Journal of
Nanomedicine & Nanotechnology

Heeba et al., J Nanomed Nanotechnol 2014, 5:4
DOI: 10.4172/2157-7439.1000215

Research Article Open Access

Nanomedical Approach to Monitor the Central Role of NO/ONOO$^-$ Imbalance in Ischemic Stroke Brain Damage – The Effects of Statins and Heme Oxygenase-1

Heeba G[1], Corbalan JJ[1], Patton S[1], Burewicz A[1,2], Stys AT[3] and Malinski T[1]*

[1]*Nanomedical Research Laboratories, Ohio University, Athens, OH 45701, USA*
[2]*Department of Chemistry, A. Mickiewicz University, 61-614 Poznan, Poland*
[3]*Sanford Cardiovascular Institute, Sanford School of Medicine, University of South Dakota, Sioux, Falls, SD 57105, USA*

Abstract

Background: There are conflicting reports concerning the role of nitric oxide (NO) and oxidative stress in brain ischemic damage. In the present study, a nanomedical approach was utilized to elucidate the role of NO and peroxynitrite ($ONOO^-$) imbalance in ischemic stroke.

Methods: Nanosensors (diameter ~ 200 nm, detection limit of 10^{-9} molL^{-1}, response time ~10 μs) were used to monitor *in situ* the concentration of NO and $ONOO^-$. Adult male Sprague Dawley rats were given permanent middle cerebral artery occlusion (pMCAO) for 3 h, 12 h or 24 h.

[NO]/[$ONOO^-$] was measured in striatum, along with, constitutive nitric oxide synthase (cNOS) enzymes and heme oxygenase-1 (HO-1) expression and infarct volume. The [NO]/[$ONOO^-$] was also monitored in pre-treated animals with simvastatin and atorvastatin in the presence of the cNOS inhibitor L-NAME. The effect of modulators of cNOS or NADPH oxidase (sepiapterin, PEG-SOD, VAS2870 and IN-7) on the [NO]/[$ONOO^-$] was elucidated.

Results: After 3 h of ischemia, NO decreased from 400 ± 20 nmolL^{-1} to 217 ± 11 nmolL^{-1} and $ONOO^-$ increased from 150 ± 9 nmolL^{-1} to 244 ± 9 nmolL^{-1}. The [NO]/[$ONOO^-$] balance shifted from 2.67 ± 0.06 to 0.89 ± 0.07 after 3 h of ischemia, indicating severe uncoupling of cNOS. The [NO]/[$ONOO^-$] imbalance shifted with time of ischemia and correlated directly with the increase in infarct volume and expression of cNOS and HO-1. Treatment with simvastatin or atorvastatin partially, but significantly, restored [NO]/[$ONOO^-$] balance and decreased infarct size in ischemic brain. Also, modulators of cNOS an NADPH restored [NO]/[$ONOO^-$].

Conclusions: The imbalance between cytoprotective NO and cytotoxic $ONOO^-$ directly correlates with brain damage in ischemic stroke. The [NO]/[$ONOO^-$] imbalance reflects on the level of uncoupled cNOS and the nitroxidative stress. [NO]/[$ONOO^-$] imbalance increases cNOS and HO-1, which contributes to or prevents further brain damage, respectively. Balancing [NO]/[$ONOO^-$] is the determinant in preventing or mollifying brain damage. Simvastatin or atorvastatin shifts favorably [NO]/[$ONOO^-$], and may provide prophylactic treatment strategy for ischemic stroke.

Keywords: Nitric oxide/peroxynitrite imbalance; Ischemic stroke; Nitric oxide synthase uncoupling; Heme oxygenase

Introduction

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase, the rate limiting step in cholesterol biosynthesis. Clinically, statins lower the risk of stroke ≤30% [1]. Experimentally, statins reduce cerebral infarct size, improve neurological function, and increase cerebral blood flow during brain ischemia [2-6]. However, mounting clinical and experimental evidence suggests that the neuroprotective effects of statins are not related to cholesterol lowering but may have an effect on constitutive nitric oxide synthase (cNOS) and/or oxidative stress [7,8]. cNOS is the enzyme that biosynthesizes the two substrates (oxygen, and L-arginine) into the three products nitric oxide (NO), water and L-citrulline [9]. There are two calcium dependent constitutive isoforms of nitric oxide synthase: endothelial NOS (eNOS) and neuronal NOS (nNOS). A third isoform, inducible NOS (iNOS) is not stimulated by calcium and can continuously produce NO. Statins increase eNOS, nNOS, and iNOS expression [5,10,11]. However, an increase in enzyme expression may not always directly correlate with enzyme activity and higher production of bioavailable NO [12].

NO produced by eNOS plays a critical role in the regulation of vascular blood flow, and inhibition of platelet/leucocytes adhesion and aggregation. nNOS can generate NO in smaller quantities than eNOS and the NO that it produces acts as a neurotransmitter. We were the first to measure, *in vivo*, *in situ* and real time, the NO generated by cNOS in the brain during ischemia [13,14]. eNOS knockout mice are hypertensive and exhibit larger cerebral infarctions after a permanent middle cerebral artery occlusion (pMCAO) [15].

Although moderately reactive reducing agent NO is made exclusively by one of the three isoforms of NOS in mammalian systems, the highly reactive oxidizing agent peroxynitrite ($ONOO^-$) has many sources [16]. NO is one of the main scavengers of superoxide (O_2^-) in a cellular environment, producing $ONOO^-$. A dysfunctional, uncoupled cNOS can produce O_2^- and NO. O_2^- and NO can react rapidly in a diffusion controlled reaction to form $ONOO^-$. O_2^- can be generated in addition to dysfunctional cNOS by several sources, especially by

***Corresponding author:** Tadeusz Malinski, Nanomedical Research Laboratories, Ohio University, 350 W. State Street Athens, OH 45701, USA, Tel: 740-274-1625; Fax: 740-597-1772; E-mail: malinski@ohio.edu

Received June 04, 2014; **Accepted** July 25, 2014; **Published** July 30, 2014

Citation: Heeba G, Corbalan JJ, Patton S, Burewicz A, Stys AT, et al. (2014) Nanomedical Approach to Monitor the Central Role of NO/ONOO$^-$ Imbalance in Ischemic Stroke Brain Damage – The Effects of Statins and Heme Oxygenase-1. J Nanomed Nanotechnol 5: 215. doi: 10.4172/2157-7439.1000215

NADPH oxidase. Therefore, the final balance/imbalance between NO and ONOO$^-$ can be affected by several sources of these molecules. Electrochemical nanosensors (length 4 to 5 μm, diameter 200-300 nm) were used to measure the concentration of NO and ONOO$^-$ released from the brain. With a time resolution better than 10 μs and detection limit of 1 nmol L^{-1}, these nanosensors are unique to monitor *in situ* and real time release of NO and ONOO$^-$ *in vivo*, *ex vivo* and *in vitro* (both from adherent cells and in suspension) in nanomedical, pharmacological, physiological and toxicological studies [17-21]. The abnormally high ONOO$^-$ concentration, which may be associated with ischemia can entail two negative consequences: exposure of brain to neurotoxic ONOO$^-$ and consumption of neuroprotective NO [16]. The ratio of maximal NO to maximal ONOO$^-$ concentrations, [NO]/[ONOO$^-$], was used in this study as a marker of the uncoupling and dysfunction of the cNOS in ischemic stroke. At high levels of NO and/or low levels of ONOO$^-$, this ratio is high (>2.0) indicating normal function of coupled cNOS. A low [NO]/[ONOO$^-$] ratio (<1.0) is associated with uncoupled, dysfunctional cNOS and NADPH oxidase [16].

