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Sevoflurane Protects Rat Mixed Cerebrocortical Neuronal–Glial Cell Cultures against Transient Oxygen–Glucose Deprivation

Involvement of Glutamate Uptake and Reactive Oxygen Species

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Background: **The purpose of this study was to clarify the role of glutamate and reactive oxygen species in sevoflurane-mediated neuroprotection on an *in vitro* model of ischemia–reoxygenation.**

Methods: **Mature mixed cerebrocortical neuronal–glial cell cultures, treated or not with increasing concentrations of sevoflurane, were exposed to 90 min combined oxygen–glucose deprivation (OGD) in an anaerobic chamber followed by reoxygenation. Cell death was quantified by lactate dehydrogenase release into the media and cell viability by reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium by mitochondrial succinate dehydrogenase. Extracellular concentrations of glutamate and glutamate uptake were assessed at the end of the ischemic injury by high-performance liquid chromatography and incorporation of L-[^{3}H]glutamate into cells, respectively. Free radical generation in cells was assessed 6 h after OGD during the reoxygenation period using 2′,7′-dichlorofluorescin diacetate, which reacts with intracellular radicals to be converted to its fluorescent product, 2′,7′-dichlorofluorescin, in cell cytosol.**

Results: **Twenty-four hours after OGD, sevoflurane, in a concentration-dependent manner, significantly reduced lactate dehydrogenase release and increased cell viability. At the end of OGD, sevoflurane was able to reduce the OGD-induced decrease in glutamate uptake. This effect was impaired in the presence of threo-3-methyl glutamate, a specific inhibitor of the glial transporter GLT1. Sevoflurane counteracted the increase in extracellular level of glutamate during OGD and the generation of reactive oxygen species during reoxygenation.**

Conclusion: **Sevoflurane had a neuroprotective effect in this *in vitro* model of ischemia–reoxygenation. This beneficial effect may be explained, at least in part, by sevoflurane-induced antiexcitotoxic properties during OGD, probably depending on GLT1, and by sevoflurane-induced decrease of reactive oxygen species generation during reoxygenation.**

VOLATILE anesthetics have been shown to reduce the volume of cerebral infarction[1,2] and to improve neurologic outcome[2,3] after experimental stroke *in vivo*. However, the mechanisms by which these neuroprotective effects are achieved have not yet been clearly established.

Volatile anesthetics were demonstrated to inhibit glutamate receptor–mediated toxicity *in vivo*[4–6] and *in vitro*[7,8] and penumbral spontaneous depolarizations.[9] Moreover, they are known to reduce the increase in extracellular glutamate levels induced by ischemia *in vivo*[10,11] and *in vitro*.[12] Do *et al.*[13] and Miyazaki *et al.*[14] have also shown that volatile anesthetics enhance the activity of glutamate transporters expressed either in *Xenopus* oocytes or in cultured astrocytes. Nevertheless, a possible antiexcitotoxic effect of volatile anesthetics based on the preservation of high-affinity glutamate transporters (EAAC1, GLAST, and GLT1) during ischemia has never been explored.

Another mechanism to explain volatile anesthetic–induced neuroprotection from ischemia could rely on antioxidant properties of volatile anesthetics. Although volatile anesthetics seem devoid of direct free radical–scavenging properties, and therefore antioxidant properties,[15] a relation between reactive oxygen species (ROS) and cerebral protection by volatile anesthetics cannot be excluded because volatile anesthetics reduce the release of deleterious quantities of ROS associated with cardiac ischemia–reperfusion.[16] However, the effect of volatile anesthetics on ROS generation during cerebral ischemia–reperfusion has not yet been explored.

Sevoflurane is a widely used volatile anesthetic in clinical practice, and its capacity to increase cerebral blood flow while preserving cerebral autoregulation makes it an attractive agent for the preservation of neuronal function.[17] The purpose of the current study was first to determine the extent of sevoflurane-induced neuroprotection on mixed cerebrocortical neuronal–glial cell cultures subjected to transient oxygen–glucose deprivation (OGD). The noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (MK-801) was used as the reference neuroprotectant.[18] Second, the possible involvement of extracellular glutamate levels, glutamate uptake activity, and ROS levels in the mechanism of the neuroprotective effect of sevoflurane was explored.

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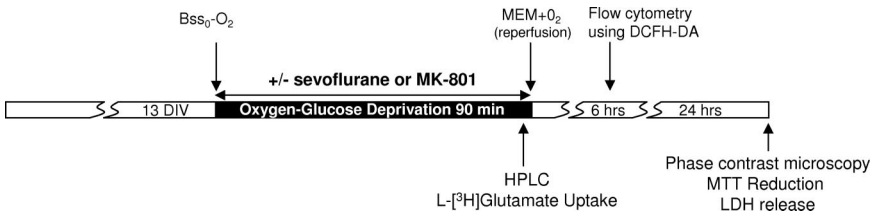

Fig. 1. Experimental paradigm. $BSS_0 - O_2$ = desoxygenated glucose-free balanced salt solution; DCFH-DA = 2′,7′-dichlorofluorescin diacetate; DIV = days *in vitro*; HPLC = high-performance liquid chromatography; LDH = lactate dehydrogenase; MEM + O_2 = oxygenated minimum essential medium with Earle salts; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Materials and Methods

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Declaration of Helsinki. Thus, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (P. Pisano; agreement No. 13-59).

