 min oxygen–glucose deprivation. The values for control LDH release in this series of experiments were in the range between 327 and 380 Berger-Broida units/ml. (*A*) *Closed circles* = xenon alone, 35.9 ± 2.2% atm; *open circles* = in the present of 0.6% isoflurane, IC_{50} = 18.9 ± 2.5% atm. (*B*) *Closed circles* = isoflurane alone, IC_{50} = 2.72 ± 0.35%; *open circles* = in the presence of 14% xenon, IC_{50} = 0.92% ± 0.13%.

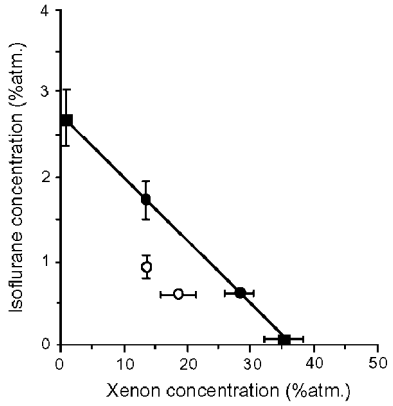

Fig. 2. Isobologram of the combinations of xenon and isoflurane on lactate dehydrogenase release induced by oxygen–glucose deprivation. The *solid diagonal line* is the "line of additivity," constructed by joining the IC_{50} ± SEM for each agent. The *open circle with horizontal bar* is the IC_{50} ± SEM for xenon when combined with 0.6% isoflurane, and the *open circle with vertical bar* is the IC_{50} ± SEM for isoflurane when combined with 14% xenon. *Closed circles* represent the predicted IC_{50}s ± SEM if their combined effect is additive. The predicted IC_{50} values assuming simple additivity differ significantly from the actual IC_{50} values for xenon and isoflurane, respectively ($P < 0.05$), in both cases, indicating that the nature of their combined protection against neuronal injury is synergistic.

To clarify the mechanisms for the neuroprotective effects of each agent, additional OGD studies were performed in the presence of a $GABA_A$ receptor agonist and antagonist. We found that 20 μM of the $GABA_A$ receptor agonist muscimol exerted a neuroprotective effect comparable to that observed with either 75% atm xenon or 3.5% atm isoflurane (fig. 3A). However, the $GABA_A$ receptor antagonist gabazine had no significant effect on the neuroprotection provided by 75% xenon, whereas it did largely reverse the neuroprotection afforded by 3.5% isoflurane (fig. 3A). None of the agents tested (xenon, isoflurane, muscimol, and gabazine) had a significant effect on the basal LDH release under control conditions (*i.e.*, in the absence of OGD) (fig. 3B).

Discussion

The neuroprotective property of isoflurane has been reported in both focal and global ischemia models.[14–18] Although isoflurane also reduced NMDA-induced neuronal damage when NMDA was microinjected directly into cerebral cortex in rats,[19] it provides little neuroprotection within clinical concentration ranges, as reported in a previous *in vitro* study.[20] Under our *in vitro* conditions, which approximate the conditions associated with excitotoxicity *in vivo*,[21] we demonstrated that isoflurane prevented neuronal injury induced by OGD and that this effect could be largely reversed by gabazine. This would suggest that the mechanism of the neuroprotection of isoflurane is at least in part a result of $GABA_A$ receptor stimulation. This is consistent with the known ability of isoflurane to potentiate $GABA_A$ receptors.[13]

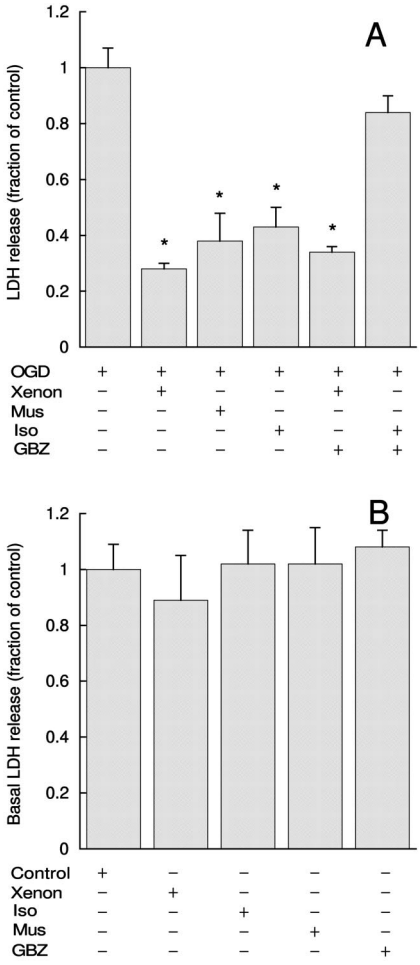

Fig. 3. (*A*) Lactate dehydrogenase (LDH) release, expressed as a fraction of the maximum LDH release (mean ± SEM, $n = 3$ or 4) caused by oxygen–glucose deprivation OGD, was reduced significantly by xenon (75% atm), the $GABA_A$ receptor agonist muscimol (Mus; 20 μM), and isoflurane (Iso; 3.5% atm). However the $GABA_A$ receptor antagonist gabazine (GBZ; 50 μM) had no significant effect on the neuroprotection afforded by 75% atm xenon but did largely reverse the neuroprotection afforded by 3.5% atm isoflurane (*$P < 0.01$). The values for control LDH release in this series of experiments were in the range between 286 and 438 Berger-Broida units/ml. (*B*) The basal LDH release under control conditions (*i.e.*, with no oxygen–glucose deprivation) was unaffected by xenon (75% atm), isoflurane (3.5% atm), muscimol (20 μM), or gabazine (50 μM). The values for control LDH release in this series of experiments were in the range between 52 and 94 Berger-Broida units/ml.

In a series of ongoing studies, we have reported that xenon exerts dose-dependent neuroprotective effects in *in vitro* and *in vivo* models of neuronal injury, as evidenced both morphologically and functionally.[9,10] Unlike other anesthetic agents, effective neuroprotection can be produced with subanesthetic concentrations.[9] The neuroprotective effect of xenon is likely to be produced *via* its antagonistic action at the NMDA subtype of the glutamate receptor.

We have shown here that xenon and isoflurane in combination have a synergistic neuroprotective effect. This is consistent with data from a recent *in vivo* study in which the combination of a $GABA_A$ receptor agonist,

muscimol, and a glutamate antagonist, MK801, produced synergistic neuroprotection in a stroke model.[22] In the context of these results, our data imply that a synergistic neuroprotective effect can be obtained by simultaneous inhibition of the NMDA subtype of the glutamate receptor together with potentiation of $GABA_A$ receptors.

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