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Putative role of nitric oxide synthase isoforms in the changes of nitric oxide concentration in rat brain cortex and cerebellum following sevoflurane and isoflurane anaesthesia

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Abstract

We have previously observed an increase in nitric oxide (NO) content in rat brain cortex following halothane, sevoflurane or isoflurane anaesthesia. This study was undertaken in order to determine whether isoform-specific nitric oxide synthase (NOS) inhibitors and inducers could modify these increases in NO contents. Rats were subjected to isoflurane and sevoflurane anaesthesia with concomitant administration of neuronal nitric oxide synthase (nNOS) inhibitor 7-Nitro-indazole (7-NI), inducible nitric oxide synthase (iNOS) inhibitor 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) or lipopolysaccharide. NO concentration in different organs was measured by electron paramagnetic resonance (EPR) spectroscopy. 7-NI significantly decreased NO concentration in cerebellum but not in brain cortex, whereas AMT decreased NO in all the organs studied. Anaesthesia significantly increased NO concentration in brain cortex and decreased that in cerebellum. AMT abolished the NO increase in brain cortex. Anaesthesia enhanced the drastic increase in NO concentration in brain cortex after intraventricular lipopolysaccharide administration. Isoflurane was found to inhibit recombinant nNOS and iNOS activities at high concentrations (EC_{50}=20 mM). Our data suggest a putative role for iNOS in the increase in NO levels produced by isoflurane and sevoflurane, whereas nNOS activity is probably inhibited during anaesthesia.

Keywords: Nitric oxide; Halogenated volatile anaesthetic; EPR spectroscopy; Inducible and neuronal nitric oxide synthase; Lipopolysaccharide; NOS inhibitor

1. Introduction

We have previously reported strong increases in nitric oxide (NO) concentrations in rat brain cortex following halothane, sevoflurane and isoflurane anaesthesia. These changes were not observed in other organs of anaesthetised rats and were eliminated by nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (Sjakste et al., 1999a,b; Baumane et al., 2002). Our data suggested that inhibition of the NOS activity and suppression of the NO neurotransmitter mediator function by the halogenated volatile anaesthetics (Johns et al., 1992, 1995; Pajewski et al., 1996; Zuo et al., 1996) can be equilibrated by increased NO production that is possibly linked to vasodilatation produced by these drugs (Harkin et al., 1997; Ogawa et al., 1997; Koenig et al., 1993; Smith et al., 1995; Staunton et al., 2000).

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It is acknowledged that NO produced by the neuronal NOS isoform (nNOS) performs both neurotransmitter mediator and vasodilating functions in the brain cortex (Staunton et al., 2000). However, it thus seems hardly possible that the same enzyme could be simultaneously inhibited and stimulated by the halogenated volatile anaesthetics. This encouraged us to test the involvement of different NOS isoforms in the halogenated volatile anaesthetics inducing NO increase.

We performed direct measurements of NO contents in tissues of rats that had been treated either with an inhibitor of neuronal NOS isoform (nNOS) or inducible NOS (iNOS), or with lipopolysaccharide. NO contents following anaesthesia were measured by trapping NO as a stable complex with iron and diethyl dithiocarbamate (DETC) and evaluating the spectrum of $Fe(DETC)_2$–NO complex by electron paramagnetic resonance (EPR) spectroscopy (Mikoyan et al., 1997). This method was chosen due to its high resolution and ability to specifically detect just NO and not its metabolites as in other methodical approaches. This report presents the data on quantitative measurements of NO contents in different organs of rats following treatments with nNOS inhibitor 7-Nitro-indazole (7-NI), iNOS inhibitor 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) or/and induction of iNOS by lipopolysaccharide with consequent sevoflurane or isoflurane anaesthesia. We detected the iNOS gene expression in brain cortex of untreated- and isoflurane-treated rats. Finally, we directly investigated the effects of the halogenated volatile anaesthetics on the activities of purified recombinant NOS isoforms in vitro.

2. Materials and methods

2.1. Animals, drug administration and sample preparation

Wistar male rats (weight 200–300 g, from "Gailezers", Riga) were used in all the experiments. All manipulations with animals were performed in accordance with regulations of the Republic of Latvia, and the permission by the Ethics Commission of the Latvian Council for Science was obtained to carry out this study. In some experiments rats were intraperitoneally injected with 7-NI, AMT, lipopolysaccharide or their combination. The chemicals came from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), except when indicated.

In a special series of experiments, lipopolysaccharide administration to brain lateral ventricles was performed as described (Klusa et al., 1998). Rats were anaesthetized with ketamine (100 mg/kg, Afasan) in combination with neuromuscular blocking drug xylasine (5 mg/kg, Dopharma, Czech Republic). An incision was made in the head skin between the ears to make an opening with 1.5 cm diameter. Connective tissues and periost were removed from the skull bone surface. A hole was drilled in the skull bone 1 mm laterally and 1.5 mm caudally from the bregma. A stainless steel canula (0.55 mm diameter) was introduced for 3 mm into the opening and was fixed on the skull bone with cement used in dentistry. The wound was treated with antibiotics and rats were kept in cages for 10 days. Lipopolysaccharide (2.5 mg/kg) was administered using a Hamilton micro-syringe.

Rats were anaesthetized with inhalation of an O_2 mixture with volatile anaesthetics (1% for isoflurane, 2% for sevoflurane, both from Abbot, Queenborough, UK) using a "Floutec" (Cyprane Ltd., Keighley, UK) vaporiser, the gas flow was kept constant (2000 cm^3/min) during the entire time of treatment. Body temperature during narcosis was constantly monitored with a rectal thermometer and maintained at 36.6–37.5 °C by covering the animals with a heating blanket. Other details are indicated in our previous publication (Baumane et al., 2002).

To determine NO content in the tissues, we used the Protocol originally elaborated by A.F. Vanin's group (Mikoyan et al., 1997). Spin trap was administered 30 min before anaesthesia. Rats were intraperitonally administered 400 mg/kg of diethyl dithiocarbamate and subcutaneously at a dose of 40 mg/kg ferrous sulphate and 200 mg/kg sodium citrate. Diethyl dithiocarbamate and ferrous citrate form a complex, $Fe(DETC)_2$, that traps NO and enables its detection as a relatively stable $Fe(DETC)_2$–NO complex easily identified by EPR spectroscopy.

Following 30 min of anaesthesia, animals were decapitated and brains were removed and cooled in a freezer for 3 min to solidify brain tissues. Brain cortex and cerebellum were dissected, and the tissue was compacted into a 30-mm-long glass tube with inner diameter of 4 mm. Samples of liver, heart, kidneys and testes were similarly obtained. Tissues were then immediately frozen and kept in liquid nitrogen.

2.2. Experimental protocols

Rats were assigned to 21 experimental groups. In Groups 1–19, animal tissues were dissected as described above and subjected to EPR spectroscopy. All animals in these groups received spin trap agents 30 min prior to decapitation. Details of experiment design in groups 1–19 are given in Table 1. Time schedule for administration of different substances is given in Fig. 1. Groups 20–21 were used to determine NO metabolites nitrate and nitrite in brain cortex using the Griess reaction, these groups were formed by five animals in each. Animals in Group 20 were intact; animals in group 21 were subjected to sevoflurane anaesthesia (2% sevoflurane, 30 min).