Preparation of Cell Cultures

Mixed cerebrocortical neuronal–glial cell cultures were prepared from cortex of 18-day-old Wistar rat fetuses. Briefly, after removal of meningeal, striatal, and hippocampal tissues and olfactory bulbs, cerebral cortices were pooled and maintained in ice-cold phosphate-buffered saline solution (Bio-Whittaker, Emerainville, France) supplemented with glucose (33 mM). Cells were mechanically dissociated from forebrains in dissociation medium by trituration through a fire-polished 9-in Pasteur pipette. After centrifugation, the pellets were resuspended in minimum essential medium with Earle salts, L-glutamine free (Life Technologies, Cergy-Pontoise, France), supplemented with 15 mM glucose, 5% fetal bovine serum (batch No. 016723; Bio-Wittaker, Cambrex, France), 5% horse serum (batch No. H0146; Sigma, Saint-Quentin Fallavier, France), 50 U/ml penicillin, and 50 μg/ml streptomycin and plated to achieve a confluent monolayer (10^5 cells/cm^2) on plastic 12-well culture plates (Costar, Brumath, France), previously coated with polyornithine (10 μg/ml). For each culture, 20–30 fetuses from two gravid animals were prepared for dissociated cultures, and cells were plated into 12-well culture plates, each well containing 2 million cells. Culture dishes were incubated at 37°C in a humidified 6% CO_2 and 94% air atmosphere. Experiments were performed on mature cultures, after synaptogenesis at 13 days *in vitro*. At that time, the cells' medium was changed from serum-rich culture medium to serum-free culture conditions. Cultures were observed every day under light microscope, and plates were discarded if neurons seemed nonviable (*i.e.*, rough appearance, irregular soma, or fragmented neurites).

OGD Followed by Reoxygenation

Mixed cultures were subjected to a transient OGD as described by Goldberg and Choi[19] with minor modification. Cells were placed in an anaerobic chamber (partial oxygen pressure was maintained below 2 mmHg), and the medium was exchanged for a prewarmed (37°C) glucose-free balanced salt solution ($BSS_0 - O_2$: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 0.025 mM phenol red, and 20 mM sucrose) previously bubbled with an anaerobic gas mix (95% N_2, 5% CO_2) for 30 min to remove residual oxygen. Cell cultures subjected to OGD were called OGD cells and were incubated in this solution at 37°C for a 90-min period to produce lethal oxygen deprivation. This period of OGD was chosen according to the experiments of Goldberg *et al.*[20] and according to preliminary results from us[21] showing that it produced injury essentially limited to neurons, with no glial cell death. Sham wash cell cultures, not subjected to OGD, were placed in $BSS_{20} + O_2$ containing 20 mM glucose and aerated with an aerobic gas mix (95% air, 5% CO_2). OGD was terminated by removing cultures from the chamber, replacing the exposure solution with oxygenated minimum essential media with Earle salts, L-glutamine–free, supplemented with 20 mM glucose and returning the multiwells to the incubator under normoxic conditions. Sevoflurane (Abbott Laboratories, Rungis, France) and dizocilpine maleate (Tocris, Fisher Bioblock Scientific, Illkirch, France) were added at the start of OGD and were removed by washout during the reoxygenation process. As shown in figure 1, 24 h after the OGD, assessment of cell injury was quantitatively performed by two biochemical tests measuring the release of cytoplasmic lactate dehydrogenase (LDH) and the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). At the end of the OGD period, before reoxygenation, extracellular glutamate concentrations and glutamate uptake activity were determined. Finally, 6 h after the reoxygenation, ROS generation was assessed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA).

LDH Release

Cell injury was quantitatively assessed by measuring cytosolic LDH release into the bathing medium, 24 h after reoxygenation (Cytotoxicity Detection Kit [LDH]; Roche Diagnostic, Meylan, France). Background LDH levels (11%) were determined in sham wash and subtracted from experimental values to yield the LDH activity specific to the experimental injury. In this system, LDH values vary little in sister cultures derived from the

same plating. However, despite a strict technical standardization of our cell culture conditions, some variability is present from plating to plating. To combine results of experiments performed on cultures from different platings, LDH values were normalized to the mean maximal LDH value in sister cultures continuously exposed to 300 μM NMDA (Tocris, Fisher Bioblock Scientific) and 1 μM glycine (Tocris, Fisher Bioblock Scientific) for a full 24-h period, which results in near-complete neuronal death without glial cell death (= 100%).[22] Results are expressed as a percentage of the maximal LDH level. Each experimental condition was done in triplicate, replicated in a minimum of four plates per culture using cells obtained from six independent cultures.

MTT Reduction Test

Cell viability was quantitatively evaluated 24 h after reoxygenation by the MTT reduction test (Sigma). This test quantifies the formation of a dark blue formazan produced by the reduction of the tetrazolium ring of MTT by the mitochondrial succinate dehydrogenase in living cells.[23] Cell viability corresponded to the value of the optical density read at 570 nm with background subtraction at 630 nm using a spectrophotometer (ELX 800; Biotek Instruments, Winooski, VT). Values obtained from cultures exposed to 300 μm NMDA and 1 μm glycine for 24 h after the OGD (= 100%) were subtracted from those measured in all experimental conditions. Results were expressed as a percentage of the optical density measured in sham wash cells. Each experimental condition was done in triplicate, replicated in a minimum of four plates per culture using cells obtained from six independent cultures.

