Downregulation of nitric oxide in the brain of mice during their hypoxic preconditioning

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Lu, Guo-Wei, and Hong-Yan Liu. Downregulation of nitric oxide in the brain of mice during their hypoxic preconditioning. J Appl Physiol 91: 1193–1198, 2001.—An animal model of hypoxic preconditioning was produced in mice by repeated exposure to autohypoxic condition. The animals’ tolerance times to hypoxia were 1.7, 1.8, 2.1, and 2.3 times longer in runs 2, 3, 4, and 5, respectively, than that in run 1, and their oxygen consumption and heart and respiration rates were progressively and significantly slowed down during the repetitive exposure to hypoxia. L-Arginine concentration, nitric oxide (NO) synthase-positive cells, NO synthase activity, and NO content in the whole brain and the subregions telencephalon, diencephalon, and brain stem were significantly increased during the first exposure and were, instead of continuing to increase, significantly decreased in run 4 after the second and third exposure. Tolerance times under the hypoxic condition were significantly shortened and prolonged when preadministration of L-arginine and its analog, respectively, was made. These results indicate that NO in the brain is downregulated under condition of hypoxic preconditioning and negatively involved in increased tolerance to hypoxia.

L-arginine; nitric oxide synthase; N-nitro-L-arginine

ISCHEMIC PRECONDITIONING was first reported in 1986 by Murry et al. (33) in terms of protection of the heart from subsequent lethal insult by previous exposure to a brief period of sublethal ischemia. A phenomenon called ischemic tolerance has been demonstrated in the brain in vitro and in vivo since the 1990s (3, 5, 12, 15–17, 24, 27, 35).

A unique model of a nonischemic but hypoxic method of “preconditioning” was introduced in mice by Lu et al. (28–30). Brief hypoxia was shown to increase superoxide dismutase and adenosine in the brain during repetitive exposure to air rebreathing from a small (125 ml) bottle, which is called “hypoxic preconditioning” (11, 40, 41).

In contrast to these findings, the content or activity of some other chemicals, namely lipid peroxides and glutamate, was decreased or downregulated during the preconditioning (11, 37, 42). Excessive release of excitatory amino acids is reported to be toxic to neuronal cells, and nitric oxide (NO) is thought to be involved in mediating the N-methyl-D-aspartate (NMDA) neurotoxicity (6, 7, 17, 21, 22, 31). The present study was designed to characterize whether the NO is downregulated or not under the condition of hypoxic preconditioning.

MATERIALS AND METHODS

Experimental subject and hypoxic exposure. Experiments were conducted at room temperature (18 ± 1°C) on adult BALB/C mice of both sexes, weighing 16.0–22.0 g. The animals were anesthetized with a 1% solution of sodium pentobarbital (5.5 ml/kg ip) and randomly divided into three basic groups: 1) blank control group with no exposure to hypoxia (H0), 2) hypoxia control group exposed to hypoxia once (H1), and 3) hypoxic preconditioning group exposed to hypoxia four or five times (H4 or H5). For dynamic observation, groups exposed to hypoxia two or three times (H2 or H3) were also added.

The animal was placed into a 125-ml jar with fresh air, and the jar was sealed with a rubber plug. The animal was removed from the jar as soon as the first gasping breath appeared and was switched to another fresh air-containing jar of similar volume. The duration of time between subsequent exposures was variable within 30 s. The jar was immediately hermetically sealed again. This procedure was performed once (H1) and repeated two or three and four or five times (H2 or H3 and H4 or H5, respectively) (28–30).

At least three factors, lowering oxygen, increasing carbon dioxide, and lowering atmospheric pressure, were thought to be involved in the airless condition in the present procedure. It is generally recognized that the main consequence is hypoxia, and the procedure is simply described as “autohypoxia” (32). To make sure, the carbon dioxide was absorbed by calcium hydroxide inside the jar, and the atmospheric pressure was kept constant with a capsule, leaving only hypoxia in the present study (Fig. 1A).