2.3. EPR spectroscopy

Before measurements of EPR spectra, the surface of the specimen was thawed to a small extent; the specimen was

Table 1
Experimental protocols of experiments with EPR-based detection of nitric oxide

Group number	Number of animals	Name of the group	Anaesthesia	LPS administration or sham ventriculostomy	NOS inhibitors
1	22	Control	–	–	–
2	5	7-NI	–	–	7-NI, 40 mg/kg, 1 h
3	5	AMT	–	–	AMT, 2 mg/kg, 1 h
4	5	Sevoflurane	2% sevoflurane, 30 min	–	–
5	5	Sevoflurane+7-NI	2% sevoflurane, 30 min	–	7-NI, 40 mg/kg, 1 h
6	5	Sevoflurane+AMT	2% sevoflurane, 30 min	–	AMT, 2 mg/kg, 1 h
7	5	Isoflurane	1% isoflurane, 30 min	–	–
8	5	Isoflurane+7-NI	1% isoflurane, 30 min	–	7-NI, 40 mg/kg, 1 h
9	5	Isoflurane+AMT	1% isoflurane, 30 min		AMT, 2 mg/kg, 1 h
10	16	LPS	–	10 mg/kg, i.p. 4 h	–
11	5	LPS+AMT 1 h	–	10 mg/kg, i.p. 4 h	
12	5	LPS+AMT 0.5 h	–	10 mg/kg, i.p. 4 h	AMT, 2 mg/kg, 30 min
13	5	LPS+sevoflurane	2% sevoflurane, 30 min	10 mg/kg, i.p. 4 h	–
14	5	LPS+isoflurane	1% isoflurane, 30 min	10 mg/kg, i.p. 4 h	–
15	8	Sham		Sham ventriculostomy	–
16	7	Sham+sevoflurane	2% sevoflurane, 30 min	Sham ventriculostomy	–
17	9	LPS i.v.	–	2.5 mg/kg, i.v. 4 h	–
18	7	LPS i.v.+sevoflurane	2% sevoflurane, 30 min	2.5 mg/kg i.v. 4 h	–
19	11	LPS i.v.+isoflurane	1% isoflurane, 30 min	2.5 mg/kg, i.v. 4 h	–

i.p.—intraperitoneal administration; i.v.—intraventricular administration; LPS—lipopolysacharide.

expelled from the tube and placed immediately in a quartz finger duar ER 167 FDS-Q (Bruker, Karlsruhe, Germany) filled with liquid nitrogen. The length of the specimen exceeded the length of the resonator bore, allowing equal amounts of tissue exposure to the magnetic field in each experiment. This fact and the identical diameter of the cylinder-shaped specimens enabled us to standardize the amounts of tissues used in our experiments. Sample preparation was rapid and equally standardized for all samples (10 min).

EPR spectra were recorded on a "Radiopan" SE/X2544 spectrometer (Radiopan, Poznan, Poland) operating at X-band. The operating conditions were as following: 25-mW microwave power (non-saturating conditions), 100-kHz modulation frequency, 5-G modulation amplitude, receiver gain 0.5×10^4, and 1-s time constant. The amounts of NO formed in tissues were estimated by measuring the third component of the EPR signal of the $Fe(DETC)_2$–NO complex ($g=2.031$).

The concentrations of NO were expressed as ng/g of tissue and were calculated on the basis of calibration curves as described previously (Shen et al., 1998). Briefly, different amounts of $NaNO_2$ (final concentrations 10, 20, 30, 40, 60, 100 μM) were mixed with DETC (33 mg/ml), $FeSO_4$, $7H_2O$ (3.3 mM), and an excess of $Na_2S_2O_4$ (2 M) was added to the mixture. The EPR spectra were taken as described above.

2.4. Determination of nitrate and nitrite ion concentrations

To measure nitrate and nitrite ions, brains were dissected as described above and weighed. A 20% tissue homogenate in 0.1 M phosphate buffer, pH 7.4, was prepared using a hand-driven glass-teflon homogeniser. The sum ($NO_2^- + NO_3^-$, NOx) of nitrite (NO_2^-) and nitrate (NO_3^-)

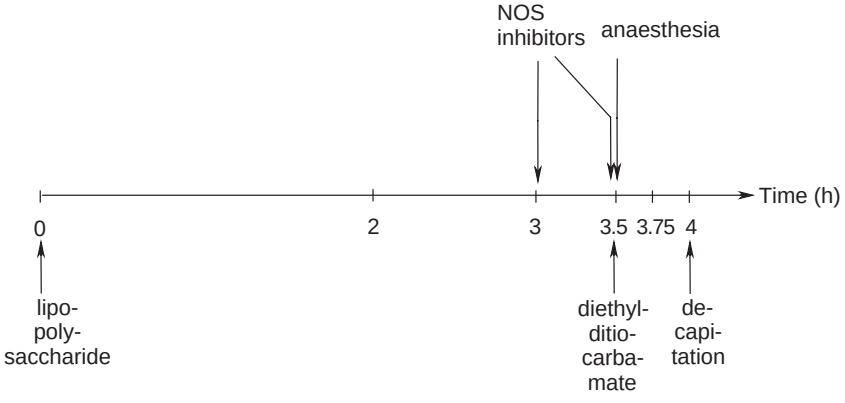

Fig. 1. Substance administration and anaesthesia time schedule.

ions was determined after incubation of the homogenate with nitrate reductase and its cofactor NADPH resulting in conversion of NO_3^- ions to NO_2^- ions. After the reduction, the proteins were precipitated with trichloroacetic acid and the samples were centrifuged for 10 min at 2000×*g* to separate the pellets. The Griess reagent was then added to the supernatant to form a coloured azo-compound and its concentration was determined spectrophotometrically at 500 nm. A calibration curve was prepared with various concentrations of $NaNO_3$ treated as described above (Calapai et al., 2000; Titheradge, 1999).

2.5. mRNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

mRNA was isolated from brain cortices of intact rats or animals subjected to 1-h isoflurane anaesthesia using Fast Track 2.0 Kit (Invitrogen, Carlsbad, CA, USA).

Synthesis of the first strand cDNA suitable for the second strand synthesis was performed using 100 ng of mRNA, sequence-specific primers and Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacture Protocol. Five microliters of aliquot containing the first strand cDNA was used as a template for PCR amplification performed in 25 μl of the reaction mixture with a final concentration of 1× PCR buffer (Fermentas, Vilnius, Lithuania), 1.5 mM of $MgCl_2$, 2 mM of each dNTP, 1 μM of forward and reverse primers and 0.612 U of Taq polymerase (Fermentas, Vilnius, Lithuania) per sample. Cycling parameters were as follows: denaturation/RT inactivation step at 94 °C during 3 min was followed by 30 cycles of three steps; denaturation at 94 °C–45 s, annealing at 55 °C–45 s, extension at 72 °C–1 min with final extension at 72 °C during 5 min.