Measurement of Extracellular Glutamate Level

Extracellular concentrations of glutamate in the culture supernatants were measured in duplicate at the end of the OGD period using high-performance liquid chromatography. An aliquot of 250 μl culture supernatant was deproteinized by 250 μl perchloric acid, 0.5N, mixed and centrifuged (30,000 rpm for 10 min) at 4°C. The supernatant was then stored at −80°C until high-performance liquid chromatography analysis. All samples were analyzed for glutamate content using high-performance liquid chromatography along with fluorometric detection (scanning fluorescence detector 474; Waters Corporation, Saint Quentin en Yvelines, France) after precolumn derivatization with *o*-phtaldialdehyde. Each sample was diluted 1:20 in 0.15 M sodium borate buffer, and 15 μl of the dilution was directly injected into a C-18 column (5 μm Spherisorb; Richard Scientific, Novato, CA; ODS2 150 × 3 mm). The mobile phase consisted of 0.1 M potassium acetate (pH 5.8) in methanol (vol/vol, 1/7). Elution was performed with a methanol gradient ranging from 12.5 to 56%. The area of the peak was determined with the Millenium32 version 3.05 software (Waters Corporation, Milford, MA) and compared with the peak area of a 1 μM glutamate standard solution to determine glutamate concentration. The limit of detection in these conditions was 1.5 pmol/15 μl. Results were expressed in micromolars. Each experimental condition was done in duplicate, replicated in a minimum of four plates per culture using cells obtained from six independent cultures.

Uptake Experiments

Uptake experiments were performed at the end of the OGD period. When a potential direct effect of sevoflurane on basal glutamate uptake was tested, 3.4 mM sevoflurane was added in sham wash 90 min before uptake measurements. After two washes of the cells with the uptake buffer (10 mM glucose, 5 mM KCl, 127 mM NaCl, 2.5 mM $CaCl_2$, 0.2 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 10 mM HEPES; pH 7.4), L-[^{3}H]glutamate (specific activity 15–25 Ci/mmol NEN; Paris, France) was added at 10^{-6} M final concentration, diluted in the buffered physiologic medium. Incubations were performed at 37°C for 2 min. The reaction was stopped by rapidly adding 1 ml cold, sodium-free buffer, followed by two washes with the same cold medium. To dissolve the cells, 1N NaOH was added to the culture dishes, and the radioactivity was assessed by liquid scintillation counting at least 12 h later. To determine the part of radioactivity not due to sodium-dependent glutamate transport, L-[^{3}H]glutamate uptake was assessed in physiologic medium in which sodium was omitted and replaced by choline. Glutamate transport was calculated as glutamate uptake rate in the presence of sodium minus glutamate uptake in the absence of sodium. Protein content was determined by the method developed by Lowry *et al.*[24] with bovine serum albumin as the standard. To estimate the role of the glial glutamate transporter GLT1 in the measured uptake rates, some incubations were performed in the presence of threo-3-methyl glutamate (3MG; Tocris, Fisher Bioblock Scientific), a rather specific inhibitor of this carrier, which was added (300 μM) at the time of the uptake experiments. Results (pmol glutamate · mg protein^{-1} · min^{-1}) were expressed as percent of the value obtained in sham wash cells. Each experimental condition was done in triplicate, replicated in a minimum of four plates per culture using cells obtained from six independent cultures.

Preparation of Sevoflurane Solutions

A 10 mM stock solution was prepared as described by Kudo *et al.*[25] by injecting 140 μl liquid sevoflurane into 103 ml BSS_0. The sealed flask was stirred for 24 h to solubilize the anesthetic agent. Immediately before use, 30 ml concentrated stock solution was poured into a 50-ml polypropylene centrifuge tube and vortexed for 5–10 s to produce the working stock. The working stock

was diluted with BSS_0 or BSS_{20} to produce the anesthetic concentration required to treat cultures.

Measurement of Dissolved Sevoflurane Concentration

The internal standard chloroform (10 μl) was added to a 1-ml medium sample containing dissolved sevoflurane. The mixture was transferred to a sealed 4.4-ml vial. The vial was vortexed for 1 min to balance the amount of volatile anesthetic in the gas and liquid phases. The analysis of the sevoflurane was performed using the Perkin Elmer GC system consisting of a chromatograph with flame ionization detector (Autosystem; Perkin-Elmer, Courtaboeuf, France) coupled to a headspace system (HS40; Perkin-Elmer). Separation was achieved using a capillary column (Optimum 624: cyanopropylphenyl-dimethylpolysiloxane column, 30 m × 0.4 mm ID, 1.40 μm film thickness; Macherey Nagel, Hoerdt, France). The carrier gas (helium) was set at 15 psi. Standards (0.01–4 mM) were prepared in undecane from liquid sevoflurane (Abbott Laboratories, Rungis, France). The percentage loss of sevoflurane in solution during the OGD period was determined by incubating samples of the working stock under identical conditions used in treating cell cultures for 90 min of OGD. The concentration was measured before and after the incubation period. The percentage loss of sevoflurane over the 90-min incubation was, in our conditions, 63%. Values presented for sevoflurane concentrations were corrected for percentage loss by averaging the concentrations at the start and end of the incubation (n = 10 observations).[25]

