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L. W. DOBRUCKI**, L. KALINOWSKI**, W. URACZ***, T. MALINSKI*

THE PROTECTIVE ROLE OF NITRIC OXIDE IN THE BRAIN ISCHEMIA

*Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio, USA
** Department of Biochemistry, Medical University of Gdansk, Medical Research Center of the Polish Academy of Science, Poland
*** Department of Pharmacology, Jagiellonian University, Cracow, Poland

A role of nitric oxide in ischemia/reperfusion (I/R) injury of brain in normotensive (Sprague-Dowley rats, SD) and stroke-prone spontaneously hypertensive rats (SHR-SP) was studied. Cerebral ischemia was produced in rats by occlusion of the middle cerebral artery (MCA). NO and O_2^- releases in the brain in response to MCA occlusion followed by reperfusion were simultaneously monitored (2h) using electrochemical microsensors. The size of infarct was evaluated in the course of I/R from images of brain slices stained with 2,3,5-triphenyltetrazolium chloride. Similar patterns of NO and O_2^- releases were exhibited for SD and SHR-SP rats in the entire course of the experiments. However, the concentration of NO release was significantly lower during I/R in SHR-SP than in SD rats (the maximal NO concentration was 2.61 ± 0.22 μmol/L for SD and 1.51 ± 0.16 μmol/L for SHR-SP rats; $^*P < 0.01$). In contrast, the concentration of O_2^- release during cerebral ischemia was significantly higher in SHR-SP than SD rats (the maximal increase was 122 ± 24 nmol/L for SD and 220 ± 44 nmol/L for SHR-SP rats; $^*P < 0.01$). The infarct sizes revealed in the course of I/R were larger in SHR-SP than SD rats (1.8 ± 0.4% vs. 1.1 ± 0.4% at 30 min., 2.84 ± 0.8% vs. 2.21 ± 0.6% at 100 min. and 9.20 ± 1.1% vs. 5.8 ± 0.6% at 180 min. of the brain weights, respectively; $^*P < 0.01$ for each time-point). These studies indicate that nitric oxide plays a protective role during I/R and deficiency of NO in SHR-SP rats is due to excess of O_2^- production. The deficiency in NO concentration correlates positively with the increase of cerebral I/R injury.

Key words: *Nitric oxide, superoxide, brain ischemia, middle cerebral artery occlusion, infarct size, MCA.*

INTRODUCTION

In the central nervous system nitric oxide (NO) is regarded as a factor involved in neurotransmitting processes (1) and regulation of cerebral blood flow (2—4). NO has also been implicated in the pathophysiology of cerebral ischemia on the basis of its actions as a mediator of tissue injury (5). During

brain ischemia, a reduction in the supply of oxygen and energy substrates results in a cascade of events in brain tissues, including alterations in the pool of oxygen free radicals and other reactive oxygen species, which may lead to development of infarct. It is also known that reperfusion of ischemic tissue during revascularizing procedures paradoxically causes tissue injury beyond the damage already developed. However, there is conflicting evidence related to the possible contribution of NO in pathogenesis of ischemia/reperfusion in brain injury (5). It has been indicated in several studies that NO may have neurotoxic effects (6—11) if present in abnormally high concentrations, which takes place during cerebral ischemia (12, 13). Postulated mechanism of brain damage would be that an excess of NO interacts with superoxide radical (O_2^-) to initiate the production of other cytotoxic oxygen species like hydroxyl (OH^-) or NO_2^- radicals. Such a mechanism has been recently indicated in the development of ischemia/reperfusion injury in skeletal muscles (14). In this regard, it has been suggested that the agents that are capable of preventing free radical production may prevent brain damage during ischemic conditions (8, 15, 16). On the other hand, a deficit in NO production in the brain of rats possessing genetic factors responsible for stroke — stroke-prone spontaneously hypertensive rats (SHR-SP) has been demonstrated (17, 18).

The aim of the present studies was to examine the role played by a deficit in NO on the ischemic injury. Ischemic injury of brain of SHR-SP rats was compared with injury of normotensive Sprague-Dowley rats (SD). In these *in vivo* experiments, NO release in the brain was measured with a porphyrinic microsensor and O_2^- concentration was monitored simultaneously with a superoxide-sensitive electrochemical microsensor.

MATERIAL AND METHODS

Animals

A group of twelve male hypertensive SHR-SP rats and twelve male control Sprague-Dowley rats was divided into two subgroups (for nitric oxide and superoxide *in vivo* measurements during brain ischemia).