Sequences of primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA were taken from Okamoto et al., 2000. iNOS primers were designed by us. The primers were as follows: iNOS forward 5′-GGAGGTCCACCTCACTGT-3′; iNOS reverse, 5′-CCTGCATTTCTTCCTGATAG-3′; GAPDH forward 5′-CACGGCAAGTTCAATGGCACA-3′; GAPDH reverse, 5′-GAATTGGAGGGAGAGTGCTC-3′. Expected size of the product was 335 bp for iNOS and 900 bp for GAPDH. Gels were documented using the histogram presentation mode for quantification of the band intensity.

2.6. Preparation and purification of recombinant NOS isoforms

Recombinant NOS I was isolated and purified from the yeast *Saccharomyces cerevisiae* transformed by a plasmide containing rat brain nNOS as previously described (Moali et al., 1998). Full length recombinant murine macrophage iNOS and bovine endothelial eNOS as well as the heme domain of rat brain nNOS ($nNOS_{oxy}$) were expressed in *Escherichia coli* and purified as described previously (Wu et al., 1996; Ghosh et al., 1998; Abu-Soud et al., 1997). These proteins were kind gifts of D.J. Stuehr (Cleveland Clinic Fundation, Cleveland, OH, USA).

2.7. NOS activity assay of [^{3}H]L-Citrulline formation

NOS-dependent oxidation of L-arginine to L-citrulline was determined according to a previously described Protocol (Bredt and Snyder, 1990). In brief, enzymatic reactions were conducted at 37 °C for 5 min in 50 mM HEPES pH 7.4, containing 5 mM dithiotreitol, 1 mM NADPH, 1 mM $CaCl_2$, 10 μg/ml calmodulin, 20 μM BH_4, 4 μM FAD, 4 μM FMN, ca. 500,000 cpm [2,3,4,5-^{3}H]L-arginine, and the indicated concentrations of L-arginine, BH_4, and the compounds tested. Due to the limited water solubility of the halogenated volatile anaesthetics, they were commonly added to the incubation mixtures as 5 μl portions dissolved in dimethyl sulfoxide (DMSO). Final incubation volumes were 100 μl. Control incubations contained similar amounts (5%) of DMSO without inhibitors, and the values are expressed relative to the DMSO controls. The reactions were started by adding protein and terminated by the addition of 500 μl cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA and 0.2 mM EGTA). Samples (500 μl) were applied to the columns containing 1 ml of Dowex AG 50W-X8 (Na^+ form, prepared from the H^+ form), pre-equilibrated with stop buffer and a total of 1.5 ml of stop buffer was added to eluate [^{3}H]L-citrulline. Aliquots were then mixed with Pico-Fluor 40 (Packard) and counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer. Control samples without NOS or NADPH were included for background determinations. Incubations in the presence of iNOS were performed similarly, but $CaCl_2$ and calmodulin were omitted.

2.8. Statistical analysis

Significance of differences between groups was evaluated according to the Student's *t*-test.

3. Results

To determine the background NO levels in rat organs, the animals were injected with DETC and ferrous citrate, and sacrificed 30 min later (Group 1). EPR spectra of the different organs had a typical Cu–DETC spectrum shape with a superposed $Fe(DETC)_2$–NO peak. No difference in the spectrum shape could be observed between different tissues (not shown, see spectra in Sjakste et al., 1999a,b). The NO contents were determined in the brain cortex, cerebellum, liver, heart, kidneys, testes and blood with highest NO levels in the cortex, cerebellum, liver and blood (46.0 ± 3.4; 27.7 ± 2.6; 27.6 ± 4.7 and 33.6 ± 12.4 ng/g of tissue, respectively, data are mean ± S.E.M.). NO contents in heart, kidneys and testes were an order of

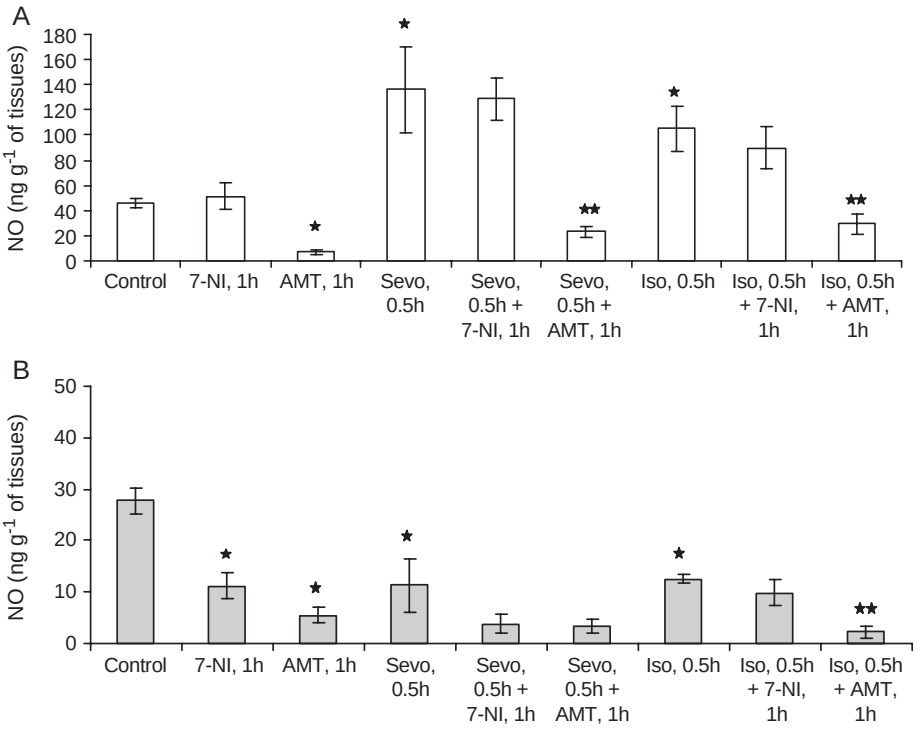

Fig. 2. Nitric oxide content in the brain cortex (A) and cerebellum (B) of rats during anaesthesia produced by isoflurane and sevoflurane combined or not with administration of nitric oxide synthase (NOS) inhibitors. Control—Group 1; 7-NI (7-Nitro-indazole)—Group 2, nNOS inhibitor (40 mg/kg^{-1}) was administered intraperitoneally 30 min prior to spin trap injection; AMT (2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine)—Group 3, iNOS inhibitor (2 mg/kg^{-1}) was administered intraperitoneally 30 min prior to spin trap injection; Sevo—Group 4, sevoflurane (2%), 30 min. Sevo+7-NI—Group 5, the inhibitor was administered 30 min before the sevoflurane anaesthesia. Sevo+AMT—Group 6, AMT was administered 30 min prior to sevoflurane; Iso—Group 7, isoflurane (1%) anaesthesia, 30 min. Iso+7-NI—Group 8, nNOS inhibitor administered 30 min before anaesthesia. Iso+AMT—Group 9, iNOS inhibitor administered 30 min before anaesthesia. Data are mean ± S.E.M. *$P<0.05$ versus the control group. **$P<0.05$ versus the group of the corresponding anaesthetic.

magnitude lower (Fig. 2; Table 2). Treatment of rats with 7-NI (Group 2), a nNOS inhibitor, decreased NO concentration in all organs except the testes, and, surprisingly, in the brain cortex. In the cerebellum, NO content was more than two-fold decreased. Treatment with the iNOS inhibitor AMT (Group 3) drastically decreased NO concentration in all organs, from a five-fold reduced level in the brain cortex and cerebellum to undetectable levels in the liver (Fig. 2; Table 2).