Monitoring of ROS Generation

Six hours after the reoxygenation procedure, sham wash or cells subjected to the OGD–reoxygenation sequence, treated or not with 3.4 mM sevoflurane, were loaded with 1 ml fresh serum-free medium containing 20 μM of the fluorogenic probe DCFH-DA (Molecular Probes, Interchim, Montluçon, France) from a 1 mM stock solution in dimethylsulfoxide. DCFH-DA reacts with intracellular radicals to be converted to its fluorescent product, 2′,7′-dichlorofluorescin, in cell cytosol. Experiments were replicated in three wells. Culture plates were replaced in the incubator at 37°C for 20 min, and cells were subsequently suspended after adding 1 ml phosphate-buffered saline solution containing 0.5% trypsin. The effects of trypsin were then blocked by the addition of 1 ml phosphate-buffered saline solution enriched with 0.5 mM Ca^{2+}–Mg^{2+}, and the cell suspensions obtained from three wells per condition were pooled in a polypropylene tube stored on ice until analyzed by means of a flow cytometer equipped with an argon laser (FACSCalibur® flow cytometer; Becton-Dickinson, Le Pont De Claix, France). In each tube, intracellular green fluorescence was measured with excitation at 488 nm and emission at 525 nm, by analyzing at least 25,000 living cells per tube. Results (percent of fluorescent living cells) were expressed as a percentage of the value obtained in sham wash cells. Intracellular levels of free radicals measured in sham wash cells were low (5.1 ± 1.7% of fluorescent living cells). Freshly prepared hydrogen peroxide (H_2O_2, 10 μM, 20 min) was used as the reference oxidant with DCFH-DA treated sham wash cells. *N*-Acetylcysteine (Sigma), which acts as a precursor for glutathione synthesis, was dissolved (50 μM) in distilled water and used as the reference antioxidant. Each experimental condition was replicated in a minimum of four tubes per culture using cells obtained from eight independent cultures.

Statistical Analysis

Statistical analysis was performed for each parameter investigated by one-way analysis of variance followed by *post hoc* Tukey test for multiple comparisons (SigmaStat® 2.0; SPSS Inc., Chicago, IL). Estimated EC_{50} values were derived from fits of our data by Hill equations using MPD 5.0 program (Micropharm, INSERM, Paris, France). Values were reported as mean ± SD. Differences were considered significant for $P < 0.05$.

Results

Sevoflurane Protects Neurons against OGD-induced Injury

Twenty-four hours after our mixed cerebrocortical neuronal–glial cell cultures were subjected to 90 min of OGD, 78.5 ± 4.4% of the neurons died, as assessed by LDH release (fig. 2A), and 21.1 ± 4.0% remained viable, as evaluated by MTT reduction (fig. 2B). Sevoflurane (0.15–3.4 mM) elicited a potent and concentration-dependent neuroprotective effect, as shown by a significant inhibition of the OGD-induced increase in LDH and decrease in MTT reduction. Twenty-four hours after OGD, the maximal neuroprotection was afforded by 3.4 mM sevoflurane and was similar when evaluated by LDH release (72%) or by MTT reduction (68%). The sevoflurane EC_{50} concentrations for neuroprotection were 0.18 ± 0.04 and 0.24 ± 0.03 mM, respectively, when using LDH or MTT test. The reference neuroprotectant MK-801 at 10 μM also mitigated the effects of OGD on LDH release and MTT reduction.

Prevention of OGD-induced Increase in Extracellular Glutamate Level by Sevoflurane

At the end of the 90-min OGD exposure, before reoxygenation, glutamate extracellular levels (fig. 3) were significantly increased compared with those measured in sham wash cells (OGD cells: 20.8 ± 7.6 μM *vs.* sham wash: 5.4 ± 2.2 μM). Sevoflurane (3.4 mM) did not influence the glutamate extracellular level in sham wash