Heme oxygenases (HO) are enzymes that catalyze the degradation of heme to form the antioxidant bilirubin, the vasodilator carbon monoxide (CO), and iron. HO-1 is inducible, but HO-2 and HO-3 are constitutively expressed [22]. Because of the antioxidant, neuroprotective, and anti-inflammatory functions of HO-1, this enzyme may augment oxidative defense mechanisms, which may be compromised by cerebral ischemia [23]. Statins also stimulate heme oxygenase-1 (HO-1) expression in endothelial and vascular smooth muscle cells [24]. The study presented here describes an application of a nanomedical system to simultaneously monitor changes of concentration of NO, ONOO$^-$ and their relation to ischemic stroke brain damage, cNOS expression and HO-1 expression and the effect of statins. The *in situ* measurement with nanosensors smaller than 300 nm allows the simultaneous monitoring of NO and ONOO$^-$ concentrations in the ischemic brain with a time resolution of about 10 μs and detection limit of 10^{-9} molL^{-1}. This study established for the first that a [NO]/[ONOO$^-$] imbalance in stroke is the crucial factor affecting the severity of brain injury. Statins and HO-1 partially restored [NO]/[ONOO$^-$] balance and mollified the brain injury.

Methods

Reagents

All reagents used in our studies were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise specified. Atorvastatin were purchased from Pfizer, Inc. (New York, NY) and simvastatin from Merck & Co., Inc. (Rahway, NJ).

Atorvastatin was dissolved in dimethyl sulfoxide and further diluted down in sterile PBS. Simvastatin was chemically activated by alkaline hydrolysis before administration as indicated by the manufacturer. Briefly, simvastatin was dissolved in ethanol; then, 0.1 molL^{-1} sodium hydroxide was added and the mixture was heated for 2 h at 50°C and finally neutralized to a pH of 7.4 with 0.1 molL^{-1} hydrochloric acid. Statin doses used in this study were based on previous studies [3,4,6].

Experimental design

All procedures used in this study were approved by the Ohio University Animal Care Committee (H07-08). In these experiments we used adult male Sprague Dawley (SD) rats weighing 250-300 g (Harlan Industries, Indianapolis, IN). Rats were divided into five experimental groups (18 rats per group) and subcutaneously injected once per day with 0.7 mL kg^{-1} of atorvastatin (10 mg kg^{-1} day^{-1}), or activated simvastatin (20 mg kg^{-1} day^{-1}), or a corresponding volume of sterile PBS (control). Another two rat groups received L-N^G-Nitroarginine methyl ester (L- NAME; 15 mg kg^{-1} day^{-1}), a cNOS inhibitor, in their drinking water along with atorvastatin or simvastatin treatment. After 14 days of experimental drug or PBS (control) pre-treatment, all rats were subjected to pMCAO with the use of the intraluminal monofilament method previously described [25]. Briefly, rats were anesthetized with an intramuscular injection of a mixture of 50 mg kg^{-1} ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg kg^{-1} xylazine (Vedco Inc., Saint Joseph, MO). Brain ischemia was induced with a 4-0 nylon monofilament with silicone rubber coating (Doccol Co., Redlands, CA). The filament was introduced into the external carotid artery, navigated into the internal carotid artery, and advanced to the anterior cerebral artery, occluding the origin of the middle cerebral artery. Rectal temperature was maintained at 37°C with a regulated heating pad and the left femoral artery was cannulated for arterial blood pressure measurements. After 3 h, 12 h, or 24 h of pMCAO rats were euthanized by decapitation. Thereafter, rat brains were removed and sliced into seven serial coronal sections of 2 mm thickness. Brain slides from 6 non-pre-treated SD rats that were not given pMCAO were used to measure [NO] and [ONOO$^-$] in non-ischemic brain.

Cerebral infarct volume by microscopy

Each slice was incubated for 60 min at 37°C in saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC), and immediately fixed by immersion in 10% formalin. The images of the stained sections were captured by digital camera (DC210, Kodak). The areas of infarction were measured in each slice using a computerized image analysis system (ImageJ, National Institute of Health, USA). The infarct volume was calculated by summation of the infarct area in each slice multiplied by the thickness of the slice and presented as percentage of infarct volume of the vehicle-treated (control) group.

eNOS, nNOS and HO-1 expressions by Western blotting

After 14 days of experimental drug or PBS (control) pre-treatment, rats were anesthetized, and pMCAO induced for 3 h, 12 h or 24 h. Thereafter, rats were euthanized by decapitation and brains removed and homogenized. Equal amounts of protein were resolved on a ready-to-use 10% polyacrylamide gel. Immunoblotting was performed with the use of an eNOS, nNOS or HO-1 monoclonal antibody (Calbiochem, Billerica, MA) diluted in non-fat milk/PBS. The membrane was subsequently probed with a secondary anti-mouse horseradish peroxidase antibody (GE Healthcare, Pittsburgh, PA) at a dilution of 1:2,000 and developed with chemiluminescence. β actin was used as an internal control. ImageJ was used for analyses.

Measurement of NO and ONOO$^-$ concentration with nanosensors

Concurrent, *ex-vivo* and real time measurements of NO and ONOO$^-$ were performed with electrochemical nanosensors. Their design was based on previously developed, well- characterized, chemically modified carbon-fiber technology [26]. Each of the nanosensors was made by depositing a sensing material on the tip of a carbon fiber (length 4 to 5 μm, diameter 200-300 nm). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. Conductive films of polymeric nickel(II) tetrakis (3-methoxy-4-hydroxyphenyl)porphyrin and polymeric manganese(III)- [2,2]paracyclophenylporphyrin were used for the NO and ONOO$^-$ sensors, respectively.

The amperometric method provided a quantitative signal (current) that is directly proportional to changes in [NO] or [$ONOO^-$]. Amperometric measurements were performed with a Gamry Reference 600™ dual potentiostat (Gamry instruments, Warminster, PA). The differential pulse voltammetry current at the peak potential for NO and $ONOO^-$ was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. For each set of analyses, linear calibration curves (current versus concentration) were constructed for each sensor from 50 nM to 400 nM before and after measurements with aliquots of NO and $ONOO^-$ standard solutions. NO and $ONOO^-$ standard solutions were prepared according to previously described methods [27].

A brain slice was placed in a dish and immersed in oxygenated physiological saline. A module of NO and $ONOO^-$ nanosensors was placed in close proximity (5 ± 2 µm) to the surface of the striate area of the brain with the help of a remote controlled micromanipulator (Sensopex, Finland) and a microscope fitted with a CCD camera (AM Scope, Irvine, CA). To stimulate maximum NO and $ONOO^-$ release, the receptor-independent cNOS agonist, calcium ionophore A23187 (CaI, 1 µmolL^{-1}), was injected into the organ chamber using a microinjector. NO and $ONOO^-$ production were also modulated by pre-incubation (15 minutes) of brain slides with L-NAME (100 µmolL^{-1}), a selective nNOS inhibitor (7-nitroindazol , 7-NI; 10 µmolL^{-1}), a selective NADPH oxidase inhibitor (VAS2870; 10 µmolL^{-1}), a precursor of cNOS cofactor tetrahydrobiopterin (sepiapterin; 200 µmolL^{-1}) and the polyethylene glycol, covalently linked to superoxide dismutase (PEG-SOD, 400 U/mL).