Tolerance time and O2 concentration determination. The appearance of the first gasping was regarded as the tolerance limit in each trial. The time period between the beginning of airtightness and the appearance of the first gasping was termed “original duration of tolerance” for each run. The standard tolerance duration in a standard jar with an effective fresh air volume of 100 ml was calculated as follows:

\[ T = \frac{(T_0/V_0)}{V_e} \times 100 \]

\[ = \frac{[(t_1 - t_0)/(V_0 - V_d)]}{V_e} \times 100 \]

\[ = \frac{[(t_1 - t_0)/(V_0 - W/D_a)]}{V_e} \times 100 \]

\[ = \frac{[(t_1 - t_0)/(V_0 - W/0.94)]}{V_e} \times 100 \]

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where $T$ is the standard tolerance time (min), $T_0$ is the original tolerance time (min), $t_0$ is the starting time of sealing, $t_1$ is the ending time of sealing, $V_e$ is the effective jar volume (ml), $V_0$ is the original jar volume (ml), $V_a$ is the animal’s volume (ml), $W_a$ is the animal’s body weight (g), and $D_a$ is the animal’s density determined from displacement $V_a$ and measured body weight. The average $D_a$ was 0.94, ranging from 0.92 to 1.00 (28–30).

Microvolume (0.2 ml) of air in the tight jar was drawn via a tube connected to the jar (Fig. 1A) at the end of each run when the first gasping occurred in the animal. A modified Scholander-Roughton technique was used to determine the concentration of oxygen in the sample (28).

**Heart and respiration monitoring.** In addition to the observation of the gasping breath and general behavioral responses, the animal’s heart and respiration activity were monitored during experimentation. An electrode was placed in the area of the xiphoid process, and the other was in the corresponding level in the back, and the signals were led to a modified electrocardiograph (ECG). To record the animal’s respiration movement, a piezoelectric crystal microphone was connected to the capsule and connected to the other lead of the ECG (Fig. 1A).

**Cerebral arginine concentration measurement.** Animals were decapitated, and their heads were kept in $-10^\circ$C liquid nitrogen right after hypoxic exposure of each group. The next day, the whole brain was isolated and weighed. Pure water (1.5 ml) was added to 400 mg of whole brain. Samples were homogenized at $<0^\circ$C and centrifuged at 13,500 rpm for 45 min under $-4^\circ$C temperature. After the addition of 100 μl of 10% trichloroacetic acid to the supernatants, they were centrifuged at 13,500 rpm for 25 min under $-4^\circ$C temperature. The supernatants were adjusted to pH 7–8 with 1 mol/l NaOH and were filtered with a 40-μm filter.

Twenty-two kinds of standard amino acids, including arginine, were measured exactly and dissolved in 0.1 mol/l acetate buffer to receive 5 mmol/l of standard solution, which was respectively diluted to 1.0 mmol/l and 500, 100, 50, and 10 μmol/l of standard solution.

The standard supernatant samples were analyzed by HPLC with the o-phthaldialdehyde precolumn derivatization technique. A reverse-phase C18 column (150 × 3.9 nm, 4 μm, Nova Pak, Waters) was used in the HPLC system (HP 1050 liquid chromatograph, Hewlett-Packard). The mobile phase, which was composed of 0.1 mol/l acetate buffer (pH 6.95) and methanol, was delivered at a flow rate of 0.8 ml/min by a high-pressure pump (HP 1050Q, Hewlett-Packard). After derivative reaction with o-phthaldialdehyde, the samples (20 μl) were injected into the HPLC system by an autosampler and eluted gradiently by HPLC. The contents of arginine in the sample were detected by a fluorescence detector with excitation = 250 nm and emission = 395 nm.

**Fig. 1.** Diagram showing apparatus for recording electrocardiogram (ECG) and respiration movement (A) and examples of these recordings (B). A: a, animal; b, balloon; e, ECG; h, ECG recording; p, piezoelectrical crystal microphone; r, respiration movement recording; t, tube for taking air from the jar. Dashed line, metal net. B: left traces are ECG, and right traces are respiration movement in runs 1 (I), 3 (III), and 5 (V). Nos. are time points (in min) at which the records were taken in runs 1, 3, and 5.