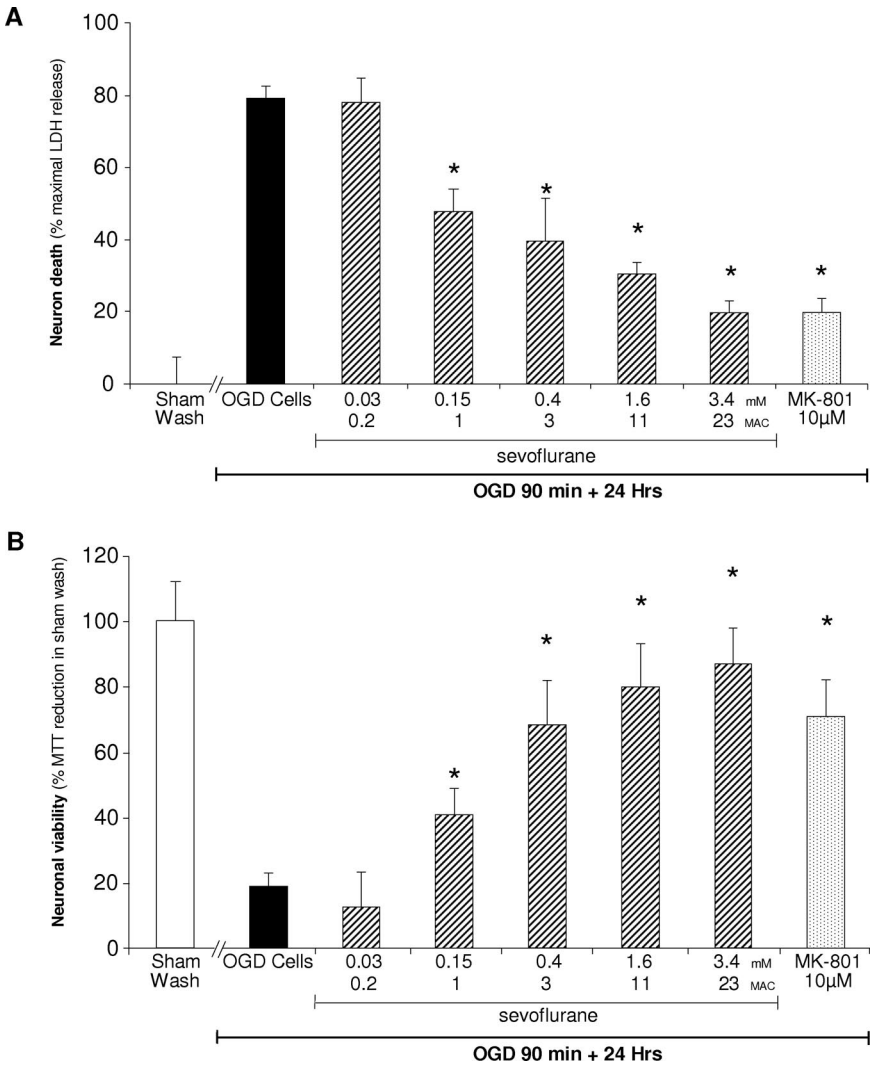

Fig. 2. Neuroprotection afforded by sevoflurane was assessed by the reduction of the increase in lactate dehydrogenase (LDH) release and the decrease in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction, both induced by oxygen–glucose deprivation (OGD). (*A*) Neuron death after 90 min of OGD was assessed by LDH release to the bathing medium. (*B*) Neuronal viability was assessed 24 h after 90 min of OGD by the densitometric analysis of MTT reduction test. Results were expressed as mean percentage of the optical density measured in sham wash cells. n = 24; * $P < 0.05$ *versus* OGD cells.

cells. However, sevoflurane counteracted the increased glutamate concentrations induced by OGD.

Prevention of OGD-induced Decrease in Glutamate Uptake by Sevoflurane

At the end of the 90-min OGD, before reoxygenation, we observed a 48.4 ± 10.6% impairment of the L-[^{3}H]glutamate uptake compared with the transport activity measured in sham wash cells (fig. 4). Sevoflurane (3.4 mM) had no effect on basal glutamate uptake measured on sham wash cells. However, sevoflurane counteracted the OGD-induced decrease in glutamate uptake. To clarify the involvement of GLT1 in the uptake activity measured with or without sevoflurane (3.4 mM), exper-

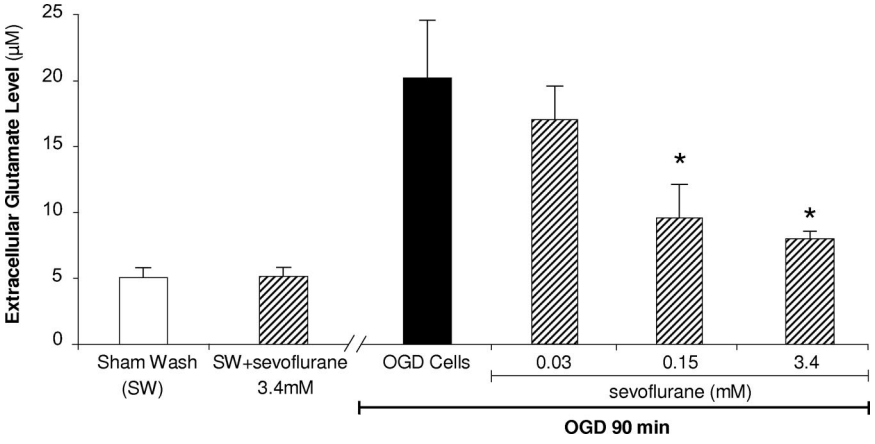

Fig. 3. After 90 min of oxygen–glucose deprivation (OGD), sevoflurane counteracted the OGD-induced increase in extracellular glutamate. Extracellular levels of glutamate in the culture supernatants were evaluated at the end of the OGD, before reoxygenation, by high-performance liquid chromatography analysis along with fluorometric detection. At the end of the 90-min OGD period, extracellular glutamate levels were significantly increased compared with those measured in sham wash (SW) cells. Sevoflurane, 3.4 mM, added to SW cells did not modify the extracellular glutamate levels. However, when added at the start of the injury, sevoflurane (0.15–3.4 mM) counteracted the OGD-induced increase in glutamate concentration. n = 20; * $P < 0.05$ *versus* OGD cells.