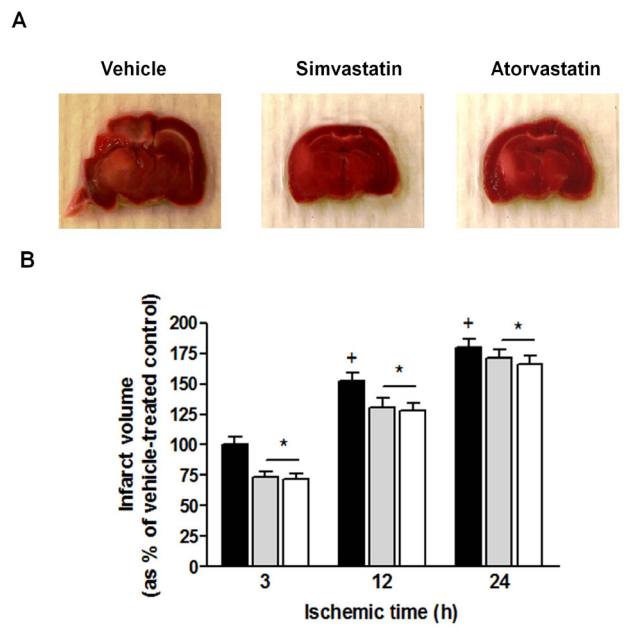

Figure 1: Evolution of focal cerebral ischemia after pMCAO in rats pre-treated with vehicle (PBS) or statins (20 mg·kg^{-1} simvastatin or 10 mg·kg^{-1} atorvastatin daily for 14 days). **A,** Representative images of coronal brain sections pre-treated with vehicle or statins, and taken after 12 h of pMCAO. **B**, Quantitative changes in infarct volume measured at different times of pMCAO and showing the effects of pre-treatment with vehicle (closed bars), simvastatin (gray bars) or atorvastatin (open bars).The percentage of infarct volume at each time is normalized versus the infarct volume (100%) measured after 3 h of untreated ischemia. All values are mean ± SEM of n=6. *P<0.05 vs. vehicle-pre-treated group, $^+$P<0.05 vs. vehicle-pre-treated group after 3 h of pMCAO.

Data analysis and statistical procedures

Data are expressed as mean ± standard error of the mean (SEM), with a value of P<0.05 considered statistically significant. Statistical analysis of the mean difference between multiple groups was determined by one-way analysis of variance and between two groups by two-tailed Student's *t*-tests. All statistical analyses were performed using GraphPad Prism (v 5.00 for Windows; GraphPad Software, San Diego, CA) and Origin (v 6.1 for Windows; OriginLab, Northampton, MA).

Results

Effect of statins on cerebral infarct volume

Representative examples of brain slices (12-hour ischemia) obtained from the PBS (vehicle) or statin pre-treated SD rats are showed in Figure 1A. The TTC method led to normal brain areas stained deep red and unstained infarct tissue. Brain slices of vehicle pre-treated rats exhibited hemispheric swelling and extensive brain infarct involving both cortex and striatum. Figure 1B shows the total volume of infarction normalized versus 3 h of pMCAO and statin pre-treated groups are expressed as a percentage of vehicle pre- treated control group. Statin pre-treatment reduced ischemic infarct by about 27% or 15% after 3 h or 12 h of pMCAO respectively, as compared to the vehicle pre-treated control group. In contrast, statin pre-treatment led to a very small reduction of infarct volume (about 5%) after 24 h of

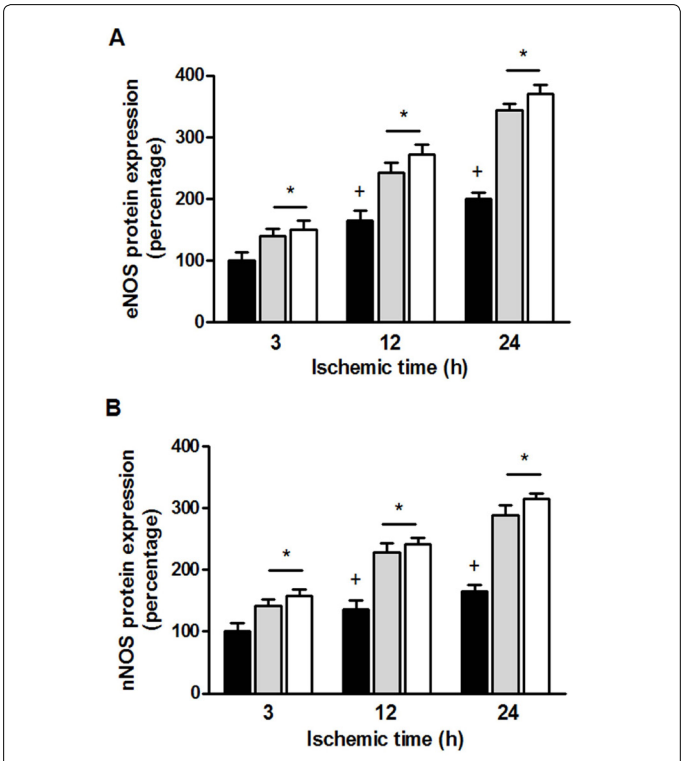

Figure 2: cNOS protein expression from ischemic brain tissue of vehicle or statin (20 mg kg^{-1} simvastatin or 10 mg·kg^{-1} atorvastatin daily for 14 days) pre-treated rats. Quantification of the expression of eNOS (A) or nNOS (B) level from ischemic brain tissue of vehicle (closed bars), simvastatin (gray bars) or atorvastatin (open bars)-treated groups. The percentage of eNOS and nNOS expression at each time is normalized versus the expression (100%) measured after 3 h of untreated ischemia. All values are mean ± SEM of n=6. *P<0.05 vs. vehicle-pre-treated group, $^+$P<0.05 vs. vehicle-pre-treated group after 3 h of pMCAO.

pMCAO. The reduction in infarct size was similar for simvastatin and atorvastatin treatment.

Effect of statins on cNOS expression

eNOS and nNOS protein expression increased with time of pMCAO (Figure 2). When compared with vehicle pre-treated controls, statin pre-treatment additionally elevated expression of both cNOS enzymes. The percentage of change in eNOS and nNOS expression from the PBS pre-treated control group is shown in Figure 2. The increase in the expression of cNOS was similar for simvastatin and atorvastatin.