The 30-min duration of anaesthesia under sevoflurane caused an increase of NO concentration in the brain cortex (up to 136.1 ± 33.9 ng/g of tissue in Group 4), whereas NO content in the cerebellum decreased more than twice (11.2 ± 2.5 ng/g of tissue as compared to 27.7 ± 2.6 in the control group). NO concentrations in other organs studied were not altered by sevoflurane anaesthesia. Isoflurane anaesthesia (Group 7) produced similar effects (Fig. 2; Table 2). Thus, both sevoflurane and isoflurane appeared

Table 2
Changes in the NO concentration in different rat organs under sevoflurane and isoflurane anaesthesia combined with NOS inhibitors

Group no.	Name of the group	Liver	Heart	Kidney	Testis
1.	Control	27.6 ± 4.7	4.8 ± 0.7	3.3 ± 0.5	13.8 ± 1.1
2.	7-NI	12.4 ± 4.5[a]	2.1 ± 0.7[a]	0.6 ± 0.6[a]	11.8 ± 1.9
3.	AMT	0.0 ± 0.0[a]	1.5 ± 0.4[a]	0.8 ± 0.4[a]	3.3 ± 1.3[a]
4.	Sevoflurane	38.5 ± 14.6	2.8 ± 0.8	3.8 ± 0.9	19.3 ± 3.5
5.	Sevoflurane+7-NI	16.3 ± 2.9	2.1 ± 1.3	1.2 ± 0.8	5.7 ± 2.7[b]
6.	Sevoflurane+AMT	0.0 ± 0.0[b]	1.2 ± 0.7	0.0 ± 0.0[b]	5.8 ± 3.9[b]
7.	Isoflurane	29.3 ± 9.3	3.7 ± 1.1	5.8 ± 1.2	18.1 ± 2.3
8.	Isoflurane+7-NI	9.4 ± 6.7	1.7 ± 1.0	0.5 ± 0.3[b]	14.9 ± 1.2
9.	Isoflurane+AMT	0.0 ± 0.0[b]	3.6 ± 1.0	0.9 ± 0.9[b]	4.5 ± 1.1[b]

Data are mean ± S.E.M.
[a] Difference with the control group is statistically significant ($P<0.05$).
[b] Difference with the sevoflurane group or the isoflurane group is statistically significant. Significance of differences between the groups was evaluated according to the Student's *t*-test. Detailed description of the groups is given in Table 1.

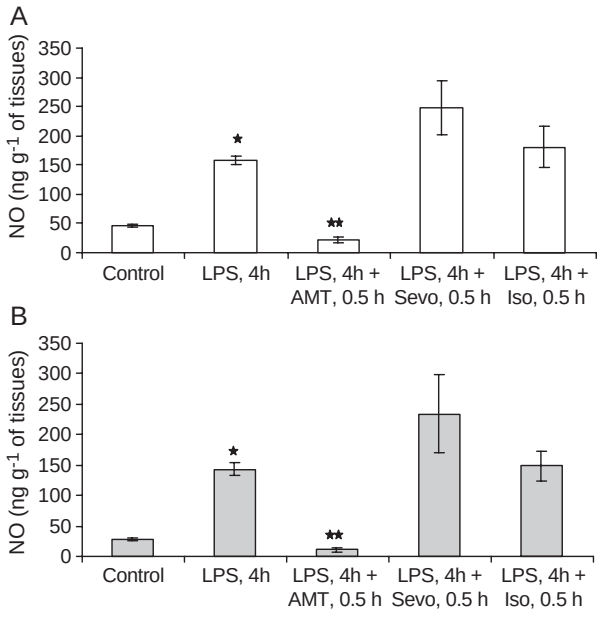

Fig. 3. Nitric oxide content in the brain cortex (A) and cerebellum (B) of rats during anaesthesia produced by isoflurane and sevoflurane, alone or in combination with NOS inhibitors and intraperitoneal administration of lipopolysaccharide. Control—Group 1; LPS (lipopolysaccharide)—Group 10, 10 mg/kg lipopolysaccharide injected intraperitoneally before 4 h; LPS+AMT 1 h—Group 11, AMT administered 3 h after lipopolysaccharide, rats were sacrificed 1 h later; LPS+AMT 30 min—Group 12, AMT administered 3.5 h after lipopolysaccharide, rats were sacrificed 30 min later. LPS+Sevo—Group 13, sevoflurane anaesthesia was given 3.5 h after the lipopolysaccharide injection; LPS+Iso—Group 14, isoflurane anaesthesia was given 3.5 h after the lipopolysaccharide injection. Data are mean ± S.E.M. *$P<0.05$ versus the control group. **$P<0.05$ versus the lipopolysaccharide group.

to increase NO concentrations in the brain cortex and to decrease them in the cerebellum. These findings were compatible with our former results (Sjakste et al., 1999a,b; Baumane et al., 2002). It should be mentioned that intensity of the Fe-DETC spectra did not change in the anaesthetized animals, the changes concerned exclusively the NO component. Thus our measurements reflected the changes in the NO production rate, but not alterations of DETC pharmacokinetics. To ensure ourselves once more that the observed changes in EPR spectra were not due to the changes in DETC tissue concentrations but rather reflected the NO concentrations per se, we determined the changes in NO metabolites ($NO_2^- + NO_3^-$) using the Griess reaction. The concentration of these ions in the brain cortex of intact animals (Group 20) was at the limit of sensitivity of the method (1288 ± 801 ng/g tissue). However, sevoflurane anaesthesia (Group 21) strongly increased it (to 6094 ± 668 ng/g tissue) and this difference was statistically significant. These results proved the validity of our data obtained applying the EPR method and in further experiments we used exclusively the EPR method. Involvement of different NOS isoforms in the anaesthetic-induced NO increase in the brain cortex was then tested using isoform-specific NOS inhibitors. Administrations of the nNOS inhibitor 7-NI 30 min before sevoflurane (Group 5) or isoflurane (Group 8) anaesthesia did not prevent from the NO increase during anaesthesia (Fig. 2; Table 2). However this increase was abolished when the animals were treated with the iNOS inhibitor AMT, and the NO concentration did not exceed the basal amounts observed in Group 3 (23.0 ± 4.1 ng/g tissue for sevoflurane, and 29.2 ± ng/g tissue for the isoflurane) (Fig. 2). Sensitivity of the anaesthetic-dependent increase in NO levels to iNOS inhibitor raised the question about a possible involvement of this enzyme into the phenomenon. iNOS mRNA was marked in the brain cortex of both intact animals and the rats subjected to isoflurane anaesthesia used in our experiments (see below). The halogenated volatile anaesthetics are known to enhance expression of the iNOS gene in vitro (Zuo and Johns, 1997), in some cases, increase of iNOS copies in neurons was observed very soon after the anaesthetic administration, probably from pre-existing mRNAs (Holtz et al., 2001). We thus hypothesized that the halogenated volatile anaesthetics would be able to enhance the activity of pre-existing iNOS copies in the brain cortex. Then we tried to increase the iNOS gene expression and the enzyme copy number by injecting lipopolysaccharide. In the case the anaesthetic-dependent NO increase was mediated by iNOS, this effect should have been strongly enhanced in the lipopolysaccharide-treated rats (Group 10). Indeed, intraperitoneal injection of the lipopolysaccharide to the animals caused a drastic increase of NO levels in all the tissues studied (Fig. 3; Table 3). To ensure that the NO levels detected after the lipopolysaccharide injection to the brain were due to increased expression in the tissue and did not reflect drastic increase

