Published in final edited form as:

Brain Res. 2010 August 12; 1348: 174–180. doi:10.1016/j.brainres.2010.05.082.

Inhibition of gp91^{phox} contributes towards normobaric hyperoxia afforded neuroprotection in focal cerebral ischemia

Xiangqi Tang^{1,2}, Ke Jian Liu^{2,3}, Jaivijay Ramu³, Qingquan Chen², Ting Li¹, and Wenlan Liu²

¹ Department of Neurology, the Second Hospital of Xiangya Medical School, Central South University, Changsha, Hunan 410078, China

² College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131

³ Department of Neurology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131

Abstract

Oxygen therapy is a promising treatment strategy for ischemic stroke. One potential safety concern with oxygen therapy, however, is the possibility of increased generation of reactive oxygen species (ROS), which could exacerbate ischemic brain injury. Our previous study indicated that normobaric hyperoxia (NBO, 95% O₂ with 5% CO₂) treatment during ischemia salvaged ischemic brain tissue and significantly reduced ROS generation in transient experimental stroke. In this follow-up study, we tested the hypothesis that suppression of NADPH oxidase is an important mechanism for NBO-induced reduction of ROS generation in focal cerebral ischemia. Male Sprague-Dawley rats were given NBO (95% O₂) or normoxia (21% O₂) during 90-min filament occlusion of the middle cerebral artery, followed by 22.5-hour reperfusion. NBO treatment increased the tissue oxygen partial pressure (pO₂) level in the ischemic penumbra close to the pre-ischemic value, as measured by electronic paramagnetic resonance (EPR), and led to a 30.2% reduction in magnetic resonance imaging (MRI) apparent diffusion coefficients (ADC) lesion volume. Real time PCR and western blot analyses showed that the mRNA and protein expression of NADPH oxidase catalytic subunit gp91^{phox} were upregulated in the ischemic brain, which was significantly inhibited by NBO. As a consequence of gp91^{phox} inhibition, NBO treatment reduced NADPH oxidase activity in the ischemic brain. Our results suggest that NBO treatment given during ischemia reduces ROS generation via inhibiting NADPH oxidase, which may serve as an important mechanism underlying NBO's neuroprotection in acute ischemic stroke.

Keywords

NADPH oxidase; neuroprotection; oxygen; free radical; stroke

1. Introduction

Tissue hypoxia plays a critical role in the primary and secondary events leading to neuronal cell death after cerebral ischemia (Zauner et al. 2002). Therefore, improving brain tissue

oxygenation is a logical and important treatment strategy for ischemic stroke. In the past several years, we and others have demonstrated that normobaric hyperoxia (NBO) treatment effectively reduces both infarct volume and neurological deficits in rodents with ischemic stroke (Singhal et al. 2002a; Singhal et al. 2002b; Liu et al. 2004; Kim et al. 2005; Liu et al. 2006; Henninger et al. 2007; Shin et al. 2007). In human studies, NBO treatment was associated with improvement in clinical deficits and survival in selected patients with acute ischemic stroke (Singhal et al. 2005; Chiu et al. 2006). Because NBO is readily available, inexpensive and can be initiated within minutes after stroke symptom onset, the observed neuroprotective effects of NBO may have important clinical implications. However, based on observations associated with reperfusion injury, there have been some concerns that increased oxygen delivery, particularly during the phase of reperfusion after stroke, could increase the generation of oxygen free radicals, which may lead to lipid peroxidation, disruption of cell membranes, and increases in blood-brain barrier damage and brain hemorrhage. Interestingly, several recent studies demonstrated that NBO treatment salvaged ischemic brain tissue without increasing the risk of oxygen free radical injury (Singhal et al. 2002b; Kim et al. 2005; Liu et al. 2006), and in fact, the generation of free radicals was reduced (Liu et al. 2006). However, how NBO affects the generation of oxygen free radical in the ischemic brain remains largely unknown.

NADPH oxidase is a principal enzyme for the production of superoxide (Cai et al. 2003). It is expressed in neural and nonneural cell types of the central nervous system, including neurons, glia, cerebral blood vessels, monocytes, and neutrophils (Anthony et al. 1997; Bianca et al. 1999; Serrano et al. 2003; Abramov et al. 2005). NADPH oxidase is a multiunit enzyme that is composed of membrane bound subunits (p22^{phox}, gp91^{phox}), cytoplasmic subunits (p40^{phox}, p47^{phox}, and p67^{phox}), and the small G protein Rac-1 (Babior 2004). Expression of gp91^{phox}, which is the catalytic subunit of NADPH oxidase, has been shown to be upregulated in ischemic stroke (Hirabayashi et al. 2000). Conversely, gp91^{phox} mutation or inhibition of NADPH oxidase resulted in reduced cerebral infarction in the ischemic brain (Walder et al. 1997; Ostrowski et al. 2006; Wang et al. 2006). These findings implicate an important role of NADPH oxidase in neuronal damage in ischemic stroke. Using electron paramagnetic resonance (EPR) oximetry, we have recently shown that NBO treatment given during ischemia raised the penumbral interstitial pO₂ levels close to the pre-ischemic value in a rat model of transient focal cerebral ischemia (Liu et al. 2006). Furthermore, NBO treatment also significantly decreased reactive oxygen species generation in the ischemic brain. In the present study, we tested the hypothesis that NBO treatment protects against cerebral infarction via inhibition of NADPH oxidase using the same rat model of focal cerebral ischemia as described in our previous study (Liu et al. 2006).

