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Involvement of Vascular NAD(P)H Oxidase-derived Superoxide in Cerebral Vasospasm after Subarachnoid Hemorrhage in Rats

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The role of vascular NAD(P)H oxidase in subarachnoid hemorrhage (SAH)-induced vasospasm in the basilar artery was examined in a rat model. Arterial vasospasm characterized by increased wall thickness and decreased lumen size was observed at 5 to 7 days after 2nd injection of blood into cisterna magna, and these changes were significantly ameliorated by pretreatment of diphenyleneiodonium (DPI, 25 μl of 100 μM), an inhibitor of NAD(P)H oxidase. To determine the time course of changes in the vascular NAD(P)H oxidase activity, cerebral vasculature was isolated at different time intervals from 12 hrs to 14 days after injection of autologous blood. At 24 hrs after the second injection of blood, the NAD(P)H oxidase activity was markedly increased with an enhanced membrane translocation of p47phox, but by 48 hours both the enzyme activity and p47phox translocation regained normal values, and were remained unchanged up to 14 days after SAH. However, no significant changes in the expression of p22phox mRNA was observed throughout the experiments. These findings suggest that the activation of NAD(P)H oxidase by which assembly of the oxidase components enhanced and subsequent production of superoxide in the early stages of SAH might contribute to the delayed cerebral vasospasm in SAH rats.

Key Words: NAD(P)H oxidase, Subarachnoid hemorrhage, Vasospasm, OFR

INTRODUCTION

Cerebral vasospasm after subarachnoid hemorrhage (SAH) is characterized by a delayed but prolonged contraction of the major cerebral arteries and seriously affects the prognosis of a patient, but the pathophysiological mechanisms of cerebral vasospasm still remains unclear. A variety of mechanisms have been proposed to explain these phenomena, and particularly oxidant stress has been implicated in the pathogenesis of cerebral vasospasm (Sano et al, 1980; Sasaki et al, 1981; MacDonald & Weir, 1991). Numerous reports have supported the significance of reactive oxygen species (ROS) causing vasospasm through activation of protein kinase C system and/or inactivation of nitric oxide (Gryglewski et al, 1986; Chakraborti & Michael, 1993).

Convincing evidences have been presented to show that production of ROS occurs in non-phagocytic cells including endothelial cells (Matsubara & Ziff, 1986), vascular smooth muscle cells (Griendling et al, 1994), and aortic adventitial fibroblasts (Pagano et al, 1998). The superoxide anion has a variety of biological functions that induce gene expression (Sen & Packer, 1996), cellular proliferation (Rao & Berk, 1992), apoptosis (Li et al, 1997; Maron et al, 1999), and hypertrophy (Zafari et al, 1998). However, both the enzymatic source and the role of vascular production of ROS in the cerebral vasospasm in SAH remain unknown.

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Among various sources for ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide (NO) synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered as a major source of ROS in the vasculature (Zalba et al, 2000). Vascular NAD(P)H oxidase is similar in structure to the neutrophil NADPH oxidase which consists of 4 major subunits; a membrane-associated cytochrome b_{558}, composing gp91phox and p22-phox and two cytosolic components, p47phox and p67phox. On activation, the cytosolic constituents are translocated to the plasma membrane where they bind to cytochrome (membrane assembly) and initiate superoxide synthesis (Chanock et al, 1994; Cross et al, 1999). In the present study, to clarify the role of vascular NAD(P)H oxidase in the delayed cerebral vasospasm in SAH, both the oxidase activity and/or activation mechanisms of the enzyme in the cerebral vasculature as well as the effect of oxidase inhibition on SAH-induced cerebral vasospasm were assessed using a rat model of SAH.

METHODS

Animal preparation

The experimental protocols used in this study were approved by the University of Pusan National University Animal Research Committee. Anesthesia was induced in the male Sprague-Dawley rats (300~400 g) with intra-

ABBREVIATIONS: SAH, Subarachnoid hemorrhage; ROS, reactive oxygen species; DPI, diphenyleneiodonium; BA, basilar artery.

peritoneal injection of thiopental sodium (50 mg/kg), and the animals were allowed to breathe spontaneously. Aided by a surgical microscope, the atlantooccipital membrane was tapped carefully into the cisterna magna with a 27-gauge needle. After aspirating 0.3 ml of cerebrospinal fluid (CSF), an equal amount of autologous blood from the femoral artery was injected during a period of 3 minutes or longer. The rats were then placed in a head-down prone position at a 30° angle for 30 min to hold the blood in the basal cisterns. For the second injection, the same procedure was repeated after 48 hours (Day 2). Sham-operated rats were injected with 0.9% sterile NaCl solution instead of blood.