Table 3
Changes in NO concentration in different rat organs under sevoflurane and isoflurane anaesthesia combined with intraperitoneal LPS administration and iNOS inhibitors

Group no.	Description of the group	*n*	Liver	Heart	Kidney	Testis
1.	Control	22	27.6 ± 4.7	4.8 ± 0.7	3.3 ± 0.5	13.8 ± 1.1
10.	LPS	16	1357.1 ± 150.5[a]	204.1 ± 13.3[a]	290.8 ± 21.4[a]	71.3 ± 6.2[a]
11.	LPS+AMT 1h	3	112.1 ± 11.6[b]	10.4 ± 6.7[b]	1.9 ± 1.9[b]	10.8 ± 5.6[b]
12.	LPS+AMT 0.5 h	5	78.7 ± 16.9[b]	5.0 ± 0.4[b]	2.9 ± 1.9[b]	9.7 ± 1.9[b]
13.	LPS+sevoflurane	10	1629.5 ± 218.3	340.6 ± 56.9[b]	377.5 ± 68.9	162.1 ± 41.0[b]
14.	LPS+isoflurane	6	837.2 ± 144.8[b]	331.2 ± 98.3	343.5 ± 86.4	130.2 ± 48.8

[a] Difference with the control group is statistically significant ($P<0.05$).
[b] Difference with the LPS group is statistically significant. Other designations as for Table 2. Detailed description of the groups is given in Table 1.

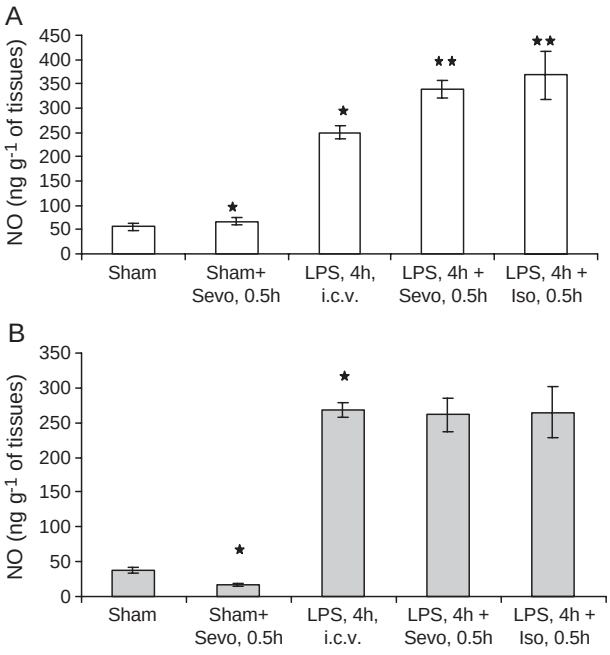

Fig. 4. Nitric oxide content in the brain cortex of rats during anaesthesia produced by isoflurane and sevoflurane combined or not with intraventricular lipopolysaccharide administration. Sham—animals with implanted canula, Group 15; Sham+Sevo—sham-operated animals subjected to the sevoflurane anaesthesia, Group 16; LPS i.c.v. −2.5 mg/g of lipopolysaccharide were administered in the brain ventricles for 4 h, Group 17; LPS i.c.v.+Sevo—Group 18, lipopolysaccharide administration in the brain ventricles was followed by sevoflurane anaesthesia; LPS i.c.v.+Iso—lipopolysaccharide administration in the brain ventricles was followed by isoflurane anaesthesia, Group 19. *$P<0.05$ versus sham group. **$P<0.05$ versus the LPS i.c.v. group.

of NO in the blood circulating in the brain blood vessels, we additionally determined the NO concentrations in the blood. Although the lipopolysaccharide injection increased $Fe(DETC)_2$–NO in the blood (from 33.6 ± 12.4 in intact animals to 463.0 ± 46.4 ng/g tissue after the lipopolysaccharide injection, $n=15$), it could not strongly contribute to the NO levels observed in the brain cortex (157.4 ± 7.6 ng/g tissue, $n=15$) and cerebellum (143.1 ± 10.5 ng/g tissue, $n=15$), taking into account the volume of blood vessels in the brain tissues. AMT administered 1 h (Group 11) or 30 min (Group 12) before decapitation eliminated the lipopolysaccharide effects in most organs (except the liver), and the NO concentrations were reduced below the levels of untreated rats (Fig. 3; Table 3). In animals subjected both to lipopolysaccharide treatment and sevoflurane anaesthesia (Group 13), we observed a further increase of NO concentration in the brain cortex, but the differences were not statistically significant (Fig. 3). It is of interest that a significant increase was also observed in the heart and testes (Table 3). A combination of lipopolysaccharide with isoflurane anaesthesia (Group 14) increased the NO levels in the brain cortex close to but not as high as those of the lipopolysaccharide-treated animals (Fig. 3).

These results obtained with intraperitoneal lipopolysaccharide injection promoted us to perform a series of experiments with intraventricular administration of lipopolysaccharide. Direct administration of lipopolysaccharide to the lateral ventricles excluded a possible impact of the transport across the blood–brain barrier on its effects. Sham operation of the canula implantation (Group 15) did not change the NO levels in any of the organs studied (Fig. 4; Table 4). When sham-operated animals were subjected to sevoflurane anaesthesia (Group 16), the changes in the NO concentrations similar to those of intact animals were observed: the NO levels increased in the brain cortex and decreased in the cerebellum (Fig. 4). Thus, implantation of canula did not interfere with the action of anaesthetics on the NO production. Four hours after ventricular administration of lipopolysaccharide (Group 17), we observed a drastic increase in the NO levels in the brain cortex (Table 4, Fig. 4). The following sevoflurane anaesthesia (Group 18) enhanced this effect (NO concentration increased from 250 ± 13 to 339 ± 18 ng/g tissue in the brain and from 336 ± 21 to 587 ± 59 ng/g in the blood, Table 4; Fig. 4). Isoflurane (Group 19) also intensified this lipopolysaccharide-dependent effect (NO level increased up to 368 ± 50 ng/g tissue, Table 4; Fig. 4).