2. RESULTS

2.1 NBO treatment increases penumbral tissue pO₂ during ischemia

Focal ischemia leads to the reduction of blood flow, and consequently, the tissue oxygenation of the affected areas. Using the EPR oximetry method that is well established in our lab to measure brain tissue pO₂ (Liu et al. 2004), we found that occlusion of the middle cerebral artery led to the reduction of cerebral tissue pO₂ from 40 mmHg to 18 mmHg in the ischemic penumbra, while NBO treatment raised the pO₂ back to the pre-ischemic level, i.e., at 38 mmHg (Figure 1B). These results further confirm our earlier report (Liu et al. 2006), and demonstrate that NBO can elevate cerebral oxygenation in the ischemic brain tissue.

2.2 NBO treatment reduces MRI ADC lesion volume in the ischemic brain

At 24 hrs after the onset of middle cerebral artery occlusion (MCAO), MRI ADCs were calculated to quantify tissue lesion volume. Typical T2 weighed (T2W) images and ADC

maps of the brains from normoxic and NBO-treated rats are shown in Figure 2A. Average ADC lesion volume was significantly lower in the NBO-treated rats ($235.7 \pm 14.8 \text{ mm}^3$, $n = 6$) compared to the normoxic rats ($374.1 \pm 36.6 \text{ mm}^3$, $n = 6$) ($P < 0.05$), representing a reduction of 37.0% (Figure 2B). These results are consistent with our previous findings that NBO treatment during 90-min MCAO significantly decreased the infarction in the ischemic brain, which was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining (Liu et al. 2006).

2.3 NBO treatment inhibits the upregulation of gp91^{phox} mRNA

Gp91^{phox}, the catalytic unit of NADPH oxidase, is upregulated and critically implicated in neuronal death during cerebral ischemia (Walder et al. 1997; Cheng et al. 2006), therefore, we examined whether NBO treatment could inhibit gp91^{phox} expression in the ischemic brain. Real time RT-PCR showed that ischemia and reperfusion significantly increased gp91^{phox} mRNA expression in the ischemic hemispheric tissue as compared to the non-ischemic hemispheric tissue (3.50 ± 0.56 versus 0.96 ± 0.04 , $n = 8$, $P < 0.05$). NBO treatment significantly inhibited gp91^{phox} mRNA upregulation in the ischemic brain tissue (2.1 ± 0.8 versus 3.5 ± 0.6 , $P < 0.05$, $n = 8$), but did not change its expression in the non-ischemic hemispheric tissue (Figure 3).

2.4 NBO treatment inhibits the overexpression of gp91^{phox} protein

We next examined whether downregulation of gp91^{phox} mRNA by NBO could result in a decreased gp91^{phox} protein expression in the ischemic brain. Western blot analysis showed that gp91^{phox} protein was expressed at a low level in the non-ischemic hemispheric tissue, but its level was remarkably elevated in the ischemic hemispheric tissue in the normoxic rats. Similar to its effect on gp91^{phox} mRNA, NBO treatment inhibited gp91^{phox} protein increase in the ischemic hemispheric tissue, but did not change its level in the non-ischemic hemisphere (Fig 4A). Gp91^{phox} protein band intensity was quantified and expressed as hemispheric gp91^{phox} ratio. The quantitative results showed that NBO significantly inhibited gp91^{phox} protein increase in the ischemic hemisphere as compared to the normoxic rats (hemispheric gp91^{phox} ratio: 4.31 ± 0.68 versus 8.04 ± 0.98 , $P < 0.05$, $n = 8$) (Fig 4B).