[NO]/[ONOO$^-$]balance/imbalance in ischemic brain

Typical amperometric curves (NO or ONOO$^-$ concentration proportional to amperometric current *vs*. time) are presented in Figure 3. The NO concentration represents the total bioavailable NO release produced by both eNOS and nNOS. NO release was observed 200-300 ms after stimulation with CaI, the receptor independent cNOS agonist, and reached maximum after about 0.9-1.2 seconds. The release of NO was followed by the increase of ONOO$^-$ with the maximal concentration being recorded after 1.2-1.4 seconds. Maximal NO and ONOO$^-$ concentrations stimulated with CaI are shown in Figure 4. In a non-ischemic brain, the maximal NO was 400 ± 20 nmolL^{-1} and ONOO$^-$ was 150 ± 9 nmolL^{-1}. In the control ischemic group, after 3 h of pMCAO, the maximal concentration of NO was reduced significantly to 217 ± 11 nmolL^{-1} and maximal concentration of ONOO$^-$ increased to 244 ± 9 nmolL^{-1}. Treatment with statins partially restored the maximal level of NO (244 ± 10 nmolL^{-1} simvastatin and 257 ± 14 nmolL^{-1} atorvastatin) and significantly reduced the maximal level of ONOO$^-$

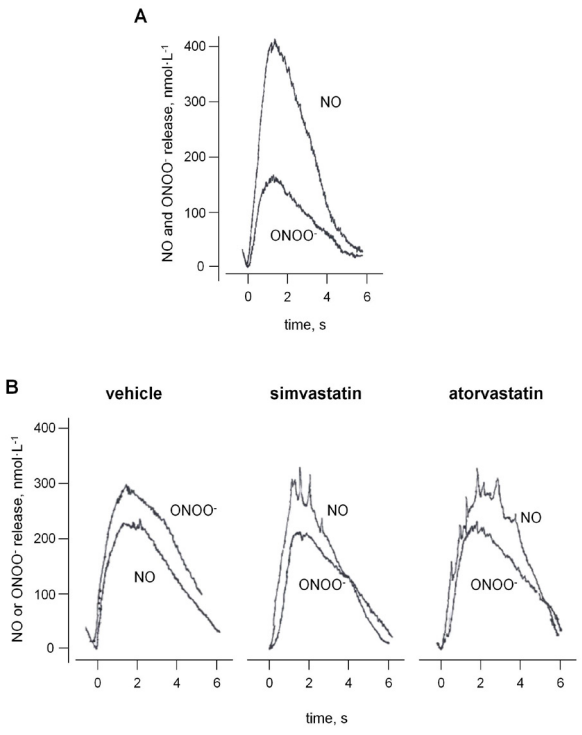

Figure 3: Typical amperograms showing changes in NO and ONOO$^-$ concentration with time after stimulation with calcium ionophore (1 μM) in non-ischemic (**A**) and ischemic (**B**) brain slides. Brain slides were obtained after 12 h of pMCAO from SD rats pre-treated with vehicle (PBS) or statins (20 mg·kg^{-1} simvastatin or 10 mg·kg^{-1} atorvastatin daily for 14 days).

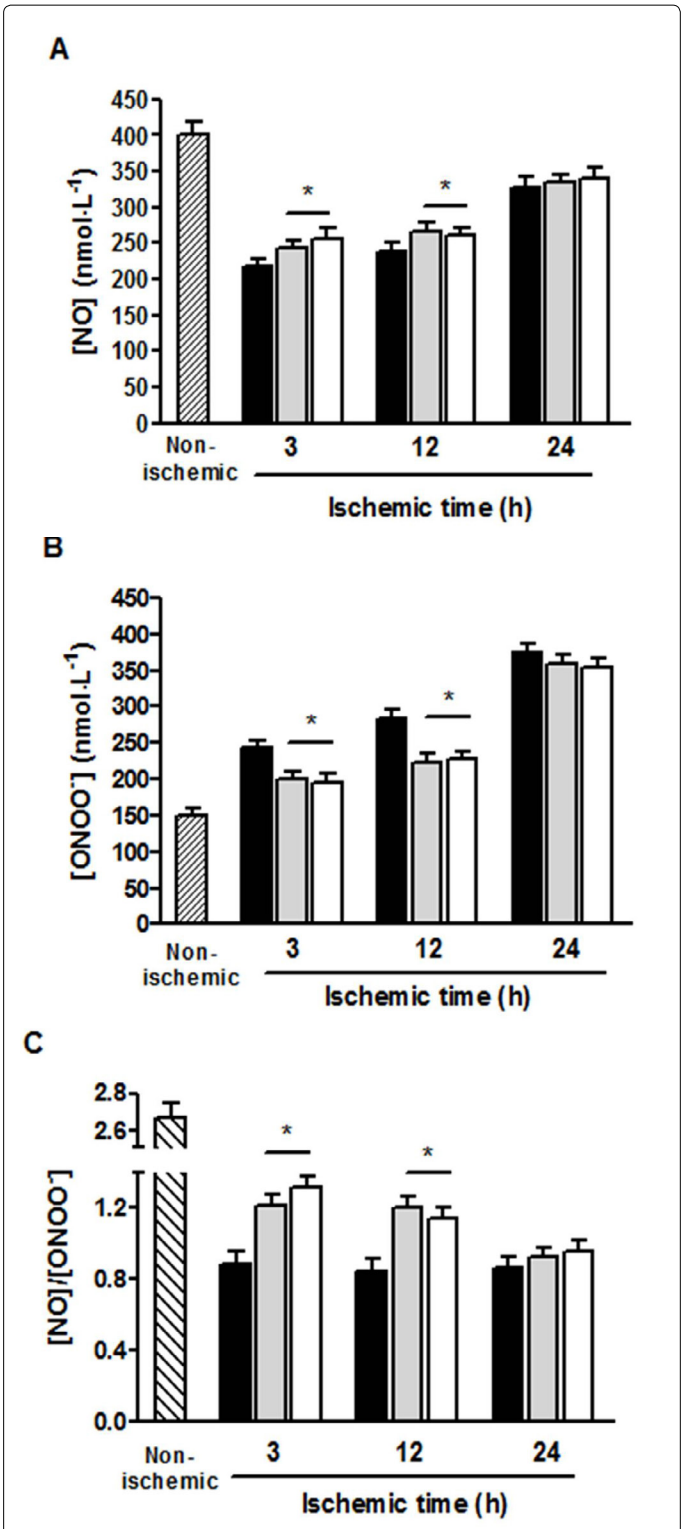

Figure 4: Calcium ionophore (1 μM)-stimulated maximal NO (**A**) and maximal ONOO$^-$ (**B**) release (maximal amperogram amplitude) from isolated brain tissues of SD rats treated with vehicle (closed bars), simvastatin (20 mg·kg^{-1} daily for 14 days, gray bars) or atorvastatin (10 mg·kg^{-1} daily for 14 days, open bars). Brain slides were obtained after 3 h, 12 h and 24 h of pMCAO. The concentration of NO and ONOO$^-$ represent maximum production from cNOS (eNOS and nNOS). **C** is the ratio of maximal NO to maximal ONOO$^-$ concentrations of the above corresponding groups. *P<0.05 vs. vehicle-pre-treated group.

(200 ± 9 nmolL^{-1} simvastatin and 194 ± 13 nmolL^{-1} atorvastatin). The increases in maximal NO concentration and the decrease in maximal $ONOO^-$ were observed with statin pre-treatment and 3 h or 12 h of pMCAO.

The ratio of [NO]/[$ONOO^-$] is shown in Figure 4C. This ratio reflects on uncoupling of eNOS or nNOS and reflects on the dysfunction of endothelial cells and neurons. After 3 h of pMCAO, the ratio was 0.89 ± 0.07 for PBS vehicle pre-treated control group, while treatment with statins significantly increased this ratio to1.22 ± 0.06 for simvastatin and 1.32 ± 0.06 for atorvastatin. However, pre-treatment with statins and 24 h of pMCAO produced insignificant changes of maximum [NO] and [$ONOO^-$] compared to PBS vehicle pre-treatment controls (0.93 ± 0.05 for simvastatin and 0.96±0.06 for atorvastatin vs 0.87 ± 0.6 for controls).