A second group of animals; SHR-SP (n = 6) and control (n = 6) rats were used for infarct size determination at distinct time points after MCA occlusion.

The experimental protocols were approved by the Institutional Animal Care Committee.

Surgical procedures

The rats were given anaesthesia (1 : 10 mixture of xylazine: ketamine, 1 ml/kg) and maintained every hour by subcutaneous injection. The animal was placed on a stereotaxic instrument to immobilize the head since head movements could influence the results by generating piezoelectric noise signals in electrochemical NO and O_2^- sensors. The right middle cerebral artery was occluded according to procedures described previously (19). Briefly, the artery was exposed using

microsurgical techniques and clamped with a microvascular clip. The position of the occlusion was localized near the origin of the striate branch of the MCA. After 120 minutes of brain ischemia, the artery clamp was released to restore the blood flow for about 60 minutes.

Nitric oxide and superoxide sensors

The NO sensor was prepared according to the procedure previously described (12, 14, 20). Briefly, polymeric film was deposited electrochemically on the carbon fiber electrode's tip from a solution of monomeric Ni(II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin. The sensor surface was then coated with a Nafion film (5% w/w in alcohol) for 15—20 s. The microsensor was calibrated by adding known volumes of NO standard (1.76 mmol/L at 0°C) prepared by saturating a 25 ml degassed phosphate buffer (pH = 7.4) with NO gas.

Similarly, a superoxide microsensor was fabricated following modified procedures described elsewhere (21). The sensor was calibrated before each measurement by adding known concentrations of aqueous solutions of xantine and xantine oxidase to generate O_2^- radicals.

A detection limit was 2 nmol/L and 1 nmol/L for NO and O_2^- sensor, respectively and the response time of sensors was 0.1 ms.

In vivo measurements of nitric oxide and superoxide

NO and O_2^- were continuously measured for 4 h (1 h of preischemia, 2 h of ischemia, 1 h of reperfusion) using amperometry. The microsensors were stereotaxically positioned with a micromanipulator 1 mm distal along the MCA from the point of occlusion. Using a standard 28-gauge needle, microsensors were implanted 1 mm deep in the parietal lobe of the brain.

In amperometric measurements, potential was kept at a constant level (0.62 V for NO sensor and −0.20 V for O_2^- sensor). A three-electrode system was used for *in vivo* measurements of NO and O_2^- before (baseline recording), during and after brain ischemia. The system consisted of the working electrode (NO or O_2^- sensor), and a standard silver-silver chloride (SSCE) reference electrode combined into one unit with a platinum counter electrode.

Measurements were carried out with an EG&G Model 283 potentiostat coupled with an IBM Pentium computer via National Instruments data acquisition board. The data were continuously collected by a LabView software package and recorded for further processing.

Infarction size

The normotensive rats (SD rats, $n = 6$) and hypertensive rats (SHR-SP, $n = 6$) were divided into three groups and decapitated at distinct time points (30, 100 and 180 minutes after MCA occlusion). Brains were immediately removed, washed with ice-cold saline solution and placed on dry ice. The frozen brains were cut into several 2 mm sagittal slices then stained with the mitochondrial activity indicator — 2,3,5-triphenyltetrazolium chloride (TTC, 2% solution in saline at 37°C) for 30 minutes as described in details elsewhere (22). The images of stained sections were captured by a DC210 Kodak Digital Camera and transferred to the computer for further processing. The brain infarct size was calculated by numeric integration of data from individual slices and presented as percentage of the total brain weight.

Statistical analysis

Results were presented as mean ± SD. Statistical analysis was done by an unpaired Student's *t* test or by ANOVA. All analyses were performed using a statistical and graphing package of Microcal Origin. Means were considered significantly different at $P < 0.05$.

RESULTS

Measurements of NO release

Nitric oxide release in the region of the infarct was recorded before (baseline), during and after MCA occlusion. *Fig. 1* shows representative tracings of these recordings from normotensive SD rats and hypertensive SHR-SP rats. A stable background was monitored after implantation of the electrode in the brain and recorded for about 1h before occlusion. After clamping the MCA, a rapid increase in NO concentration was observed in both SHR-SP and SD rats. The NO concentration was detectable 120 ± 31 s after occlusion for SD rats and after 210 ± 45 s for SHR-SP rats. The maximum concentration of NO was observed after 7 ± 2 min for normotensive rats and after 12 ± 3 min of the occlusion. The maximal concentration of NO in the brain of hypertensive rats (*Fig. 1b*) after MCA occlusion was 1.51 ± 0.16 µmol/L ($n = 6$). This concentration was significantly smaller than observed in control rats (2.61 ± 0.22 µmol/L, $n = 6$, $P < 0.01$) (*Fig. 1b*).