2.5 NBO treatment impedes NADPH oxidase activity

Our results above showed that NBO inhibited the increase of gp91^{phox} mRNA and protein expression in the ischemic hemisphere (Figure 3 and 4), we next examined whether gp91^{phox} inhibition by NBO could result in a reduction in NADPH oxidase activity. Lucigenin-enhanced chemiluminescence was used to measure NADPH oxidase activity. Since no obvious difference of gp91^{phox} expression was observed (Figure 4) in the non-ischemic hemispheres between the normoxic or NBO-treated rats, we only compared NADPH oxidase activity in their ischemic hemispheres. In consistent with the lower level of gp91^{phox} observed in the NBO-treated rats, its NADPH oxidase activity was also significantly lower than that of the normoxic rats (5054.4 ± 533.6 versus 7798.3 ± 948.7 RLU/min/mg, $P < 0.05$, $n = 6$) (Figure 5).

3. Discussion

Tissue hypoxia is a key event leading to cell death after cerebral ischemia (Zauner et al. 2002). Therefore, improving brain tissue oxygenation could be a logical and important stroke treatment strategy. A growing body of evidence has shown that normobaric hyperoxia (NBO) treatment is neuroprotective in experimental cerebral ischemia across a wide range of species including mice, rats, rabbits and baboons (Branston et al. 1976; Kaminogo 1989; Singhal et al. 2002a; Singhal et al. 2002b; Liu et al. 2004; Kim et al. 2005; Liu et al. 2006; Henninger et al. 2007; Shin et al. 2007). However, a potential safety concern with oxygen

therapy is the possibility of increased generation of reactive oxygen species (ROS), which, in the setting of ischemic stroke, could exacerbate brain edema, post-ischemic hemorrhage and neuronal death (Singhal 2007). Additionally, oxygen's effect on ROS generation in ischemic stroke is still not conclusive because controversial results have been reported about ROS production in stroke animals exposed to hyperbaric hyperoxia (HBO) (Mink and Dutka 1995; Elayan et al. 2000; Sunami et al. 2000; Oter et al. 2005) or NBO (Mickel et al. 1987; Singhal et al. 2002b; Kim et al. 2005). There are several factors potentially contributing to these inconsistent results, such as severity and duration of cerebral ischemia, timing and duration of hyperoxia treatment, oxygen pressure and concentration, and animal species. In our recent study, we showed that NBO (95% O₂ with 5% CO₂) treatment during ischemia increased brain tissue pO₂ level, but significantly reduced ROS generation in a rat model of middle cerebral artery occlusion (MCAO) (Liu et al. 2006). As a follow-up study, we herein further investigated the mechanism underlying the inhibitory action of NBO on ROS generation using the same rat model of ischemic stroke. Our results demonstrate that NBO significantly improves tissue oxygenation in the peri-infarct area and reduces infarction size. Cerebral ischemia and reperfusion induced gp91^{phox} mRNA and protein expression in the ischemic brain, which was significantly inhibited by NBO treatment. As a consequence of gp91^{phox} inhibition, NBO treatment reduced NADPH oxidase activity in the ischemic brain. These results suggest that inhibition of NADPH oxidase may be an important mechanism for NBO-induced reduction of ROS generation in focal cerebral ischemia.

ROS may be excessively generated when tissue oxygen is too high (hyperoxic status) or too low (hypoxic status) (Kulkarni et al. 2007). Consistent with our previous results (Liu et al. 2006), we found that cerebral ischemia decreased peri-infarct tissue pO₂ level to approximately 40% of the pre-ischemic level at 30 min after MCAO. NBO treatment during ischemia significantly reversed this ischemia-induced decrease of tissue pO₂ level, maintaining tissue pO₂ level close to the pre-ischemic value (Fig 1). In another study using a mouse stroke model of distal MCAO, NBO (100% O₂) treatment partially recovered brain tissue oxygenation as manifested by the increase of oxyhaemoglobin in the ischemic core and peri-infarct area (Shin et al. 2007). Under these experimental conditions, NBO treatment significantly improved the hypoxic status of the ischemic brain, but did not cause an overshoot of tissue pO₂. Therefore, it is conceivable that NBO treatment would decrease, rather than increase, ROS generation in the ischemic brain.

We further investigated the mechanism of the inhibitory action of NBO on ROS generation in cerebral ischemia. Accumulating experimental evidence indicates that NADPH oxidase is an important contributor to the oxidative damage in ischemic stroke. Several studies have demonstrated that gp91^{phox}, the catalytic subunit of this enzyme, is upregulated in ischemic stroke, and inhibition of NADPH oxidase limits infarction size and blood brain barrier disruption in different animal stroke models (Walder et al. 1997; Hirabayashi et al. 2000; Cheng et al. 2006; Ostrowski et al. 2006; Wang et al. 2006; Kahles et al. 2007; Liu et al. 2008; Chen et al. 2009; Kim et al. 2009). In addition, NADPH oxidase has been suggested as a possible oxygen sensor in cells (Schumacker 2003). Therefore, brain tissue pO₂ alternation due to ischemia and NBO treatment could change the expression and activity of NADPH oxidase. We tested this hypothesis by analyzing gp91^{phox} expression and NADPH oxidase activity in the present study. Our results indicate that NBO treatment significantly inhibited ischemia-induced upregulation of gp91^{phox} mRNA and protein expression in the ischemic brain. As a consequence of gp91^{phox} inhibition, NADPH oxidase activity was also inhibited by NBO. Given the important role of NADPH oxidase in ROS generation in the ischemic brain (Suh et al. 2008), inhibiting NADPH oxidase may represent an important mechanism underlying NBO's protection against oxidative damage in the ischemic brain, reflected as reduced 8-OHdG formation in NBO-treated rats in our earlier study (Liu et al. 2006). However, further research is necessary to precisely determine the importance of