Morphological findings with light microscope

In a separate experiment, after planned death by an overdose of anesthetic agents, the hearts were cannulated and perfused with a phosphate buffer solution containing 2% glutaraldehyde maintaining at 7.8 ml/min by using peristaltic pump (Cole-Parmer Inc., Chicago, IL, USA). The brain was fixed with 2% glutaraldehyde and embedded in paraffin, sliced, and stained with hematoxylin and eosin for light microscopy. The basilar artery (BA) was devided into three portions. The wall thickness and cross-sectional areas of the vessel lumen were measured by use of an image analysis system (Image-Pro Plus, Media Cybernetics, Maryland, USA).

Measurement of NAD(P)H oxidase activity

NAD(P)H oxidase activity was measured with the lucigenin assay which is specific for superoxide anion. Briefly, cerebral vasculature (approximately 30 mg) was homogenized with a motor-driven tissue homogenizer for 2 min in 50 mM phosphate buffer containing 0.01 mM EDTA. The homogenate was centrifuged at 600×g for 10 min to remove unbroken cells and debris. Protein content was determined by using the BCA protein assay kit (Sigma). The assay was performed in 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 250 μM lucigenin as the detector, and NAD(P)H as the substrate (final volume 150 μl). NADH and NADPH were used at a final concentration of 100 μM, and the reaction was started by the addition of 25 μg of protein. The photon emission was measured every 15 s for 10 min in a microtiterplate luminometer (Microlumat LB96P, EG&G Berthold, Germany).

Membrane translocation of p47phox

Cerebral vasculature was initially homogenized in Tris-HCl buffer containing the protease inhibitors (1 mM PMSF, and 1 μg/ml each of antipain, aprotinin, bestatin, leupeptin, soybean trypsin inhibitors, pepstatin A, and 0.1% 2-mercaptoethanol). The supernatant from the low speed centrifugation was subjected to 30,000×g centrifugation for 45 min at 4°C to separate membrane and cytosolic fractions, and the both fractions containing 30 μg of protein were used for immunoblotting. Antibodies used were polyclonal goat anti-human p47phox (Santa Cruz Biotech) and peroxidase-labeled goat anti-goat IgG (Santa Cruz Biotech).

Reverse transcription and PCR

Total RNA was extracted from cerebral vasculature with the use of TRIzol reagent (Gibco/BRL Life Technologies) according to the manufacturer's instructions. After ethanol precipitation, RNA was resuspended in DEPC-treated H_2O. RNA (3 μg) was converted to cDNA with Moloney Murine leukemia virus reverse transcriptase (Promega), and PCR amplification was performed with *Taq* polymerase (Promega). For the PCR, a set of primers generated from the rat vascular smooth muscle cell p22phox sequence (Fukui et al, 1995) was used to amplify a 485-base pair fragment of the p22phox gene product (5′-GACGCTTCACGCAGTGGTACT-3′ and 5′-CACGACCTCATCTGTCACTGG-3′) and also for amplification of β-actin, sense, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and antisense, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′ were used as an internal control generating 285-base pair fragment. The amplification products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide. The optical density of PCR fragment was estimated densitometrically (GS-710 Calibrated imaging densitometer, Bio-RAD).

Statistical analysis

Data were presented as means±SEM or percentage of control. Statistical comparisons between groups were performed with two-tailed Student's *t*-test for unpaired data. A value of $p<0.05$ was considered statistically significant.

RESULTS

The physiological parameters measured before and after SAH are listed in Table 1. There were no significant differences among the groups in terms of mean arterial blood pressure, pO_2, pCO_2, and arterial pH. Furthermore, none of the rats were observed to develop neurologic deficits.

Histological changes in response to SAH

The wall thickness and lumen area of the basilar artery (BA) were quantitatively analyzed by image analysis of photomicrographs obtained with light microscopy. The baseline values of the wall thickness and lumen area were 18.7±2.5 μm and 40570±2237 μm^2, respectively. In the two-hemorrhage model of SAH rats, arterial vasospasm characterized by increased wall thickness and decreased lumen area was observed (Fig. 1). The lumen area of the BA started to decrease on Day 1, peaked on Day 5 (26370±3274 μm^2) after second injection of autologous blood into cisterna magna. However, no significant change in the lumen area of the BA was observed in any of the one-

Table 1. Physiological variables of mean arterial blood pressure (MABP), blood gas, and pH analysis before and after SAH

Variables	Before SAH	After SAH, days		
		1	7	14
Number of rats	20	5	7	7
MABP, mmHg	113.4±7.8	102.7±3.0	98.6±2.7	104.9±4.3
PCO_2, mmHg	32.5±5.7	34.0±2.4	35.9±1.2	37.6±2.6
PO_2, mmHg	103.9±10.4	95.4±0.9	97.1±1.2	100.3±2.2
pH	7.39±0.22	7.49±0.02	7.46±0.03	7.42±0.02