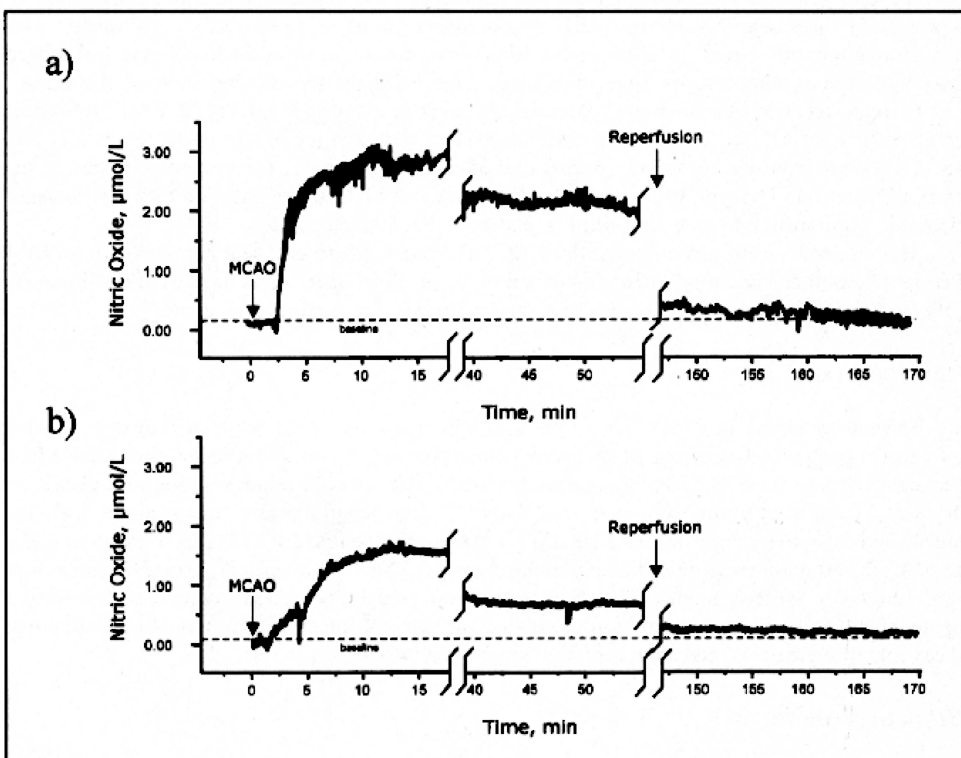

Fig. 1. Amperograms of nitric oxide release recorded during cerebral ischemia/reperfusion in the striatum of (a) SD and (b) SHR-SP rat.

The plateau observed during NO release persisted for several minutes and than decayed at the rates of -0.02 ± 0.006 μmol/L·s^{-1} and -0.01 ± 0.004 μmol/L·s^{-1} for SD and SHR-SP rats, respectively. A reperfusion (120 minutes after MCA occlusion) caused a drop in NO concentration below the basal level which was more significant for SHR-SP rats.

Measurements of O_2^- release

The measurements of O_2^- concentration were conducted in a similar manner to NO measurements. Before MCA occlusion a baseline was recorded followed by measurements of O_2^- during ischemic conditions.

Fig. 2 shows O_2^- concentrations recorded as a series of single measurements every 20 minutes. The kinetics of O_2^- release differs from the kinetics of NO. The superoxide concentration reached a semiplateau about 40 minutes after onset of brain ischemia, peaking at 122 ± 24 nmol/L for SD rats and 220 ± 44 nmol/L for SHR-SP rats. During reperfusion a significant initial