NADPH oxidase in mediating 8-OHdG formation, as well as oxidative stress mediated signaling pathways, in transient cerebral ischemia.

In conclusion, the present study suggests that NBO therapy with 95% O₂ given during ischemia reduces ROS generation in the ischemia brain via inhibiting NADPH oxidase, which may serve as an important mechanism mediating the neuroprotective effect of NBO in acute ischemic stroke.

4. Experimental Procedure

4.1 Rat model of focal cerebral ischemia and reperfusion

All experimental protocols are approved by the Laboratory Animal Care and Use Committee of the University of New Mexico. Sprague–Dawley rats (Charles River Laboratory) weighing 290 to 320 g were anesthetized with isoflurane (4% for surgical induction, 1.75% for maintenance) in N₂:O₂ (70%:30%) during surgical procedures. Body temperature was monitored continuously with a rectal probe and maintained at 37.5°C ± 0.5°C using a heating pad. Middle cerebral artery occlusion (MCAO) was established by proximal occlusion of the right middle cerebral artery with a 4-0 silicone-coated nylon monofilament, as we previously described (Liu et al. 2008). After 90-min occlusion, reperfusion was accomplished by careful withdrawal of the monofilament and the rats were returned to their cages for 22.5 h before sacrificing. The normoxic and NBO rats were ventilated (3 liter/min) with medical air (21% O₂) or a gas mixture of 95% O₂ + 5% CO₂, respectively, during the 90-min ischemic period.

4.2 Electron paramagnetic resonance (EPR) oximetry measurement of tissue pO₂

Interstitial partial pressure of oxygen (pO₂) in the ischemic penumbra was measured using Bruker EleXsys E540 EPR spectrometer equipped with an L-band bridge (Bruker Instruments, Billerica, MA, USA) exactly as described in our previous studies (Liu et al. 2004). In brief, lithium phthalocyanine crystal (approximate diameter 0.2 mm) was placed at 0.5 mm posterior, 2 mm lateral to bregma, and 5.5 mm down from skull 72 h before induction of cerebral ischemia. Interstitial pO₂ was measured with the EPR spectrometer using the following acquisition parameters: microwave power 10 mW, microwave frequency 1.07 GHz, magnetic field center 380 G, scan range 2.0 G, and modulation amplitude at less than one-third of the EPR line-width. The lithium phthalocyanine material was a gift from Dr Harold Swartz (NIH *In Vivo* EPR Center, Dartmouth College, NH, USA). Three tissue pO₂ measurements were made for each rat at the same location before and during ischemia as indicated in Figure 1A.

4.3 Magnetic resonance imaging measurements for infarction volume

Thirty minutes before the end of reperfusion, rats were subjected to MRI analysis on a 4.7T Biospec dedicated research MR scanner (Bruker-Biospin, Billerica), equipped with 500 mT/m (rise time 80–120 μs) gradient set (for performing small animal imaging) and a small bore linear RF coil (ID 72 mm). Infarction volume was estimated by calculating the apparent diffusion coefficient (ADC) of water on ADC maps obtained with MRI, as previously described (Sood et al. 2009). In brief, a tripilot imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. T2 weighted (T2W) and diffusion weighted imaging (DWI) were acquired with the following parameters: T2W TR/TE 4s/65ms, FOV 3.2cm × 3.2 cm, matrix 256 × 128, number of slices 20, slice thickness 2 mm, number of averages 22; DWI- TR/TE 2s/50ms, number of averages 15, d = 5ms, D = 20ms, b=0 and 927 s/mm². The DWI images were slices matched to the T2 weighted images. At the end of the MRI experiments, the raw MRI data was transferred to an offline workstation. ADC maps were reconstructed from DWI using commercial software

MRVision (MRVision, Winchester, MA). Lesion size was measured using various ADC thresholds and was defined as area with ADC values <80% of the mean contralateral hemisphere values on the ADC map. The analysis was performed by a single operator and the procedure was repeated twice. Lesion size was calculated as number of pixels $\times$ pixels size, where the number of pixels correspond to pixels with values less than the ADC threshold value. Infarction volume was calculated as the sum of lesion size on each prescribed slices.