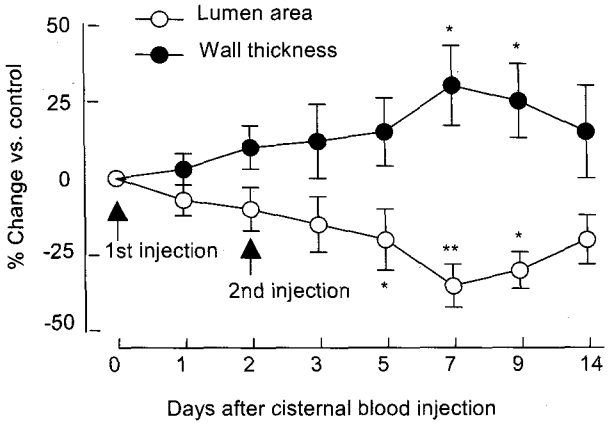

Fig. 1. Time course changes in wall thickness and cross-sectional area of lumen of the rat basilar artery after subarachnoid administration of autologous blood. Values are expressed as means ±SEM from 6 animals. *, $p<0.05$; **, $p<0.01$ are significantly different from the baseline values on day 0.

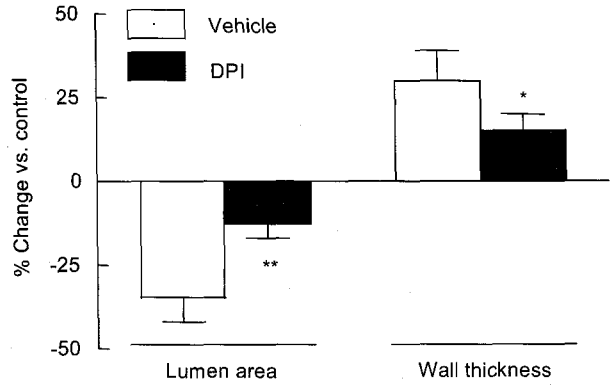

Fig. 2. Effect of diphenyleneiodonium (DPI, 25 μl of 100 μM) on the SAH-induced changes for the wall thickness and lumen area of basilar artery. Values were obtained from 5 days after the second injection of blood, and were expressed as means ± SEM from 6 animals. *, $p<0.05$; **, $p<0.01$ vs Vehicle of SAH.

hemorrhage group.

Effect of DPI on the SAH-induced cerebral vasospasm

Quantitative light microscopic examination of the BA in the SAH groups revealed substantial corrugation of the internal elastic lamina, whereas arterial corrugation was less prominent in animals treated with DPI (25 μl of 100 μM). As shown in Fig. 2, 5 days after second injection of autologous blood into cisterna magna, the cross-sectional wall thickness and lumen area of the BA were quantitatively measured. The SAH group exhibited large and statistically significant vasospasm when compared with the sham-operated group. The magnitude of cerebral vasospasm was significantly attenuated in animals treated with DPI (25 μl of 100 μM).

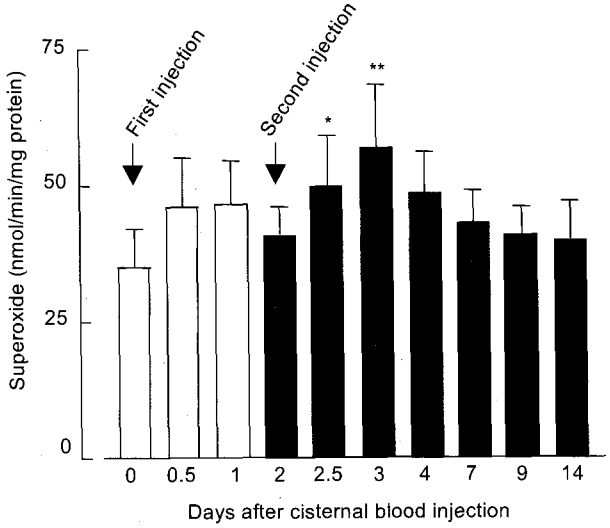

Fig. 3. The time course changes of NAD(P)H oxidase activity in the cerebral vasculature from one-hemorrhage (open bars) and two-hemorrhage (filled bars) rats. Arrows indicate time of intracisternal administration of autologous blood. Values are expressed as means ± SEM from 5 measurements. *, $p<0.05$; **, $p<0.01$; Significantly different from the baseline values on day 0.