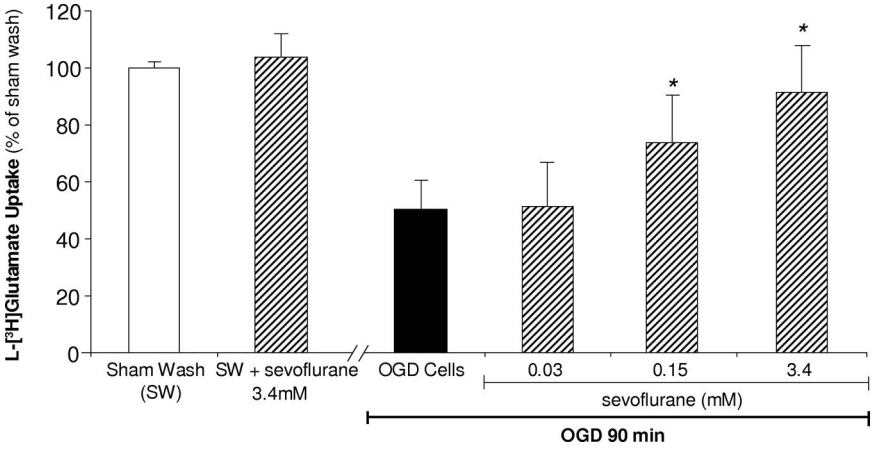

Fig. 4. After 90 min of oxygen–glucose deprivation (OGD), sevoflurane (0.15–3.4 mM) counteracted the OGD-impaired glutamate uptake. Uptake experiments were performed at the end of the injury, before reoxygenation by incorporation of L-[^{3}H]glutamate. Sevoflurane did not modify the glutamate transport apparent rate of sham wash (SW), but it counteracted the OGD-induced decrease in glutamate uptake when it was added at the start of the injury. n = 24; * $P < 0.05$ *versus* OGD cells.

iments were performed in the presence of 3MG, which preferentially inhibits this carrier (fig. 5). 3MG (300 μM) induced a 37.5 ± 10.5% decrease in uptake rate measured in sham wash but did not further affect this activity after OGD (percent of sham wash; OGD: 51.6 ± 10.6%; OGD + 3MG: 47.1 ± 11.4%). The protective effect of 3.4 mM sevoflurane on the glutamate uptake rate measured in OGD cells was significantly decreased in the presence of 3MG (percent sham wash; OGD + sevoflurane: 91.3 ± 17.4%; OGD + sevoflurane + 3MG: 57.4 ± 14.3%; $P < 0.05$).

Sevoflurane's Effects on Generation of ROS

Six hours after the 90-min OGD, we observed a significant increase in DCFH-DA fluorescence intensity (237 ± 63%) compared with sham wash cultures (fig. 6). A similar increase was obtained in the presence of 10 μM H_2O_2 (290 ± 70%). In normoxic cultures treated by sevoflurane (3.4 mM) or *N*-acetylcysteine (50 μM), DCFH-DA fluorescence intensity respectively increased (161 ± 16%) and decreased (66 ± 22%) in a significant manner compared with sham wash cultures. However, both sevoflurane (0.15–3.4 mM) and *N*-acetylcysteine, when added at the start of the injury, reversed the OGD-reoxygenation–induced increase in ROS generation.

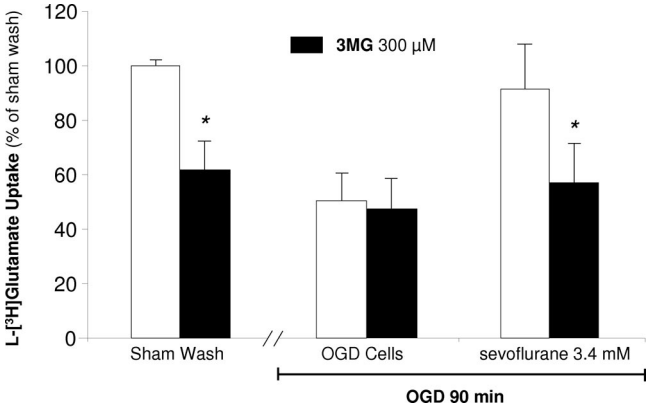

Fig. 5. After 90 min of oxygen–glucose deprivation (OGD), the protective effect of sevoflurane on glutamate uptake seemed to be mediated by the glial glutamate transporter (GLT1). Uptake experiments were performed at the end of the injury, before reoxygenation. Experiments were performed in the presence or absence of 300 μM threo-3-methyl glutamate (3MG), a rather selective inhibitor of GLT1. 3MG decreased the glutamate uptake rate but did not further affect this activity after 90 min of OGD. Moreover, the glutamate uptake rate measured in OGD cells treated with 3.4 mM sevoflurane decreased significantly in the presence of 3MG. n = 12; * $P < 0.05$ *versus* without 3MG.

Discussion

Using a transient OGD on mixed cerebrocortical neuronal–glial cell cultures, we have shown here that sevoflurane is a potent neuroprotective agent *in vitro*. It substantially reduced OGD-reoxygenation–induced neuron death 24 h after injury. This beneficial effect can probably be explained by the prevention of an extracellular glutamate concentration increase at the end of OGD and by inhibition of ROS generation during the reoxygenation process.