4.4 Postischemic tissue processing

Rats were sacrificed by decapitation at the end of reperfusion. Brains were quickly removed and chilled in ice-cold PBS for 5 minutes. A 8-mm thick brain region 3 mm away from the tip of the frontal lobe were then cut into four 2-mm thick coronary slices, which contained the main infarction area according to our earlier studies with 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining (Liu et al. 2004). The 2-mm thick brain slices were carefully cleaned of meninges, and then a longitudinal cut was made 2 mm away from the midline between two hemispheres to exclude tissue primarily supplied by the anterior cerebral artery. Non-ischemic and ischemic hemispheric tissue were then collected from each brain slice, and used for analyzing the protein and mRNA expression of gp91^{phox} and the enzymatic activity of NADPH oxidase.

4.5 SYBER® Green real time RT-PCR analysis for gp91^{phox} mRNA

Brain tissue collected as described above was homogenized in Trizol reagents (Invitrogen, Carlsbad, CA). Total RNA was isolated and purified using the DNA-Free kit (Ambion, Austin, TX) according to manufacturer's protocols. 1 μ g RNA was reverse-transcribed with random primers in a 20 μ l final reaction volume using TaqMan® Reverse Transcription Kits (Applied Biosystems, Foster City, CA). 1.0 μ l RT products were amplified with the 7900HT Fast Real-time PCR system (PerkinElmer Life Sciences, Boston, MA) in a 10 μ l final reaction volume in 361-well plate using SYBR® Green PCR Master Mix (Applied Biosystems). The thermal cycling condition comprised an initial incubation at 95 °C 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Oligonucleotide primers (Integrated DNA Technologies, Inc., Coralville, IA) for gp91^{phox} (Accession number: AF298656) and ribosomal protein 32 (rpl 32) (Accession number: NM_013226) were as follows: gp91^{phox}, 5'-CAGCCTGCCTGAATTTCAACT-3' (sense), 5'-GGAGAGGAGATTCCGACACACT-3' (antisense); rpl 32, 5'-AGACCTGAATGTGAAGGAAG-3' (sense), 5'-CCTTGGGATTGGTGACTCTGA -3' (antisense). No template control or melting curve analysis was used to eliminate non-specific reaction. The fluorescence threshold value (C_t value) was calculated using the 7900HT Fast Real-time PCR system software. Triplicate determinations were averaged for each data point. The relative value of mRNA expression was calculated by the comparative $\Delta\Delta C_t$ method described in the 7900HT manual. In brief, mean C_t values were normalized to the internal control rpl 32 and the difference was defined as ΔC_t . The difference between the mean ΔC_t values of compared samples was calculated and defined as $\Delta\Delta C_t$. The comparative mRNA expression level was expressed as $2^{-\Delta\Delta C_t}$.

4.6 Western blot analysis for gp91^{phox} protein expression

Non-ischemic and ischemic hemispheric brain tissue was homogenized in RIPA buffer containing protease inhibitor cocktail (Sigma). After centrifugation at 14,000 g for 15 min at 4 °C, the supernatant was stored at -80 °C until use. Protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL). Samples (75 μ g of total protein) were boiled for 5 min and then electrophoresed in 10% SDS-PAGE acrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad), and incubated for 1 hr in TBS-T (Tris-buffered saline and 0.1% Tween 20) containing 5% nonfat milk at room temperature.

Membranes were then incubated overnight with monoclonal anti-gp91^{phox} antibody (1:1000 dilution; BD Transduction Laboratories, Forest Grove, OR), washed in TBS-T, incubated for 1 hr at room temperature with HRP-conjugated anti-mouse antibody (1:1000; Santa Cruz Biotech). The protein was detected using the SuperSignal West Pico chemiluminescent kit (Pierce, Rockford, IL) according to the manufacturer's instructions, and the bands were visualized and quantified on a Kodak Image Station 4000 Digital Imaging System. To control sample loading and protein transfer, the membranes were stripped and rehybridized for β -actin (1:4000, Sigma, St. Louis, MO). Gp91^{phox} protein expression was standardized to an equivalent β -actin protein amount and expressed as a hemispheric ratio of band intensity (ischemic hemisphere versus non-ischemic hemisphere).