Effect of statins on HO-1 expression and the role of [NO]/[$ONOO^-$] balance on this expression

The expression of HO-1 protein in PBS vehicle and statin pre-treated groups was investigated in the brain tissue after 3 h, 12 h and 24 h of pMCAO using Western blotting. The expression of HO-1 increased significantly with the time of duration of ischemia and was about 60% higher after 24 h than after 3 h. Treatment with statins resulted in a small but significant increase in HO-1 expression when compared to vehicle-treated control group (Figure 5). After 3 h of pMCAO, HO-1 expression increased about 24% (simvastatin) and 30% (atorvastatin) as compared to the vehicle-treated group.

We investigated whether a change in NO and $ONOO^-$ production can affect a statin-induced HO-1 expression. Rats were pre-treated with statins in the presence and absence of non-selective NOS inhibitor (L-NAME) for 14-days. Figure 6A shows the changes of HO-1 expression in ischemic rats, treated with statins in the presence or absence of L-NAME.

L-NAME treatment alone increased [NO]/[$ONOO^-$] ratio from about 0.8 to 1.2 (3h ischemia). Both, simvastatin and atorvastatin decreased the expression of HO-1 in the brain of L-NAME treated SD rats.

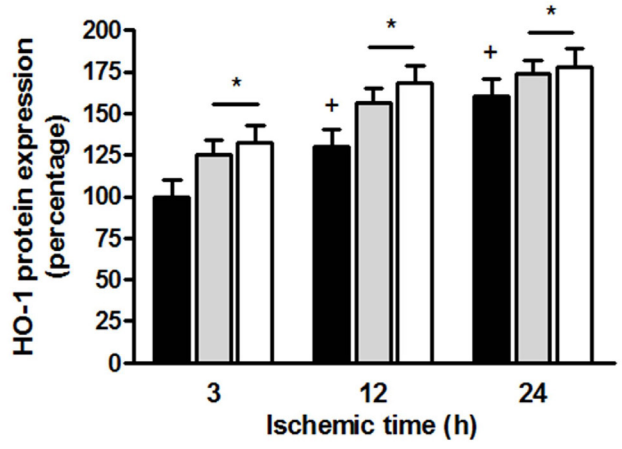

Figure 5: Expression of HO-1 protein level from ischemic brain tissue of PBS-treated vehicle (closed bars) or statin (20 mg·kg^{-1} simvastatin or 10 mg·kg^{-1} atorvastatin daily for 14 days)-treated rats after 3, 12 and 24 h of pMCAO. The percentage of HO-1 expression at each time is normalized versus the expression (100%) measured after 3 h of untreated ischemia. All values are mean ± SEM of n=6. *P<0.05 vs. vehicle-pre-treated group after 3h of pMCAO.

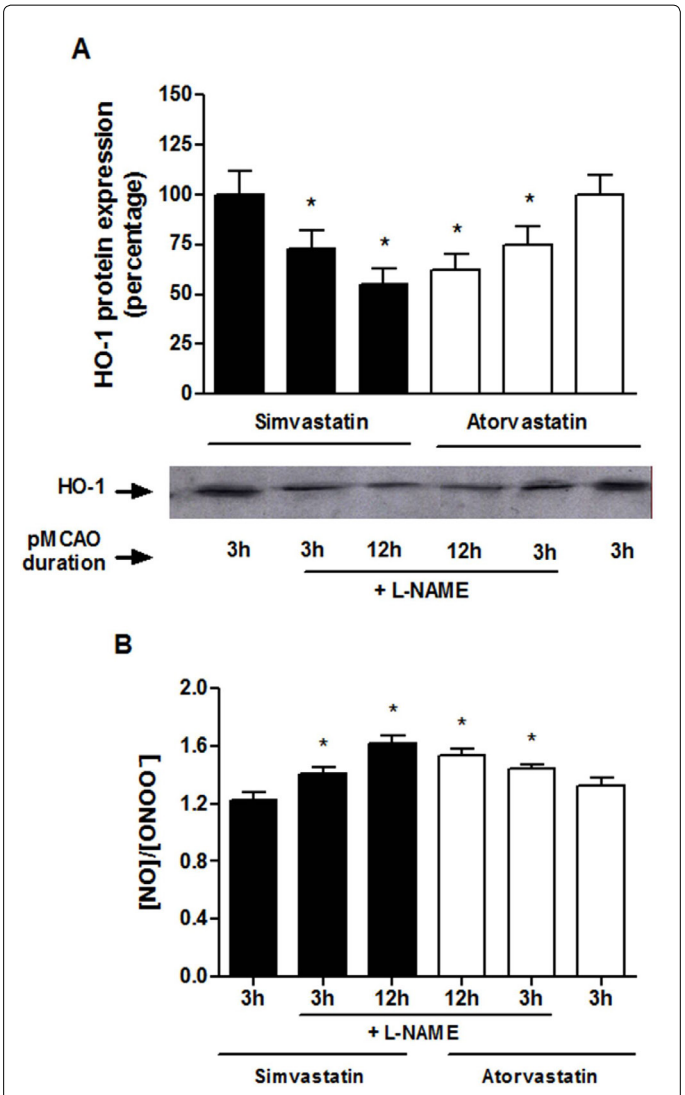

Figure 6: (**A**) Immunoblots showing the expression of HO-1 protein level from ischemic brain tissue of simvastatin (20 mg kg^{-1} daily for 14 days, closed bars) or atorvastatin (10 mg kg^{-1} daily for 14 days, open bars) with or without L-NAME (15 mg·kg^{-1} daily for 14 days)-treated groups after 3 h and 12 h of pMCAO. (**B**) [NO]/[ONOO] from ischemic brain tissue of simvastatin (closed bars) or atorvastatin (open bars) with or without L-NAME-treated groups after 3 h and 12 h of pMCAO. All values are mean ± SEM of n=6. *P<0.05 vs. 3 h of pMCAO of either simvastatin- or atorvastatin-treated group.

Effect of Modulators of cNOS and NADPH oxidase on [NO]/[ONOO-] balance

To elucidate the basis for decreased NO bioavailability and elevated $ONOO^-$ in ischemic stroke (3 h of pMCAO) we compared the concentration of NO and $ONOO^-$ produced in the presence of modulators of eNOS and nNOS function. As expected, in the presence of L-NAME, a nonselective inhibitor of both eNOS and nNOS, the concentration of NO decreased by about 75% while $ONOO^-$ decreased more than 80% (Figure 7). 7-NI, a selective inhibitor of nNOS, decreased NO and $ONOO^-$ by about 35%. In the presence of VAS2870, an inhibitor of NADPH oxidase, NO level increased by about 40% with subsequent decrease of $ONOO^-$. A similar increase in NO with a subsequent decrease of $ONOO^-$ was observed in the presence of sepiapterin, a precursor of tetrahydrobiopterin and also in the presence