Sensitivity of the anaesthetic-induced increase in the NO levels to iNOS-specific inhibitor AMT, and its enhancement by the lipopolysaccharide administration strongly supported the involvement of iNOS into the effects of the halogenated volatile anaesthetics. RT-PCR confirmed iNOS expression in control and isoflurane-treated animals in our experiments (Fig. 5). A short-time delay between onset of anaesthesia and registration of the NO increase (30 min in our

Table 4
Changes in NO concentration in different rat organs under sevoflurane and isoflurane anaesthesia combined with intraventricular lipopolysaccharide (LPS) administration and iNOS inhibitors

Group no.	Name of the group	n	Liver	Heart	Kidneys	Testes
1.	Control	22	27.6 ± 4.7	4.8 ± 0.7	3.3 ± 0.5	13.8 ± 1.1
15.	Sham	8	52.0 ± 11.9	4.4 ± 2.5	3.4 ± 1.7	15.0 ± 4.4
16.	Sham+sevoflurane	7	25.3 ± 3.2	1.6 ± 0.5[a]	6.0 ± 1.2	9.4 ± 2.2
17.	LPS i.v.	9	1763.6 ± 190.1[a]	256.0 ± 37.7[a]	362.6 ± 41.4[a]	78.1 ± 9.9[a]
18.	LPS i.v.+sevoflurane	8	2317.3 ± 289.3	388.0 ± 42.3	546.4 ± 58.8	92.5 ± 9.3
19.	LPS i.v.+isoflurane	11	1987.5 ± 204.2	352.5 ± 45.0[b]	462.1 ± 65.0[b]	103.5 ± 19.4

[a] Difference with the sham group is statistically significant ($P<0.05$).
[b] Difference with the lipopolysaccharide group is statistically significant. Other designations as for Table 2. Detailed description of the groups is given in Table 1.

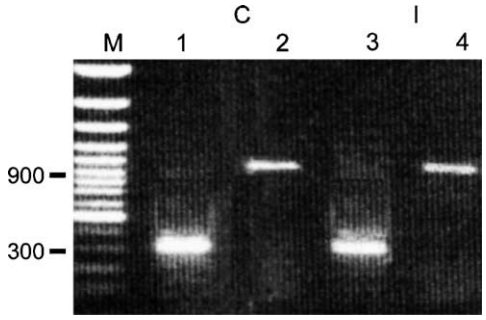

Fig. 5. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) products of iNOS (Lanes 1, 3) and GAPDH (Lanes 2, 4). mRNA was extracted from the brain cortex of intact rats (Lanes 1, 2; C) and the animals subjected to isoflurane anaesthesia for 1 h (Lanes 3, 4; I). 100 ng of mRNA isolated form the brain cortex of individual animal were taken for each reaction. M—molecular weight marker, marker positions are indicated in base pairs (bp).

experiments) suggested that additional induction of iNOS gene was unlikely, similar intensity of the histograms of the PCR product bands confirmed this consideration (not shown). However this may be due to the increase in the activity of pre-existing enzyme. This conclusion raised up a possible modification of NOSs activities by the halogenated volatile anaesthetics. We thus tried experiments with purified recombinant NOSs to investigate their direct interaction with the halogenated volatile anaesthetics. Isoflurane and sevoflurane inhibited the conversion of labelled L-arginine to L-citrulline catalyzed by the three NOSs isoforms at very high concentrations only (EC_{50}=18 mM for isoflurane in the presence of nNOS and 25 mM in the presence of iNOS), and sevoflurane appears as an even weaker inhibitor (Fig. 6). Effects on eNOS were similar (not shown). At the first glance, the EC_{50} for inhibition of nNOS by isoflurane seems to be very high, but it should be kept in mind that the halogenated volatile anaesthetics accumulate in synapses almost up to millimole concentrations (Eckenhoff and Eckenhoff, 1998). Thus it appeared important to more deeply investigate the interaction of nNOSs with the halogenated volatile anaesthetics. Decrease of L-arginine concentration in the incubation medium for nNOS activity (from 10 to 1 μM) did not affect the inhibiting activities of the anaesthetics; almost identical results were obtained when calmodulin concentration decreased by a factor of 50 times (not shown). Thus NOS inhibition by the anaesthetics was not due to the drug competition with substrate or calmodulin. Subsequent studies using the oxygenase domain of nNOS ($nNOS_{oxy}$) showed that these compounds did not modify the optical properties of the heme, suggesting that they did not bind to the active site (data not shown). In addition, the two compounds had no effect on the NADPH consumption and cytochrome *c* reduction catalyzed by the three isoforms (data not shown). Thus, the site of the halogenated volatile anaesthetics interaction with NOSs and fine mechanism of the inhibition remain to be established.

4. Discussion

This study is in full agreement with our previous results indicating that the anaesthesia-produced halogenated volatile anaesthetics enhance NO accumulation and detection in the rat brain cortex (Sjakste et al., 1999a,b; Baumane et al., 2002). It was shown that increased levels of NO related to a

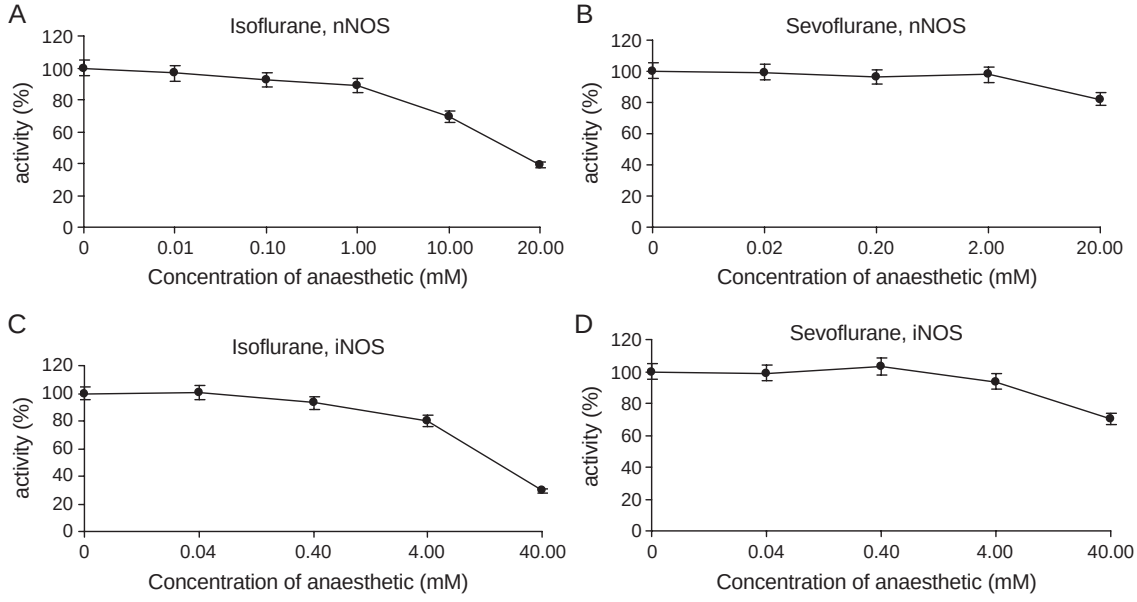

Fig. 6. Effects of the addition of increasing sevoflurane and isoflurane concentrations on the nNOS and iNOS activities determined by the arginine to citrulline conversion assay. (A) Effect of isoflurane on the oxidation of L-arginine to citrulline catalyzed by nNOS. (B) Effect of sevoflurane on the oxidation of L-arginine to citrulline catalyzed by nNOS. (C) Effect of isoflurane on the oxidation of L-arginine to citrulline catalyzed by iNOS. (D) Effect of sevoflurane on the oxidation of L-arginine to citrulline catalyzed by iNOS. Data are mean ± S.E.M. from three independent experiments.