4.7 Measurement of NADPH oxidase activity

NADPH oxidase activity was determined based on superoxide-induced lucigenin photoemission as described previously (Ostrowski et al. 2006). Briefly, ischemic hemispheric brain tissue was homogenized in ice-cold modified Krebs HEPES buffer of the following composition (in mmol/L): (NaCl 119, HEPES 20, KCl 4.6, MgSO₄ 1.0, CaCl₂ 1.2, Na₂HPO₄ 0.15, KH₂PO₄ 0.4, NaHCO₃ 5, and glucose 5.5 (pH 7.4) with phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Sigma). To assay NADPH oxidase enzymatic activity in each sample, 20 μ L homogenate was transferred to a luminometer tube, which contained 80 μ L Krebs buffer supplemented with 6.25 μ M of lucigenin (Sigma). The reaction was initiated by the addition of 1 μ L NADPH solution (Sigma) to a final concentration of 0.1 mmol/L. Chemiluminescence counts were recorded every 30 seconds continuously for 5 min using a Turner Designs TD20/20 luminometer. The respective background counts were subtracted and chemiluminescence was expressed in relative light units (RLU) per min per mg protein.

4.8 Statistical analysis

The Data are presented as means $\pm$ SEM. Statistical analysis was performed using ANOVA and Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Acknowledgments

The work was supported in part by grants from National Institutes of Health (P20 RR15636 and R01AG031725), American Heart Association (0555669Z and 0765461Z), and PhD Programs Foundation of the Ministry of Education of China (Grant NO.20070533060).

List of abbreviations

NBO	Normobaric hyperoxia
ROS	Reactive oxygen species
MCAO	middle cerebral artery occlusion
TTC	2,3,5-triphenyltetrazolium chloride
pO₂	tissue oxygen partial pressure
EPR	electronic paramagnetic resonance
MRI	magnetic resonance imaging
T2W	T2 weighted
ADC	apparent diffusion coefficient
DWI	diffusion weighted imaging

References

- Abramov AY, Jacobson J, Wientjes F, Hothersall J, Canevari L, Duchen MR. Expression and modulation of an NADPH oxidase in mammalian astrocytes. *J Neurosci*. 2005; 25:9176–9184. [PubMed: 16207877]
- Anthony DC, Ferguson B, Matyzak MK, Miller KM, Esiri MM, Perry VH. Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. *Neuropathol Appl Neurobiol*. 1997; 23:406–415. [PubMed: 9364466]
- Babior BM. NADPH oxidase. *Curr Opin Immunol*. 2004; 16:42–47. [PubMed: 14734109]
- Bianca VD, Dusi S, Bianchini E, Dal Pra I, Rossi F. beta-amyloid activates the O-2 forming NADPH oxidase in microglia, monocytes, and neutrophils. A possible inflammatory mechanism of neuronal damage in Alzheimer's disease. *J Biol Chem*. 1999; 274:15493–15499. [PubMed: 10336441]
- Branston NM, Symon L, Crockard HA. Recovery of the cortical evoked response following temporary middle cerebral artery occlusion in baboons: relation to local blood flow and PO₂. *Stroke*. 1976; 7:151–157. [PubMed: 1265808]
- Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*. 2003; 24:471–478. [PubMed: 12967772]
- Chen H, Song YS, Chan PH. Inhibition of NADPH oxidase is neuroprotective after ischemia-reperfusion. *J Cereb Blood Flow Metab*. 2009; 29:1262–1272. [PubMed: 19417757]
- Cheng T, Petraglia AL, Li Z, Thiagarajan M, Zhong Z, Wu Z, Liu D, Maggirwar SB, Deane R, Fernandez JA, LaRue B, Griffin JH, Chopp M, Zlokovic BV. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. *Nat Med*. 2006; 12:1278–1285. [PubMed: 17072311]
- Chiu EH, Liu CS, Tan TY, Chang KC. Venturi mask adjuvant oxygen therapy in severe acute ischemic stroke. *Arch Neurol*. 2006; 63:741–744. [PubMed: 16682544]
- Elayan IM, Axley MJ, Prasad PV, Ahlers ST, Auker CR. Effect of hyperbaric oxygen treatment on nitric oxide and oxygen free radicals in rat brain. *J Neurophysiol*. 2000; 83:2022–2029. [PubMed: 10758112]
- Henninger N, Bouley J, Nelligan JM, Sicard KM, Fisher M. Normobaric hyperoxia delays perfusion/diffusion mismatch evolution, reduces infarct volume, and differentially affects neuronal cell death pathways after suture middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab*. 2007; 27:1632–1642. [PubMed: 17311078]
- Hirabayashi H, Takizawa S, Fukuyama N, Nakazawa H, Shinohara Y. Nitrotyrosine generation via inducible nitric oxide synthase in vascular wall in focal ischemia-reperfusion. *Brain Res*. 2000; 852:319–325. [PubMed: 10678758]
- Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H, Busse R, Neumann-Haefelin T, Brandes RP. NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke*. 2007; 38:3000–3006. [PubMed: 17916764]
- Kaminogo M. The effects of mild hyperoxia and/or hypertension on oxygen availability and oxidative metabolism in acute focal ischaemia. *Neurol Res*. 1989; 11:145–149. [PubMed: 2573848]
- Kim GS, Jung JE, Niizuma K, Chan PH. CK2 is a novel negative regulator of NADPH oxidase and a neuroprotectant in mice after cerebral ischemia. *J Neurosci*. 2009; 29:14779–14789. [PubMed: 19940173]
- Kim HY, Singhal AB, Lo EH. Normobaric hyperoxia extends the reperfusion window in focal cerebral ischemia. *Ann Neurol*. 2005; 57:571–575. [PubMed: 15786465]
- Kulkarni AC, Kuppusamy P, Parinandi N. Oxygen, the lead actor in the pathophysiologic drama: enactment of the trinity of normoxia, hypoxia, and hyperoxia in disease and therapy. *Antioxid Redox Signal*. 2007; 9:1717–1730. [PubMed: 17822371]
- Liu S, Shi H, Liu W, Furuichi T, Timmins GS, Liu KJ. Interstitial pO₂ in ischemic penumbra and core are differentially affected following transient focal cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 2004; 24:343–349. [PubMed: 15091115]
- Liu S, Liu W, Ding W, Miyake M, Rosenberg GA, Liu KJ. Electron paramagnetic resonance-guided normobaric hyperoxia treatment protects the brain by maintaining penumbral oxygenation in a rat