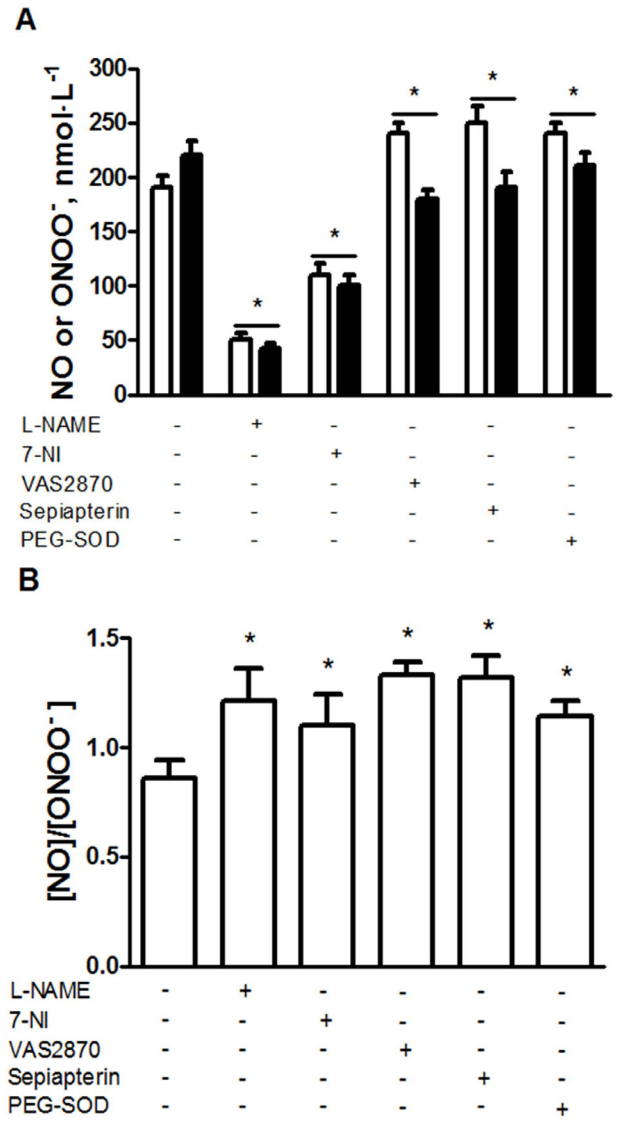

Figure 7: (**A**) Calcium ionophore (1 μM)-stimulated maximal NO and maximal ONOO$^-$ release from isolated brain tissues of rats treated with simvastatin (20 mg·kg^{-1} daily for 14 days, open bars) or atorvastatin (10 mg·kg^{-1} daily for 14 days, close bars). Brain slides were obtained after 3 h, 12 h and 24 h of pMCAO. Brain slides were pre-incubated (15 minutes) with L-NAME, 7-NI, VAS2870, sepiapterin or PEG-SOD prior to stimulation of NO and ONOO$^-$ release. **C** is the ratio of maximal NO to maximal ONOO$^-$ concentrations of the above corresponding groups. *P<0.05 vs. vehicle-pre-treated group.

of membrane permeable, superoxide dismutase PEG-SOD. A favorable change in the balance of [NO]/[ONOO$^-$] was observed in the presence of modulators of cNOS function. The inhibition of eNOS and nNOS decreased both NO and ONOO$^-$ production, however the decrease in ONOO$^-$ was more pronounced than NO. Therefore the overall effect of inhibition of cNOS by L-NAME favorably shifts [NO]/[ONOO$^-$] balance (Figure 7).

Discussion

The main finding of this study is that the cellular redox balance/imbalance between NO and ONOO$^-$ (but not the absolute level of NO or ONOO$^-$ concentration) plays a pivotal role in the damage of the brain during ischemic stroke. Also, [NO]/[ONOO$^-$] balance is a measure of cNOS uncoupling and a determinant of changes in the expression of eNOS, nNOS and HO-1. An elevated level of eNOS and nNOS in ischemic brain provides the potential for higher production of the cytoprotective, vasorelaxant NO. However, under ischemic conditions, eNOS and nNOS enzymes are highly uncoupled and become the generators, not only of NO, but concomitantly O_2^-. NO and O_2^- can react in rapid diffusion controlled reaction to produce ONOO$^-$. Therefore, the balance between the bioavailable, cytoprotective NO and the cytotoxic ONOO$^-$, may be significantly shifted under the condition of [NO]/[ONOO$^-$] ratio dropping below 1.0. The [NO]/[ONOO$^-$] imbalance, below 1.0, can impose a significant oxidative stress in the brain, during an ischemic event, in both endothelial cells and neurons [18,28]. At low [NO] and high [ONOO$^-$], the redox environment is mainly controlled by the ONOO$^-$ with little NO protection. ONOO$^-$ is a more powerful oxidant than NO or O_2^-. Therefore, ONOO$^-$ at high concentrations with miniscule levels of NO may trigger a cascade of events leading to significant change of the cellular environment, including oxidation of DNA and lipoproteins, activation or inactivation of some enzymes, as well as, oxidation of cNOS cofactors like tetrahydrobiopterin. Also, a severe [NO]/[ONOO$^-$] imbalance can trigger apoptosis and necrosis. The study reported here clearly indicates that, in the ischemic brain, there is a reverse correlation between the size of the brain infract and the ratio of [NO]/[ONOO$^-$]. The [NO]/[ONOO] imbalance affects not only brain damage but also the elevation of protein expression of cNOS and HO-1.

Based on these findings, one can speculate that any efficient process of the prevention, mollification, of the ischemic brain injury should involve pre-treatment/treatment which would lead to the increase of bioavailable NO and/or to decrease the ONOO$^-$ level so the favourable balance in [NO]/[ONOO$^-$] can be efficiently maintained or restored. One of the possible self-defense mechanisms against ischemia is based on the restoration of oxygen supply by the increase of bioavailable NO. This can be achieved by the increase in eNOS and nNOS protein expression. However, under ischemic conditions, the increase in cNOS expression is proven to be a self-destructive process. This is because a large cNOS protein expression requires a high level of substrates (L-arginine and oxygen) and both are in short supply (especially oxygen) under ischemic conditions. Also, the process of NO production requires a high concentration of cNOS cofactors, like tetrahydrobiopterin, which can be rapidly oxidized by both O_2^- and ONOO$^-$ in the ischemic brain. A deficiency of substrates and/or cofactors can cause cNOS uncoupling and dysfunction associated with a low level of bioavailable NO and high production of ONOO$^-$. Therefore, in ischemia, uncoupled cNOS may produce high levels of O_2^-, which converts NO, to cytotoxic ONOO$^-$. Therefore, uncoupled cNOS not only produces ONOO$^-$, but concomitantly reduces the level of bioavailable NO, exponentially decreasing [NO]/[ONOO$^-$] balance. Under ischemic conditions, this is a self-destructive biological process leading to severe [NO]/[ONOO$^-$] imbalance and generation of a highly aggressive redox environment which initiates a cascade of oxidation processes in the biological milieu, eventually leading to apoptosis, necrosis and irreversible brain damage. Paradoxically, the increase in cNOS was supposed to be a natural positive response to ischemic insult; however the contrary is true, it becomes a negative response that, in fact, further contributes to brain damage.