Experimental ischemic injury induces damage *via* multiple pathways[26] such as an increase in the tissue level of glutamate and lactate followed by a decrease in pH and increased activity of the Na^+–H^+ exchanger, but, in our experimental conditions, the potent neuroprotective effect of the NMDA receptor antagonist MK-801 against OGD suggests a major role for glutamate excitotoxicity mechanisms in neuron death. Volatile anesthetics have been shown to exert antiexcitotoxic effects because they block NMDA-induced excitotoxicity *in vivo*[6] and *in vitro*.[7,8] They also inhibit NMDA receptor function in various electrophysiologic studies, such as those on NMDA-gated currents from cultured cerebrocortical neurons.[27] During ischemia, these properties could have counteracted the excitotoxic mechanisms mediated by the dramatically increased glutamate levels. However, during ischemia, volatile anesthetics could also have antiexcitotoxic effects, because they reduced the glutamate accumulation in the extracellular space observed *in vivo*[10] and *in vitro*.[28] Extracellular glutamate accumu-

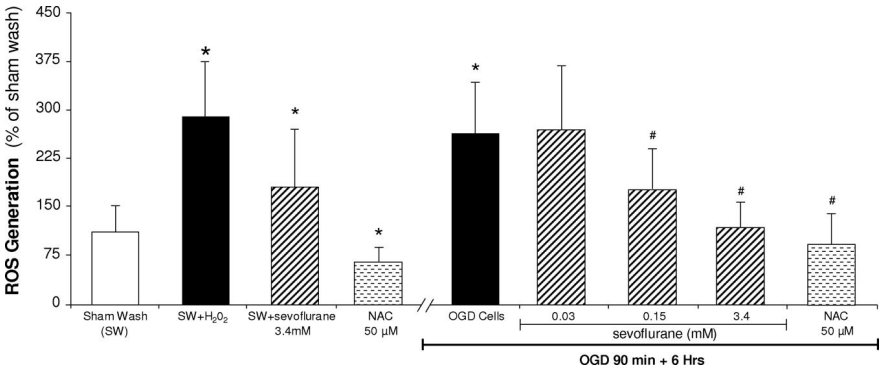

Fig. 6. Sevoflurane decreased reactive oxygen species (ROS) generation during reoxygenation. ROS generation was measured, 6 h after reoxygenation, by flow cytometry using the fluorescent dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The oxygen-glucose deprivation (OGD)–reoxygenation sequence induced a significant increase in fluorescence intensity of DCFH-DA. Sevoflurane 3.4 mM induced a significant increase in fluorescence intensity of DCFH-DA on sham wash (SW), but reduced (0.15–3.4 mM) the OGD-reoxygenation–induced increase in ROS generation when added during the OGD. *N*-acetylcysteine (NAC; 50 μM) decreased ROS levels in both SW and OGD cells. n = 32; * $P < 0.05$ *versus* SW; # $P < 0.05$ *versus* OGD cells.

lation after *in vivo* or *in vitro* experimental ischemia is now a well-documented phenomenon[26] depending on an overrelease[29] coupled with a decreased[30] or reversed[31] glutamate uptake process. At the end of the OGD, we also observed an OGD-induced increase in extracellular glutamate levels which was similar to that reported on *in vivo*[29] and *in vitro*[32] models of cerebral ischemia. In agreement with data obtained *in vivo* by Engelhard *et al.*[11] and Toner *et al.*,[12] we report here that sevoflurane, although lacking an effect on basal extracellular glutamate levels in sham wash cells, counteracted the OGD-induced increase in extracellular glutamate levels. This effect is probably due to the inhibition of glutamate release, because Bickler *et al.*[28] reported a volatile anesthetic-induced reduction in glutamate release from cerebral cortical slices during anoxia. However, in our experiments, the maintenance of low glutamate levels during ischemic conditions in the presence of sevoflurane could also be related to the drug-induced preservation of the glutamate transport activity. Indeed, we report here that, although without any effect on glutamate uptake in normoxic conditions, sevoflurane curtailed the OGD-induced glutamate uptake inhibition in cultures subjected to OGD. Although the effect of volatile anesthetics on glutamate transport activity during ischemia is not yet fully documented, reports on the effect of these drugs on basal activity of glutamate transporters remain inconclusive. Indeed, Fang *et al.*[33] found that sevoflurane did not affect the glutamate uptake by the glial GLT1 transporter isolated from rat hippocampus, whereas Do *et al.*[13] and Miyazaki *et al.*[14] found that volatile anesthetics enhanced the activity of glutamate transporters expressed either in *Xenopus* oocytes or in cultured astrocytes. To clarify the involvement of GLT1 in the protective effect of sevoflurane, we have shown that, as we previously reported,[21] 3MG, a rather specific GLT1 inhibitor, was able to dramatically reduce the uptake rate of the sham wash, indicating that the glutamate transport process in our normoxic experimental conditions relied largely on GLT1. However, 3MG did not further decreased glutamate transport during OGD, suggesting that GLT1-mediated glutamate uptake was totally inhibited by ischemic conditions. Finally, 3MG reduced the sevoflurane-induced preservation of glutamate uptake during ischemia. This GLT1-mediated effect of sevoflurane could represent a link between the sevoflurane's protective effect against OGD and the putative role of glutamate transporters. This could therefore support the glutamate hypothesis of this protective effect in a more direct manner. Whatever the mechanism involved, the protective effect of sevoflurane on glutamate uptake under ischemic conditions reported here may explain, at least in part, the prevention of an extracellular glutamate concentration increase during OGD and therefore the anti-ischemic properties of this drug.