- model of transient focal cerebral ischemia. *J Cereb Blood Flow Metab.* 2006; 26:1274–1284. [PubMed: 16421507]
- Liu W, Sood R, Chen Q, Sakoglu U, Hendren J, Cetin O, Miyake M, Liu KJ. Normobaric hyperoxia inhibits NADPH oxidase-mediated matrix metalloproteinase-9 induction in cerebral microvessels in experimental stroke. *J Neurochem.* 2008; 107:1196–1205. [PubMed: 18786175]
- Mickel HS, Vaishnav YN, Kempinski O, von Lubitz D, Weiss JF, Feuerstein G. Breathing 100% oxygen after global brain ischemia in Mongolian Gerbils results in increased lipid peroxidation and increased mortality. *Stroke.* 1987; 18:426–430. [PubMed: 3564100]
- Mink RB, Dutka AJ. Hyperbaric oxygen after global cerebral ischemia in rabbits does not promote brain lipid peroxidation. *Crit Care Med.* 1995; 23:1398–1404. [PubMed: 7634811]
- Ostrowski RP, Tang J, Zhang JH. Hyperbaric oxygen suppresses NADPH oxidase in a rat subarachnoid hemorrhage model. *Stroke.* 2006; 37:1314–1318. [PubMed: 16556878]
- Oter S, Korkmaz A, Topal T, Ozcan O, Sadir S, Ozler M, Ogur R, Bilgic H. Correlation between hyperbaric oxygen exposure pressures and oxidative parameters in rat lung, brain, and erythrocytes. *Clin Biochem.* 2005; 38:706–711. [PubMed: 15904909]
- Schumacker PT. Current paradigms in cellular oxygen sensing. *Adv Exp Med Biol.* 2003; 543:57–71. [PubMed: 14713114]
- Serrano F, Kolluri NS, Wientjes FB, Card JP, Klann E. NADPH oxidase immunoreactivity in the mouse brain. *Brain Res.* 2003; 988:193–198. [PubMed: 14519542]
- Shin HK, Dunn AK, Jones PB, Boas DA, Lo EH, Moskowitz MA, Ayata C. Normobaric hyperoxia improves cerebral blood flow and oxygenation, and inhibits peri-infarct depolarizations in experimental focal ischaemia. *Brain.* 2007; 130:1631–1642. [PubMed: 17468117]
- Singhal AB. A review of oxygen therapy in ischemic stroke. *Neurol Res.* 2007; 29:173–183. [PubMed: 17439702]
- Singhal AB, Dijkhuizen RM, Rosen BR, Lo EH. Normobaric hyperoxia reduces MRI diffusion abnormalities and infarct size in experimental stroke. *Neurology.* 2002a; 58:945–952. [PubMed: 11914413]
- Singhal AB, Wang X, Sumii T, Mori T, Lo EH. Effects of normobaric hyperoxia in a rat model of focal cerebral ischemia-reperfusion. *J Cereb Blood Flow Metab.* 2002b; 22:861–868. [PubMed: 12142571]
- Singhal AB, Benner T, Roccatagliata L, Koroshetz WJ, Schaefer PW, Lo EH, Buonanno FS, Gonzalez RG, Sorensen AG. A pilot study of normobaric oxygen therapy in acute ischemic stroke. *Stroke.* 2005; 36:797–802. [PubMed: 15761201]
- Sood R, Yang Y, Taheri S, Candelario-Jalil E, Estrada EY, Walker EJ, Thompson J, Rosenberg GA. Increased apparent diffusion coefficients on MRI linked with matrix metalloproteinases and edema in white matter after bilateral carotid artery occlusion in rats. *J Cereb Blood Flow Metab.* 2009; 29:308–316. [PubMed: 18941468]
- Suh SW, Shin BS, Ma H, Van Hoecke M, Brennan AM, Yenari MA, Swanson RA. Glucose and NADPH oxidase drive neuronal superoxide formation in stroke. *Ann Neurol.* 2008; 64:654–663. [PubMed: 19107988]
- Sunami K, Takeda Y, Hashimoto M, Hirakawa M. Hyperbaric oxygen reduces infarct volume in rats by increasing oxygen supply to the ischemic periphery. *Crit Care Med.* 2000; 28:2831–2836. [PubMed: 10966258]
- Walder CE, Green SP, Darbonne WC, Mathias J, Rae J, Dinanier MC, Curnutte JT, Thomas GR. Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. *Stroke.* 1997; 28:2252–2258. [PubMed: 9368573]
- Wang Q, Tompkins KD, Simonyi A, Korthuis RJ, Sun AY, Sun GY. Apocynin protects against global cerebral ischemia-reperfusion-induced oxidative stress and injury in the gerbil hippocampus. *Brain Res.* 2006; 1090:182–189. [PubMed: 16650838]
- Zauner A, Daugherty WP, Bullock MR, Warner DS. Brain oxygenation and energy metabolism: part I—biological function and pathophysiology. *Neurosurgery.* 2002; 51:289–301. discussion 302. [PubMed: 12182767]