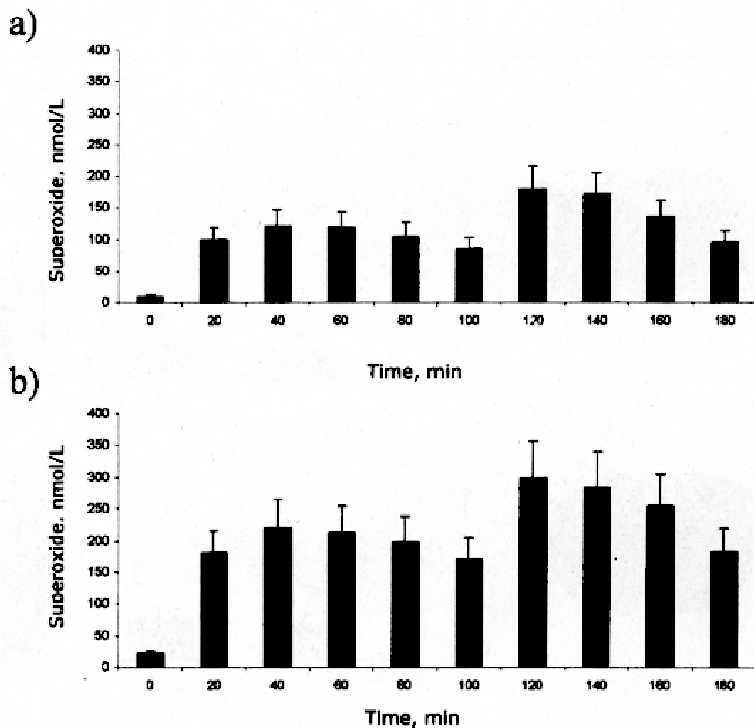

Fig. 2. Superoxide concentrations recorded sequentially during ischemia/reperfusion in the striatum of (a) SD and (b) SHR-SP rats.

increase in O_2^- concentration was observed reaching 180 ± 36 nmol/L for SD rats and 297 ± 59 nmol/L for SHR-SP rats. After that, the concentration of O_2^- gradually decayed.

Infarct size determination

The infarct sizes determined in SD and SHR-SP rats after brain ischemia are depicted in *Fig. 3*. Infarct sizes were expressed as a percentage of total brain weight. About 30 minutes after MCA occlusion TTC staining showed very small brain damage in both control ($1.1 \pm 0.4\%$) and hypertensive ($1.8 \pm 0.4\%$) rats. After 100 minutes of cerebral ischemia, small but significant increase in brain damage was noticed; $2.2 \pm 0.6\%$ for SD rats and $2.8 \pm 0.8\%$ for SHR-SP rats. The difference between the infarct size of SD and SHR-SP rats was statistically insignificant. The largest infarct size was observed at 180 minute reaching $5.8 \pm 0.6\%$ in normotensive and $9.2 \pm 1.1\%$ in hypertensive rats ($^*P < 0.01$).