This neuroprotective effect of sevoflurane could also be related to ROS generation because sevoflurane, added at the start of OGD, elicited an inhibition of reoxygenation-induced ROS generation from the different cell populations present in our system. However, unlike propofol,[15] volatile anesthetics have been shown to be devoid of direct antioxidant properties. First, they were unable to protect primary nervous cell cultures from H_2O_2-induced oxidative stress.[25] Second, we, on normoxic nervous cell cultures, and others, in normoxic cardiac tissue,[34] showed that sevoflurane induces ROS generation. This increase in ROS generation has been suggested as one of the triggers of the preconditioning effect of volatile anesthetics against cardiac ischemia.[35] Nevertheless, sevoflurane may have indirect antioxidant properties, because it induced antiexcitotoxic properties during OGD that could lead to a decrease in ROS generation during reoxygenation. Experimental evidence repeatedly suggests a causal relation between glutamate excitotoxicity and an increase in ROS generation during the early reperfusion period on various models of cerebral ischemia.[36,37] For example, cultured cortical neurons overexpressing the free radical–scavenging enzyme superoxide dismutase are resistant to glutamate toxicity.[38] Ciani *et al.*[39] have also demonstrated that glutamate-induced excitotoxic neuron death is counteracted either by inhibition of free radical generation or by

scavenging the excess of free radicals produced. In the same way, the depletion of the antioxidant glutathione enhances glutamate neurotoxicity.[40] Finally, electron paramagnetic resonance spectroscopy provides direct evidence that NMDA receptor activation leads to the generation of superoxide radicals.[41] All this suggests that sevoflurane, by inhibiting the OGD-induced increase in extracellular glutamate level, may also inhibit the OGD-induced increase in ROS level after reoxygenation. Whether sevoflurane exerts these effects preferentially on neurons or astrocytes remains to be determined. However, because the regulation of extracellular glutamate level and ROS generation are two of the astrocytic functions that are known to influence neuronal survival during ischemia,[42] one hypothesis could be that sevoflurane helps astrocytes to decrease extracellular glutamate level *via* astrocytic glutamate uptake. This leads to an increase in astrocytic glutamate content that, in turn, increases the glutathione level in astrocytes. ROS generation is therefore limited in astrocytes, and neuronal oxidant stress is reduced. Another hypothesis could be that sevoflurane sustains the increase in astrocytic lactate level during ischemia[43] and therefore the astrocyte–neuron lactate shuttle, which could serve as fuel for neurons during recovery.[44]

It must be underlined that neuroprotection produced by volatile anesthetics is multifactorial and may not only imply glutamate and ROS but also actions at γ-aminobutyric acid type A ($GABA_A$) receptors. Bickler *et al.*,[45] in organotypic cultures of rat hippocampus, showed that protection by isoflurane of CA1, CA3, and dentate neurons from death caused by OGD involves $GABA_A$ receptors. Similarly, several $GABA_A$-potentiating compounds such as topiramate are neuroprotectants against cerebral ischemia.[46] Finally, a direct agonist effect of volatile anesthetics on $GABA_A$ receptors was suggested by Nishikawa *et al.*[47] These authors showed that specific amino acid residues in the putative second transmembrane segment of the $GABA_A$ receptor play a critical role in the enhancement of $GABA_A$ receptor function by several volatile anesthetics.

Certain methodologic issues in this study must be discussed. First, because we used a recovery interval of 24 h in cultured cells exposed to OGD, we cannot exclude a transient neuroprotective effect for sevoflurane as reported by Kawaguchi *et al.*[48] or Elsersy *et al.*[3] *in vivo*. However, it was difficult to transpose such long-lasting studies *in vitro* because the neuronal death induced by OGD did not allow the experiments to be performed at times longer than 24 h after reoxygenation. Second, cocultures cannot duplicate all the features of ischemia and recovery that are present in the intact brain. Particularly, the contribution of leukocytes to inflammation could limit the neuroprotective qualities of sevoflurane in intact animals compared with cocultures. Indeed, it is possible that, in intact animals, this or other processes might kill neurons initially protected by sevoflurane. However, *in vitro* model systems provide the opportunity to examine mechanisms involved in drug-induced neuroprotection in a controlled experimental setting. Third, our *in vitro* model of OGD was chosen to selectively injure neurons, but we cannot exclude that, except astrocytes, which have been shown to be resistant to longer OGD durations, other cell types present in our cultures (microglia, endothelial cells, and so forth) have contributed to LDH release. However, these cell types represent less than 12% of the total cell number. Finally, the concentrations of sevoflurane that we used have been reported to be in the anesthetic range by some authors[8] but may considered to be high.

In conclusion, this study confirms a neuroprotective effect of sevoflurane on an *in vitro* model of ischemia–reoxygenation. It can be assumed that this beneficial effect of sevoflurane on neuronal viability is mediated, at least in part, by a restoration of the GLT1-mediated glutamate transport activity, which is dramatically impaired by the OGD, thus allowing extracellular glutamate levels to be maintained below excitotoxic concentrations. Our results also indicate that this sevoflurane-induced protection of glutamate uptake during OGD may induce a decrease in ROS generation during the reoxygenation period. Further research is needed to explore the relation between ROS and cerebral protection by volatile anesthetics.

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