**Fig. 2.** Tolerance time (A), ending oxygen concentration in the jar (B), and heart (HR) and respiration rate (RR; C) in different runs of exposure. Values are means ± SD; $n$ = 15 (A, B) and 20 animals (C). c/min, Cycles/min (beats/min for heart rate and breaths/min for respiration rate); HR-i, initial HR; HR-e, ending HR; RR-i, initial RR; RR-e, ending RR. Significant difference compared with run 2 (A) and with corresponding value in run 1 (C), *$P < 0.05$; significant difference compared with preceding run (A and B) and corresponding value in run 1 (C), **$P < 0.01$. 

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H0–H4, hypoxia exposure for 0–4 times, respectively. Values are means ± SD; n = 10 animals for each group. Significant difference compared with H0: *P < 0.05 and **P < 0.01; significant difference compared with H1, *P < 0.05.

**NADPH-diaphorase and NO synthase histochemistry (43).** At the completion of experiments, the mice were anesthetized with a lethal dose of 0.4% solution of sodium pentobarbital and were perfused intracardially with phosphate-buffered saline. The animals were then perfused with 200–300 ml of fixative containing 4% paraformaldehyde. The animal’s whole brain was removed and immersion fixed in 30% solution of sucrose overnight.

On the next day, horizontal vibrotome sections (20 μm) of the brain were cut; immersed and incubated in PBS solution containing 1 mmol/l β-NADPH, 1.2 mmol/l nitro blue tetrazolium, and 0.3% Triton X-100 (pH 8.0) at 37°C for 1 h; then transferred into PBS solution (pH 7.4) to end the reaction; and then counterstained with neutral red.

**Photometric determination of NO synthase activity (25).** Animals were decapitated, and their brains were removed and placed quickly into liquid nitrogen. The animal’s brain was transferred into PBS solution containing 1 mmol/l L-arginine, 11 μmol/l NADPH, 50 μmol/l L-valine, and 40 mmol/l KPO3 (pH 7.2). Absorption value was recorded for 3 min at 401 and 421 nm for every 30 s. NOS activity was expressed in moles per minute of NO and calculated as follows

\[
\text{NOS activity (mol/min)} = \frac{[(a - b) - (a' - b')] \times 194.3}{2}
\]

where a and b are absorption values at 30 s at 401 and 421 nm, respectively, and a’ and b’ are values at 90 s at 401 and 421 nm, respectively.

**Fluorometric determination of NO (34, 36).** The animal’s whole brain and subregions of telencephalon, diencephalon, brain stem, and cerebellum were taken out, and 2 ml of PBS solution were added and homogenized under ice bath. One-half milliliter of the sample was kept for measurement of protein. Trichloroacetic acid (10%) was added to the remaining sample and centrifuged to make it protein free. One-half milliliter of 0.04% 4-hyocoumarin reagent was added to 1 ml of supernatant in a 10-ml stopped test tube. The tube was left in an ice bath for 5 min. The reaction mixture was added 0.1 ml of 8% sodium sulfate, which was left for 10 min at room temperature, and reduced mixture was made alkaline with 1.0 ml of 1.5 mol/l NaOH and left for 10 min at room temperature.

The method is based on the nitrosation of 4-hydroxyxocoumarin in acidic medium and subsequent reduction to 3-amino-4-hydroxyxocoumarin, which is fluorescent in alkaline medium. The fluorescent intensity is proportional to the nitrite concentration in the range of 3 ng/ml to 1 μg/ml in the sample solution, with a relative standard deviation of 0.5% (50 ng/ml) (34). The fluorescence intensity was measured with excitation at 347 nm and emission at 453 nm. A standard curve was made with NaNO2. NO values were expressed in picomoles per milligram of protein for subregions of the brain.

**Administration of L-arginine and its analog.** Mice were divided randomly into groups of L-arginine, L-arginine analog, and normal saline. L-Arginine (50 mg/kg), N-nitro-L-arginine (L-NNA; 50 mg/kg), and normal saline (90 mg/kg) were administrated intraperitoneally to the three respective groups. Thirty minutes later, animals were exposed to hypoxia repeatedly as shown in the model. The standard tolerance times of runs 1–4 were compared correspondingly among the three groups.