Figure 1.

NBO during ischemia raises the cerebral tissue oxygenation pO₂ in the ischemic penumbra to the pre-ischemic level. **A)** The sketch shows that the penumbral pO₂ values were measured in vivo by EPR oximetry in the anesthetized rats 40 min before ischemia (FiO₂: 21% O₂, Pre-I), 30 min after the start of ischemia (FiO₂: 21% O₂, Normoxia-I), and then 30 min after the start of NBO treatment (FiO₂: 95% O₂, NBO-I). FiO₂: inspired oxygen fraction. **B)** Bar graph shows the changes of the pO₂ levels in the ischemic penumbra. Data were expressed as mean $\pm$ SEM, n = 5. *p < 0.05 versus Pre-I, #p < 0.05 versus Normoxia-I.

Figure 2.

NBO treatment significantly reduced infarction volume in the ischemic brain after 90-min MCAO with 22.5 hrs of reperfusion. NBO (95% O₂) was delivered during 90-min ischemia.

A) Representative T2W images and ADC color maps showing tissue infarction in the ischemic hemispheres (right) of the normoxic and NBO-treated rats. **B)** Total infarction volume was quantified as described in the text. Data were expressed as mean $\pm$ SEM, $n = 7$ for the normoxic group, and $n = 6$ for the NBO group. * $P < 0.05$ versus normoxic group.

Figure 3.

The effect of NBO treatment on gp91^{phox} mRNA expression in the ischemic hemisphere after 90-min MCAO with 22.5 hrs of reperfusion. NBO (95% O₂) was delivered during 90-min ischemia. Gp91^{phox} mRNA level was analyzed by real time RT-PCR on extracted total RNA. Threshold cycles (Ct values) were normalized to rpl 32 and the comparative mRNA levels were determined by the $\Delta\Delta C_t$ method. Data were expressed as mean $\pm$ SEM, n =8. **P* < 0.05 versus non-ischemic hemisphere, #*P* < 0.05 versus normoxic group.

Figure 4. NBO treatment significantly reduced the increase of gp91^{phox} protein in ischemic brain tissue after 90-min MCAO with 22.5 hrs of reperfusion. NBO (95% O₂) was delivered during 90-min ischemia. Gp91^{phox} protein level was analyzed by western blot. The membrane was stripped and re-blotted with β -actin antibody. A) Representative western blots of gp91^{phox} and β -actin. B) Hemispheric ratio of band intensity of gp91^{phox} protein in the ischemic hemisphere versus non-ischemic hemisphere after normalization to β -actin. * P < 0.05 versus normoxic group, n = 8.

Figure 5.

NBO treatment significantly reduced the activity of NADPH oxidase in ischemic brain tissue after 90-min MCAO with 22.5 hrs of reperfusion. NBO (95% O₂) was delivered during 90-min ischemia. Data are expressed as mean $\pm$ SEM, n = 6, * P < 0.05 versus the normoxic group.