NOS activity as unspecific NOS inhibitor reduced them, and were specific upon halogenated volatile anaesthetic action but not of anaesthesia per se as intravenous anaesthetic such as ketamine had no effect (Sjakste et al., 1999a,b; Baumane et al., 2002). The present study extends these observations in several ways. We clearly demonstrate that halogenated volatile anaesthetics produce opposite effects in the brain cortex and cerebellum with increased NO concentrations in the brain cortex but reduced ones in the cerebellum. The data on NO levels in the brain cortex obtained by the EPR method are well correlated by detection of the nitrite and nitrate ions (NO metabolites) using the Griess reaction. Changes of NO*x* ion concentration in the brain cortex after sevoflurane anaesthesia followed the same trend as NO measured by the EPR method. Differences in the quantity of NO and its metabolites in the brain cortex tissue appear to be striking (46.0 ± 3.4 ng/g tissue NO and 1288 ± 801 ng/g tissue for NO*x*). However, our measurements are in good agreement with the previously published data on both NO and its metabolite concentrations in the brain tissues, determined by different methods (Hlatky et al., 2003; Starkey et al., 2001). Apparently NO metabolites accumulate in the brain tissue over time; this is not the case of the Fe–NO–DETC complex (Sjakste et al., 1999a,b), the NO EPR spectrum really reflects the NO production rate. Moreover, NO and NO*x* concentrations can be modified in opposite ways during some pathological processes, for example, during the brain ischemia NO*x* concentration increases even when NO production is decreased (Zhang et al., 2004). Thus NO*x* measurements confirmed the data on NO increase in the rat brain cortex under the halogenated volatile anaesthetic-produced anaesthesia.

Different changes in the NO levels measured in the brain cortex and cerebellum suggested a possible involvement of different NOS isoforms. It is known that the nNOS content and activity measured in vitro in the cortex are the lowest among the brain compartments, whereas the same parameters are the highest in the cerebellum (Barjavel and Bhargava, 1995; Singh et al., 2000). Decrease of the NO level in the cerebellum can be easily explained by the inhibitory effect of the halogenated volatile anaesthetics on nNOS and NO signal transduction function (Johns et al., 1992, 1995; Pajewski et al., 1996; Zuo et al., 1996). This seemed to be hardly the case in the cortex.

The identification of the NOS isoform responsible for the increases in the NO levels during the halogenated volatile anaesthetics-induced narcosis was the main goal of the present investigation. We thus tried to solve this problem by using isoform-specific inhibitors. Activity of the specific nNOS and iNOS inhibitors appeared to be organ-specific even when administered to an intact animal. The nNOS inhibitor 7-NI decreased sharply the NO concentration in the cerebellum, this result was quite expected because nNOS is strongly marked in this brain compartment (Barjavel and Bhargava, 1995; Singh et al., 2000), apparently, this enzyme isoform activity determines the cerebellum NO level. 7-NI did not decrease the NO level in the testes. Probably, this drug fails to inhibit the testis-specific isoform of nNOS (Wang et al., 2002). Alternatively, the NO level in this organ can be determined by activity of other isoforms, as all the three NOS isoforms are found in testes (Ha et al., 2004). Moderate decrease of NO production by 7-NI in liver, heart and kidneys is quite understandable, as nNOS is pronounced in these organs (Wei et al., 2002; Fernandez et al., 2003; Sears et al., 2004). iNOS-specific inhibitor AMT efficiently decreased the NO concentration under study, this is not surprising as iNOS is the main isoform marked in the liver (Wei et al., 2002), it is also pronounced in the testes (Ha et al., 2004), kidneys (Fernandez et al., 2003), heart (Papapetropoulos et al., 1999). Certainly, 7-NI and AMT are not absolutely isoform-specific; their efficiency in different organs depends on pharmacokinetics of the drugs, vascularization of organs and other factors. However the above data are compatible with generally accepted point of view on tissue distribution of the NOS isoforms. Data on the brain cortex appear to contradict the idea about a pivotal role of nNOS in NO production in this tissue (Staunton et al., 2000), as 7-NI failed to reduce the NO concentration in the cortex. On the contrary, AMT appears to reduce the NO concentration in the brain cortex quite efficiently. The literature data indicate that the nNOS markedness in the brain cortex is the lowest among the brain compartments (Barjavel and Bhargava, 1995; Singh et al., 2000). Inducible NOS is pronounced in the tissue, it is found in neurons, glia, macrophages and brain blood vessels (Petrov et al., 2000) and is involved in several physiological and pathological processes (Heneka and Feinstein, 2001; Ferrini et al., 2001; Madrigal et al., 2001). Moreover, its activity unlike nNOS activity does not depend on the functional status of the cortex (Clement et al., 2004). Our experimental data also confirm iNOS gene expression in the brain cortex of intact rats. Thus the NO level in the rat brain cortex appears to be determined by the iNOS activity. Unfortunately, we had no sufficiently specific eNOS inhibitor to compare with 7-NI and AMT. However the latter isoform does not seem to be very important in the brain cortex, as even vasodilatation in the brain cortex is mediated by the nNOS activity (Staunton et al., 2000). Thus the choice between nNOS and iNOS appeared to be the most important for further studies.

When NOS inhibitors were administered to anaesthetized rats, the efficiency of different isoform inhibitors was also different. The nNOS inhibitor 7-NI was unable to abolish the increase in the NO levels during anaesthesia. Thus it is possible to conclude that this effect did not apparently relate to the changes in the nNOS activity. On the opposite, the iNOS inhibitor AMT strongly eliminated this NO increase suggesting that iNOS can be a suitable candidate for the origin of the NO increase triggered by the halogenated volatile anaesthetics. iNOS gene expression was detected in the brain cortex of anaesthetized rats. The experiments with halogenated volatile anaesthetic-produced narcosis in addi-

tion to lipopolysaccharide administration in the brain ventricles also confirmed this suggestion. Both sevoflurane and isoflurane produced stronger effects in the animals with an increased expression of iNOS in the brain. We could not expect that isoflurane would trigger iNOS mRNA transcription during short-term anaesthesia in our experiments, this process beginning after a 6-h anaesthesia only (Kapinya et al., 2002). Perhaps, the increase in the NO levels by iNOS could be achieved by intensification of the enzyme translation from pre-existing mRNAs. It was reported that an increase in the iNOS copy number derived from pre-existing mRNA in neurons can be detected 15 min after the stimulus (Holtz et al., 2001). Existence of iNOS mRNAs in the brain cortex from untreated rats favours this hypothesis. Okamoto et al. (2000) made a similar conclusion after observation of an increased hyperaemic effect of halothane following the lipopolysaccharide treatment. Other mechanisms of iNOS activation are also possible. Evidently, the expected effect of iNOS activation by the halogenated volatile anaesthetics on the background of intacerebroventricular administration of lipopolysaccharide is not as drastic as one could expect from activation of the pre-existing enzymes or translation of pre-existing mRNAs. Indeed, in intact rat brain cortex sevoflurane causes an almost three-fold increase in NO concentration, in the lipopolysaccharide-treated brain the concentration increases only by 1.3 times. In our opinion, this might be due to a general distortion of functions of the brain structures under the conditions of acute inflammation, however alternative mechanisms without involvement of the iNOS activation should be also discussed. Several possibilities exist in theory.