**Statistical analysis.** ANOVA and Duncan’s test from the SYSTAT program were used for analysis of experimental data. A level of 0.05 or less was accepted as an indicator of significance.

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Fig. 4. Nitric oxide synthase (NOS)-positive neurons on hemispheres (A) and hippocampus (B) in groups H0 (1), H1 (2), and H4 (3). Magnification ×400 (A) and ×100 (B).
RESULTS

During the preconditioning, the animals’ respiration gradually quickened, cyanosis gradually increased, and finally spasmlike activity and gasping breath appeared in the first run of exposure to hypoxia. Similar manifestations were evident during the second run. Starting with the third run, the animal remained quiet most of the time, and its respiration become slow, deep, but regular in pattern (Fig. 1B). Cyanosis become more apparent in the fourth run, and the eyeballs showed a black-violet color.

Changes in tolerance time and chamber oxygen level. The increment of each run in standard tolerance time was progressive when the animals were under anesthesia, and the preconditioning procedure was taken at room temperature of 18 ± 1°C. The tolerance time was 1.7, 1.8, 2.1, and 2.3 times longer in runs 2, 3, 4, and 5, respectively, than in run 1 (Fig. 2A). The oxygen concentration inside the jar at the moment of appearance of the first gasping was 6.6% in run 1 and progressively decreased to 3.2% in run 5, which was 49% of that in run 1 (Fig. 2B).

The rhythm of ECG was kept regular all the way from run 1 through 5 (Fig. 1B). The average initial heart rate at the beginning of each run progressively decreased from 744 beats/min in run 1 to 180 beats/min in run 5, and the ending one at the first grasping was reduced from 469 beats/min in run 1 to 157 beats/min in run 5, which was one-fifth of initial rate in run 1 (Fig. 2C).

Changes in cerebral L-arginine content. As shown in Fig. 3, the content of L-arginine in whole brain was 347.182 ± 49.354, 430.864 ± 41.770, 307.303 ± 55.690, 296.033 ± 41.211, and 293.080 ± 51.830 nmol/g in groups H0, H1, H2, H3, and H4, respectively. The content significantly increased in group H1, progressively decreased in groups H2–H4, and even was significantly less than that in group H0.

Changes in NOS-positive neurons and NOS activity in the brain. No apparent difference in number and shape of NOS-positive neurons in the cerebral cortex

![Fig. 5](image-url)  Fig. 5. NOS activity (A), nitric oxide content in whole brain (B), and different brain subregions (C) in groups H0, H1, and H4. Values are means ± SD; n = 16 (A), 15 (B), and 10 animals (C). Significant difference compared with H0: *P < 0.05 and **P < 0.01; significant difference compared with H1: *P < 0.05 and **P < 0.01.

![Fig. 6](image-url)  Fig. 6. Standard tolerance time of different runs in group control, L-Arg, and L-Arg analog. Values are means ± SD; n = 15 animals for each group. Significant difference compared with corresponding value in preceding run, *P < 0.05; significant difference compared with control value within run, *P < 0.05.
was seen among groups $H_0$, $H_1$, and $H_4$. However, the diameter of processes of positive neurons was clearly more enlarged in group $H_1$ compared with both groups $H_0$ and $H_4$, the intensity of cell stain in group $H_1$ was more positive than that in both groups $H_0$ and $H_4$, and no apparent difference was shown between the latter two groups (Fig. 4A). The number of NOS-positive neurons in the hippocampus was 5, 42, and 39 on average in groups $H_0, H_1$, and $H_4$, respectively. Instead of continuing to increase, the number tended to decrease in group $H_4$ (Fig. 4B).

NOS activity in the whole brain dramatically increased in group $H_1$ and, instead of continuing to increase, it decreased significantly and tended toward the control level in group $H_4$ (Fig. 5A).