We showed here that the degree of cNOS uncoupling can be changed with several possible interventions. L-NANE decreased total NO production but increased cNOS coupling, leading to a more significant decrease in ONOO$^-$ production. This resulted in a small but significant increase in bioavailable NO and most importantly in

the overall increase in [NO]/[ONOO$^-$] ratio. Also, in the presence of sepiapterin, which is a precursor of tetrahydrobiopterin, the uncoupled cNOS was partially restored following the favorable shift in [NO]/[ONOO$^-$] balance. Our previous study described in detail the kinetics of stimulated NO release by cNOS [19]. Generally, the production of NO is always accompanied by the production of ONOO$^-$. With slow and low NO release, the uncoupling of cNOS is minimized, as well as, the production of O_2^- and ONOO$^-$. This is due to the fact that the process of NO release requires a subsequent fast transport of substrates and cofactors to cNOS. This transport is based on the diffusion process, limited by the concentration gradient. Therefore, with rapid production of NO, as observed during initial phase of ischemia, the diffusion controlled transport of substrates and/or cofactors can be too slow to stabilize cNOS coupling Also, during prolonged ischemia, the availability of cNOS substrates and cofactors will be substantially reduced.

In the presence of 7NI, a selective nNOS inhibitor, the total concentration of NO decreased by about 35%. Therefore, one can assume that nNOS delivers a relatively small amount to the pool of NO generated during ischemia. cNOS is one, but not the only, generator of O_2^- in endothelial cells. Another major source of O_2^- in the brain can be NADPH oxidase. VAS2870, a selective inhibitor of this enzyme, increased NO production in the ischemic brain tissue by about 40% with concomitant reduction in ONOO$^-$ concentration by about 37%. An analysis of the different contributing sources for the total content of bioavailable NO and ONOO$^-$ in the brain during the first 3 hours of the ischemic event, we found that most (about 60%) of NO is produced by eNOS. We estimated that inducible NOS probably supplies less than 5% of NO to the total pool, which may be produced during the initial period (3 h) of ischemia.

Based on the study presented here, showing the central role of [NO]/[ONOO$^-$] imbalance on brain injury in ischemic stroke, one can propose a strategy to mollify ischemic damage. This strategy includes interventions to limit the generation of O_2^- and ONOO$^-$ and/or increase the level of bioavailable NO. The favorable shift (an increase) in [NO]/[ONOO$^-$] balance can be potentially achieved by an increase in cNOS coupling, an increase in cNOS expression, scavenging of ONOO$^-$, dismutase/scavenging of O_2^- or by the delivery of NO from other sources than cNOS. Among these different possibilities, the stabilization, or restoration/coupling of cNOS, may be the most effective. We have shown here that the inhibition of cNOS can increase the coupling of the enzymes involved and can favorably shift the [NO]/[ONOO$^-$] balance. Also, pretreatment with statins can significantly increase [NO]/[ONOO$^-$] ratio and mollify a brain injury by increasing NO production and simultaneously decreasing ONOO$^-$. This is a significant finding of great importance.

Statins improved the [NO]/[ONOO$^-$] and reduced the extent of brain damage during ischemic insult and this protective effect of statins was dependent on the duration of ischemia. Longer time (24 h) of an ischemic event decreases the chance of protection by statins. The cyto-/neuroprotective effect of statins during ischemic stroke is based on the restoration of cNOS coupling and functional activity, which in turn increases [NO]/[ONOO$^-$] balance. Experimental investigations carried out on animal models of cerebral ischemia have suggested the beneficial role of statins in stroke [29,30]. But, the molecular mechanisms underlying this protective action were not completely understood. It was also suggested that this mechanism may differ according to the type of insult (global, focal, transient or permanent) [29]. Based on our study, one can suggest that the protection mechanism of statins is probably the same for any type of ischemic insult. However, the degree of cNOS uncoupling and the level of [NO]/[ONOO$^-$] imbalance may vary among different types of ischemic insult, as can the protective effects of statins. Previous reports indicated that the neuroprotective effect of statins was dependent on the treatment duration (7, 14 and 28 days) and also it was dependent on the dose of statins used [2,31].

This study confirmed that treatment with statins increased HO-1 expression, as compared to the PBS vehicle-treated control group. Previous studies demonstrated that statins enhance *in vitro* HO-1 expression in human endothelial and vascular smooth muscle cells [24,32]. Another study reported that statins induce *in vivo* expression of HO-1, which appears to be a class effect of statins, and occurs not only in vasculature but also extra-vascularly [33].

It has been demonstrated that up-regulation of HO-1 expression renders endothelial cells resistant to oxidative stress by increasing other antioxidant proteins, including SOD and catalase which in turn, decreases O_2^- production [34]. Moreover, up-regulation of HO-1 gene expression was shown to decrease the availability of the heme-mediated activation of NADPH oxidase and O_2^- generation [35]. This could explain the antioxidant properties of the HO-1 and the beneficial effects observed in different conditions such as stroke. Suggestion also has been made that, HO-1 may augment the oxidative defense mechanism and pharmacological stimulation of HO-1 activity may constitute a novel therapeutic approach in the amelioration of ischemic injury during the acute period of stroke [36]. Moreover, it was demonstrated that HO-1-transgenic mice exhibited resistance to the neuro-degeneration induced by focal ischemia, and neurons over expressing HO-1 inhibited oxidative stress-induced cell death [37,38].

Importantly, the data from our study revealed that, the expression of HO-1 was reduced with L- NAME co-treated with statins when compared to treatment with statins alone. Since L-NAME blocks cNOS activity with subsequent decrease in NO release, but also with a substantial increase in [NO]/[ONOO$^-$] ratio, the decrease in HO-1 expression in combined pre-treatment with L-NAME and statins suggests that [NO]/[ONOO$^-$] might act as a signaling system for a change of the expression of this enzyme. The low [NO]/[ONOO$^-$] ratio can be associated with the increase in HO-1 expression, while the increase in [NO]/[ONOO$^-$] seems to have the opposite effect.

Conclusions

A rapid, dynamic, shift of balance between the concentration of cytoprotective NO and the concentration of cytotoxic ONOO$^-$, directly correlates with the infarct volume and brain damage during ischemic stroke. The ratio of [NO] to [ONOO$^-$] reflects on, and is a measure of, cNOS uncoupling, cNOS dysfunction and the level of oxidative/nitroxidative stress. High [NO]/[ONOO$^-$] balance is the determinant in preventing brain damage, while [NO]/[ONOO$^-$] imbalance triggers an increase in the protein expression of cNOS, which contributes to a more severe imbalance of [NO]/[ONOO$^-$] and increases brain damage.

The increase in HO-1 expression observed at low [NO]/[ONOO$^-$] ratio has a beneficial effect on the reduction of nitroxidative stress and the partial restoration of [NO]/[ONOO$^-$] balance and a reduction in brain injury. The beneficial effects of simvastatin and atorvastatin on the reduction of infarct volume is based on the restoration of cNOS coupling and the favorable shift in [NO]/[ONOO$^-$] balance. This effect of statins may be superimposed with the effect of elevated HO-1 expression.

We propose here that statins may be used prophylactically to

prevent/limit ischemic damage to the brain. Also, we suggest, that the treatment or mollification of ischemic damage can be designed based on the maintenance/restoration of [NO]/[ONOO$^-$] balance to a level higher than one. This can be achieved by limiting NO production, scavenging ONOO$^-$, scavenging/dismutase of O_2^-, decreasing cNOS expression, increasing HO-1 expression, or increasing cNOS coupling by supplementation of substrate(s) and/or cofactors of cNOS enzyme.