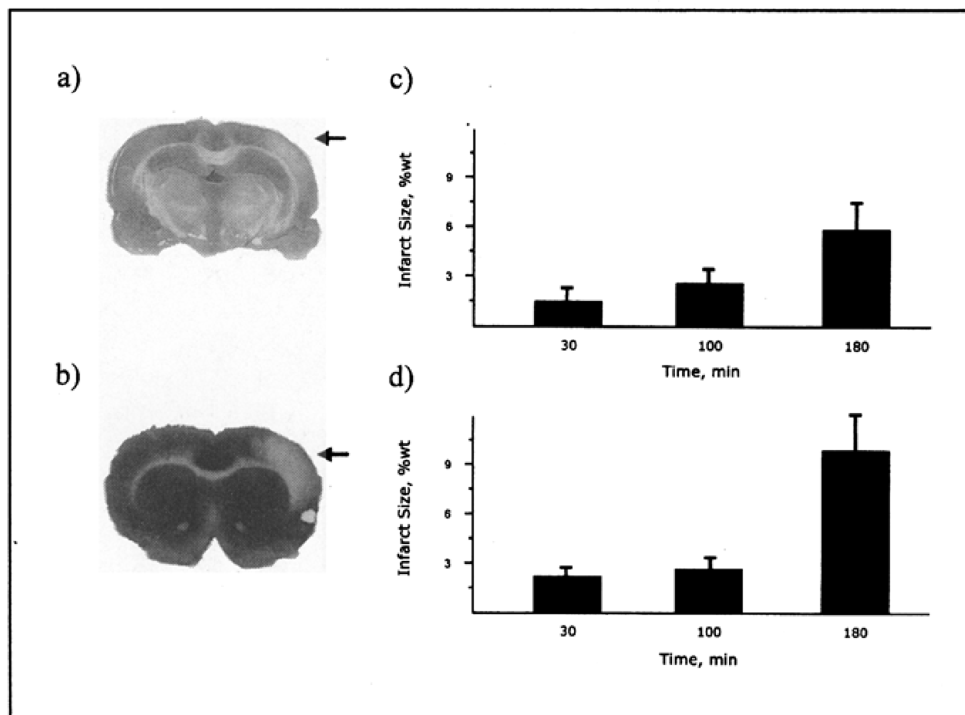

Fig. 3. Brain slices stained after 180 min of cerebral ischemia of (a) SD and (b) SHR-SP rats. Change of infarct size during cerebral ischemia/reperfusion of (c) SD and (d) SHR-SP rats.

DISCUSSION

The present study provides the correlation between NO and O_2^- production, and infarct size during transient focal ischemia (2 h) and reperfusion (1 h).

The rationale underlying our studies is that a deficit in NO release during I/R of the brain is due to significant generation of O_2^- followed by the production of peroxynitrite. This hypothesis is supported by two observations described previously (18): 1) the measured increase in NO concentration in the brain in response to MCA occlusion in SHR-SP was almost identical to the increase in NO concentration observed in NO-deficient (L-NNA-treated) rats and 2) the size of the infarct resulting from MCA occlusion was 63% greater in NO-deficient (L-NNA-treated) rats than in control (untreated) rats. NO in the brain is released rapidly in response to ischemia. The function of NO release is to decrease the cerebrovascular resistance and to restore blood flow to the ischemic region. An analysis of the kinetics of NO and O_2^- release during I/R indicates a rapid increase in NO concentration followed by O_2^- generation within striatum shortly after MCA occlusion. Superoxide was produced in relatively high concentrations and that overproduction was in the reverse proportion to NO release. Parallel to the increase of O_2^- concentration, which reached maximum at 40 min from the onset of ischemia, the rapid decrease in concentration of NO was observed (*Fig. 1* and *2*). During the reperfusion, the concentration of O_2^- reached maximum causing a significant drop in NO concentration. Nitric oxide and superoxide when produced in close proximity may react together forming the stable peroxynitrite ($ONOO^-$). Peroxynitrite when produced at low concentration usually undergoes protonation and isomerization to form hydrogen cation and nitrate anion. However, under conditions of high $ONOO^-$ production, the protonated peroxynitrite ion can diffuse for a significant distance of several cells and undergoes both homolytic and heterolytic cleavage resulting in formation of hydroxyl ($OH^\cdot$) and nitrogen dioxide ($NO_2^\cdot$) radicals and a nitronium cation (NO_2^+). These molecules are among the most reactive and damaging species in biological systems.

During the first minutes of reperfusion, NO release depends not only on availability of substrates for cNOS (L-arginine and O_2) but also on the amount of accumulated O_2^-. Recent studies have shown that the O_2^- generation can be inhibited by certain L-arginine derivatives what suggests that like NO, production of O_2^- during pathological conditions is calcium-dependent (14). There are also several calcium-independent sources of O_2^-, which may contribute to the basal level of O_2^- such as prostaglandin metabolism, cytochrome P-450, and processes stimulated by protein kinase C or xantine oxidase.

Recently it was shown that increased concentration of NO might inhibit O_2^- generation from natural sources (23). This may suggest that lower NO generation during the latter period of ischemia may allow increased O_2^- release.

These observations are in good agreement with results obtained from infarct size measurements. During the initial phase of cerebral ischemia (at 30 min) the observed infarct size was very small and similar for both SD and SHR-SP rats. 100 minutes after MCA occlusion, shortly before the reperfusion, the infarct size in both groups of rats increased but was still relatively small. However, after 180 minutes of I/R, infarct size measurement revealed significant changes between brain damages of SD and SHR-SP rats.

This is probably due to the increase of O_2^- generation and the decrease of NO concentration.

Evidences presented here support the hypothesis that NO produced during ischemia plays a beneficial role as a neuroprotectant. Damage of the brain with the hindered NO release (SHR-SP rats) is much higher than in the brain with high NO production (SD rats).

In conclusion, we demonstrated that NO deficiency and overproduction of superoxide anion by calcium-independent and dependent sources contribute to larger brain infarct size.

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Author's address: Tadeusz Malinski, Ph. D. Department of Chemistry and Biochemistry, Ohio University Athens, OH 4570-2979, USA.