**Changes in cerebral NO content.** Similar to the changes in NOS activity, the content of NO in whole brain also dramatically increased in group $H_1$ and significantly decreased to control level in group $H_4$ (Fig. 5B). The value of NO content was $6.294 \pm 2.337$, $18.879 \pm 10.491$, and $8.280 \pm 2.130$ pmol/mg protein in groups $H_0, H_1$, and $H_4$, respectively.

Similar changes were shown in the subregion of the telencephalon, diencephalons, and brain stem (Fig. 5C). The NO content in these subregions markedly increased in group $H_1$ and tended to reduce to the group $H_0$ level in group $H_4$ (Fig. 5C).

**Effects of extraneous arginine and its analog.** Behaviorally, more severe responses and significant shortening in tolerance time were shown during the second, third, and fourth run of exposure in the arginine group. The downregulation of these indexes may thus be beneficial to the increase in the animals’ tolerance to hypoxia. This seems to be confirmed by the effect of preadministration of extraneous L-arginine and its analog. The animals’ tolerance time under the condition of hypoxia is significantly shortened and prolonged by administration of the NO donor L-arginine and the L-arginine analog L-NNA, respectively.

NMDA neurotoxicity is markedly decreased after treatment with NOS inhibitors and in cultures from nNOS-deficient mice (8, 10). The increase in tolerance to hypoxia by arginine analog administration and the negative correlation between the increment in tolerance and decrease in NOS-positive neurons and activity, as well as in NO production, might be related to the decrease in NMDA neurotoxicity.

NO itself may not be toxic and becomes toxic only when it reacts with superoxide and is converted to peroxynitrite (1). Instead of a continuing increase in lipid peroxides during hypoxic preconditioning, its content became less and tended to reach control level (11). In addition, the increased activity of superoxide dismutase during repetitive exposure to hypoxia (11) might also be beneficial to the reduction of peroxides. The decrease in NO toxicity may also be involved in the development of hypoxic preconditioning and tolerance to hypoxia.

**DISCUSSION**

Similar to our laboratory’s previous findings (28–30), the present study shows that the animal is able to tolerate progressively lowered levels of oxygen and to keep alive in a lowered state of life activity for longer and longer time periods during repeated exposure to hypoxia. The progressive lowering of life activity, as shown by slowed oxygen consumption and heart and respiration rate, seems to be beneficial to the progressive increase in tolerance.

NO is a putative neurotransmitter in the neuronal systems and is synthesized from L-arginine by the enzyme NOS (2, 19). Based on existing knowledge, it is most likely that neuronal NOS (nNOS) is responsible for the observed phenomenon (12). However, it is not possible to rule out the contribution of the endothelial isoform.

NO may act as both a neuroprotective and a neurodestructive agent in the hypoxic and ischemic injuries (1, 18, 38). NO donors or NO produced by constitutive nNOS limit apoptosis induced by trophic factor deprivation in PC12 cells and primary neurons (13, 14, 23, 26). NO from constitutive hippocampal NOS may be involved in the neuroprotection afforded by preconditioning (4), and NO production and activity are critical to the induction of ischemic tolerance in vivo (15).

In contrast, NO is able to lead to neuronal cell death when it is produced in excess (8–10, 18, 39). Several reports of increased NO production after ischemia have been published (22, 31). Stroke damage is diminished in nNOS−/− mice (20, 21), the damage is also reduced by NOS inhibitors (22), and nNOS activation is linked to neural damage (12).

What we have seen in the present study is consistent with these reports. The content of the NO donor L-arginine, NOS-positive cells, NOS activity, and NO content in the brain increased initially in run 1 and then decreased in run 4 after repeated hypoxic exposure, whereas tolerance increased steadily with each trial. The decreased NOS activity and NO content seem to be related to the increase in tolerance to hypoxia.

The downregulation of these indexes may thus be beneficial to the increase in the animals’ tolerance to hypoxia. This seems to be confirmed by the effect of preadministration of extraneous L-arginine and its analog. The animals’ tolerance time under the condition of hypoxia is significantly shortened and prolonged by administration of the NO donor L-arginine and the L-arginine analog L-NNA, respectively.

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