Acknowledgements

We are grateful to Collin Arocho for his technical assistance in the preparation of this manuscript. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR-014575-01 from the National Center for Research Resources, National Institutes of Health.

References

1. Jonsson N, Asplund K (2001) Does pretreatment with statins improve clinical outcome after stroke? A pilot case-referent study. Stroke 32: 1112-1115.
2. Amin-Hanjani S, Stagliano NE, Yamada M, Huang PL, Liao JK, et al. (2001) Mevastatin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nitric oxide synthase in mice. Stroke 32: 980-986.
3. Endres M, Laufs U, Huang Z, Nakamura T, Huang P, et al. (1998) Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. Proc Natl Acad Sci U S A 95: 8880-8885.
4. Sironi L, Cimino M, Guerrini U, Calvio AM, Lodetti B, et al. (2003) Treatment with statins after induction of focal ischemia in rats reduces the extent of brain damage. Arterioscler Thromb Vasc Biol 23: 322-327.
5. Heeba G, Moselhy ME, Hassan M, Khalifa M, Gryglewski R, et al. (2009) Anti-atherogenic effect of statins: role of nitric oxide, peroxynitrite and haem oxygenase-1. Br J Pharmacol 156: 1256-1266.
6. Laufs U, Gertz K, Huang P, Nickenig G, Böhm M, et al. (2000) Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice. Stroke 31: 2442-2449.
7. Gaddam V, Li DY, Mehta JL (2002) Anti-thrombotic effects of atorvastatin--an effect unrelated to lipid lowering. J Cardiovasc Pharmacol Ther 7: 247-253.
8. Antonopoulos AS1, Margaritis M, Shirodaria C, Antoniades C (2012) Translating the effects of statins: from redox regulation to suppression of vascular wall inflammation. Thromb Haemost 108: 840-848.
9. Sessa WC (1994) The nitric oxide synthase family of proteins. J Vasc Res 31: 131-143.
10. Laufs U, La Fata V, Plutzky J, Liao JK (1998) Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 97: 1129-1135.
11. Nakata S, Tsutsui M, Shimokawa H, Yamashita K, Tanimoto A, et al. (2007) Statin treatment upregulates vascular neuronal nitric oxide synthase through Akt/NF-kappaB pathway. Arterioscler Thromb Vasc Biol 27: 92-98.
12. Kalinowski L, Dobrucki LW, Brovkovych V, Malinski T (2002) Increased nitric oxide bioavailability in endothelial cells contributes to the pleiotropic effect of cerivastatin. Circulation 105: 933-938.
13. Malinski T, Bailey F, Zhang ZG, Chopp M (1993) Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 13: 355-358.
14. Zhang ZG, Chopp M, Bailey F, Malinski T (1995) Nitric oxide changes in the rat brain after transient middle cerebral artery occlusion. J Neurol Sci 128: 22-27.
15. Huang Z, Huang PL, Ma J, Meng W, Ayata C, et al. (1996) Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab 16: 981-987.
16. Soneja A, Drews M, Malinski T (2005) Role of nitric oxide, nitroxidative and oxidative stress in wound healing. Pharmacol Rep 57 Suppl: 108-119.
17. Gerová M, Mesaros S, Kristek F, Kittová M, Malinski T (1998) NO concentration in the periendothelial area of the femoral artery of the dog measured *in vivo*. Physiol Res 47: 169-175.
18. Mason RP, Jacob RF, Kubant R, Ciszewski A, Corbalan JJ, et al. (2012) Dipeptidyl peptidase-4 inhibition with saxagliptin enhanced nitric oxide release and reduced blood pressure and sICAM-1 levels in hypertensive rats. J Cardiovasc Pharmacol 60: 467-473.
19. Mason RP, Jacob RF, Corbalan JJ, Szczesny D, Matysiak K, et al. (2013) The favorable kinetics and balance of nebivolol-stimulated nitric oxide and peroxynitrite release in human endothelial cells. BMC Pharmacol Toxicol 14: 48.
20. Corbalan JJ, Medina C, Jacoby A, Malinski T, Radomski MW (2012) Amorphous silica nanoparticles aggregate human platelets: potential implications for vascular homeostasis. Int J Nanomedicine 7: 631-639.
21. Corbalan JJ, Medina C, Jacoby A, Malinski T, Radomski MW (2011) Amorphous silica nanoparticles trigger nitric oxide/peroxynitrite imbalance in human endothelial cells: inflammatory and cytotoxic effects. Int J Nanomedicine 6: 2821-2835.
22. Otterbein LE, Soares MP, Yamashita K, Bach FH (2003) Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 24: 449-455.
23. Poss KD, Tonegawa S (1997) Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci U S A 94: 10925-10930.
24. Lee TS, Chang CC, Zhu Y, Shyy JY (2004) Simvastatin induces heme oxygenase-1: a novel mechanism of vessel protection. Circulation 110: 1296-1302.
25. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, et al. (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265: 1883-1885.
26. Malinski T, Taha Z (1992) Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358: 676-678.
27. Beckman JS, Wink DA, Crow JP (1996) Methods in Nitric Oxide Research. John Wiley & Sons Inc., New York.
28. Mason RP, Jacob RF, Corbalan JJ, Kaliszan R, Malinski T (2014) Amlodipine increased endothelial nitric oxide and decreased nitroxidative stress disproportionately to blood pressure changes. Am J Hypertens 27: 482-488.
29. Cimino M, Balduini W, Carloni S, Gelosa P, Guerrini U, et al. (2005) Neuroprotective effect of simvastatin in stroke: a comparison between adult and neonatal rat models of cerebral ischemia. Neurotoxicology 26: 929-933.
30. Kawashima S, Yamashita T, Miwa Y, Ozaki M, Namiki M, et al. (2003) HMG-CoA reductase inhibitor has protective effects against stroke events in stroke-prone spontaneously hypertensive rats. Stroke 34: 157-163.
31. Laufs U, Gertz K, Dirnagl U, Böhm M, Nickenig G, et al. (2002) Rosuvastatin, a new HMG-CoA reductase inhibitor, upregulates endothelial nitric oxide synthase and protects from ischemic stroke in mice. Brain Res 942: 23-30.
32. Uchiyama T, Atsuta H, Utsugi T, Ohyama Y, Nakamura T, et al. (2006) Simvastatin induces heat shock factor 1 in vascular endothelial cells. Atherosclerosis 188: 265-273.
33. Hsu M, Muchova L, Morioka I, Wong RJ, Schröder H, et al. (2006) Tissue-specific effects of statins on the expression of heme oxygenase-1 *in vivo*. Biochem Biophys Res Commun 343: 738-744.
34. Turkseven S, Kruger A, Mingone CJ, Kaminski P, Inaba M, et al. (2005) Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. Am J Physiol Heart Circ Physiol 289: H701-707.
35. Taillé C, El-Benna J, Lanone S, Dang MC, Ogier-Denis E, et al. (2004) Induction of heme oxygenase-1 inhibits NAD(P)H oxidase activity by down-regulating cytochrome b558 expression via the reduction of heme availability. J Biol Chem 279: 28681-28688.
36. Maines MD (2002) Heme oxygenase 1 transgenic mice as a model to study neuroprotection. Methods Enzymol 353: 374-388.
37. Panahian N, Yoshiura M, Maines MD (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J Neurochem 72: 1187-1203.
38. Chen K, Gunter K, Maines MD (2000) Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem 75: 304-313.