Increase of EPR signal in the rat brain under anaesthesia might be also due to the increased Fe–DETC accessibility to NO under the action of the halogenated volatile anaesthetics. However, Cu–DETC spectra in our experiments indicated that we had a large excess of Fe–DETC complex over NO and even distribution of the spin traps. Additional arguments for invalidity of such a hypothesis proposal were published previously (Sjakste et al., 1999a,b; Baumane et al., 2002). Moreover, the experiments with NO metabolite determinations performed in this study provide unambiguous arguments for real NO increase in the brain cortex of rats under the halogenated volatile anaesthetic-induced narcosis.

Modification of the NO transport between the blood and brain tissue is also doubtful, as NO transport is mostly passive. Increase of tetrahydrobiopterin biosynthesis in the brain cortex under the action of anaesthetics could be assumed in theory, but the data of Galley et al. (2001) indicate that there are no changes of tetrahydrobiopterin concentration in the brain cortex of rats subjected to halothane or isoflurane anaesthesia. Specific induction by the halogenated volatile anaesthetics of L-arginine transporters or some other enzymes involved in arginine metabolism seems to be unlikely, as in the brain tissues these enzymes are co-induced with iNOS (Kawahara et al., 2001). Increase in L-arginine transport from the brain nuclei to cortex cannot be excluded in principle. A special investigation should be performed to test this hypothesis (Wiesinger, 2001). Changes in caveole conformation in the brain endothelium under the anaesthetic action can also contribute to the increase in the NO production; the question needs a special study.

The observed increase in the NO concentration appears to be related to the hyperaemic and not anaesthetic action of the halogenated volatile anaesthetics. AMT and nitroarginine (Baumane et al., 2002) eliminate this NO increase but do not interfere with anaesthesia. In this case it should be also related to iNOS activity, as this isoform is responsible for the hyperaemic action of the halogenated volatile anaesthetics (Okamoto et al., 2000). Interestingly, the nitric oxide produced by iNOS turned out to be very important in the development of another type of unconsciousness, namely, the influenza-virus induced sleep (Chen et al., 2004).

We think that our data do not contradict the hypothesis about the ability of the halogenated volatile anaesthetics to inhibit NOS activities (Johns et al., 1992, 1995; Pajewski et al., 1996). The above hypothesis postulates inhibition of the NMDA–nNOS–cGMP pathway. Seeking for confirmation of the hypothesis, the potential inhibition of NOSs by the halogenated volatile anaesthetics was tested using the arginine to citrulline conversion method in cells or ex vivo tissue homogenates. The studies resulted in conflicting data. Some authors reported decrease in the NOSs activities in the presence of the anaesthetics (Tobin et al., 1994; Galley et al., 1995), whereas others claimed that these drugs do not alter the NOSs activities (Rengasamy et al., 1995; Tagliente et al., 1997; Galley et al., 2001), and increase in the NOSs activities was also reported (Rengasamy et al., 1997). The data concerning the influence of the halogenated volatile anaesthetics on the cGMP content in neurons are much more coherent, and a decrease was always observed (Rengasamy et al., 1997; Galley et al., 2001). Recent publication showed that the halogenated volatile anaesthetics inhibit the cGMP-dependent protein kinase Iα (Tao et al., 2000). We also provide some evidence in favour of this hypothesis and observed a decrease in the NO cerebellum concentration. Thus, the nNOS-inhibiting activity of the halogenated volatile anaesthetics cannot be excluded. It appeared that isoflurane and sevoflurane are able to inhibit recombinant NOSs. Although at extremely high drug concentrations (IC_{50} around 20 mM), this effect could have a biological significance as local concentrations of the halogenated volatile anaesthetics in synapses may reach at least a millimole level (Eckenhoff and Eckenhoff, 1998).

Thus, halogenated volatile anaesthetic effects on NOS activities and regulation in the brain are multiple and probably depend on the enzyme isoforms. Apparently, two opposite NO-related processes are triggered in the brain cortex. In synapses the anaesthetics accumulate in high concentrations and inhibit nNOS. The NMDA–nNOS–

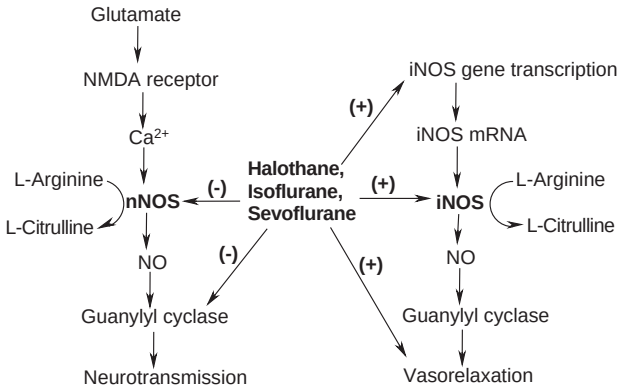

Fig. 7. Summary of the interactions of HVAs with enzymatic systems in the brain cortex (+): stimulation, (−): inhibition. The scheme is based on the literature data and data of the present communication.

cGMP pathway is blocked; this inhibition contributes to the narcosis development. However, nNOS produces a relatively insignificant quantity of NO in the brain cortex, thus inhibition of the enzyme does not decrease much the overall NO quantity produced in the brain cortex. Other isoforms and mainly iNOS produce the main quantity of the radical; its level is determined by the activity of this enzyme. Isoflurane and sevoflurane do not inhibit iNOS activity in vivo (Zuo et al., 1996), probably these anaesthetics increase activity of iNOS by triggering its translation from pre-existing mRNAs or by some other mechanism. This results in increased NO production, the latter produces a hyperaemic effect. The above considerations are summarized in Fig. 7.

The consequences of the NO concentration increase under isoflurane and sevoflurane anaesthesia for clinics should be studied in detail. First of all, the level of NO in the brain cortex should be determined in the patients undergoing anaesthesia. A rapid progress in methodological approaches related to NO chemistry and physiology makes this project feasible. If the NO concentration increases also in the patients under isoflurane or sevoflurane narcosis, the clinical significance of the phenomenon should be studied. As the above increase is not obligatory to narcosis development, possible negative effects of NO increase should be revealed.

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