Time course of the changes in the vascular NAD(P)H oxidase

Fig. 3 shows the time course of the changes in vascular NAD(P)H oxidase in the cerebral vasculature following injection of autologous blood into the cisterna magna. At 12 and 24 hrs after the second injection of blood, the activity of NAD(P)H oxidase in the cerebral vasculature was markedly increased ($p<0.05$ at 12 hrs; $p<0.01$ at 24 hrs), and subsequently, the enhanced oxidase activity returned to the control values after 48 hrs maintaining up to 12 days after second injection of blood.

Membrane translocation of p47phox

The results of quantitative analysis of changes in the amount of p47phox in the cytosol and the membrane fractions are summarized in Fig. 4. At 24 hrs after the second injection of autologous blood into cisterna magna, the value in the cytosol fraction (n=5) was significantly lower than that in the sham-operated group ($p<0.01$). In the membrane fractions, the value was significantly higher than that in the sham-operated group ($p<0.01$). Furthermore, the time course of the changes in the amounts of membrane-associated p47phox was similar to those of the changes in the oxidase activity.

Expression of vascular p22phox mRNA

Since p22phox, one of the membrane-associated components of the vascular NAD(P)H oxidase, has been shown to present and functions for the vasculature, we elucidated the p22phox mRNA expression in sham-operated and SAH rats by semiquantitative RT-PCR analysis. As shown in Fig. 5, the level of p22phox mRNA expression in SAH rats was not different from that of the sham-operated rats. This

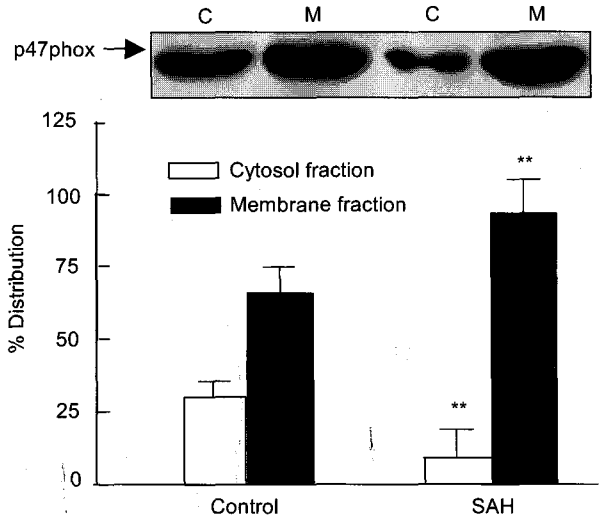

Fig. 4. The changes in the amounts of p47phox in the cytosol (C) and membrane (M) fractions of cerebral vasculature from sham-operated and SAH rats. The results are expressed as a percentage of the total amount of p47phox. In the SAH rats, cerebral vasculature was isolated 24 hrs after secondary cisternal injection of autologous blood. Values were expressed as mean±SEM from 5 measurements. **, $p<0.01$ vs corresponding values from sham-operated group.

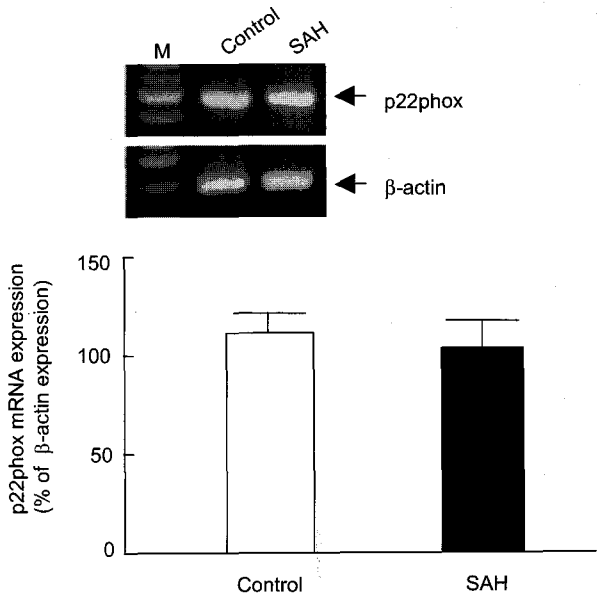

Fig. 5. Expression of p22phox mRNA in the cerebral vasculature from sham-operated and SAH rats. The bar graph summarizes data obtained from 5 experiments, expressed as the percent change over the levels of β-actin expression (mean±SEM). In the SAH rats, cerebral vasculature was isolated 24 hrs after secondary cisternal injection of autologous blood. M indicates molecular size marker.

data suggest that the expression of p22phox mRNA may not be responsible for the increased NAD(P)H oxidase activity in the cerebral vasculature in SAH rats.

DISCUSSION

Although cerebral vasospasm in SAH patients is a major cause of morbidity and mortality, the precise mechanisms for the delayed cerebral vasospasm is not clear. Cerebral vasospasm after SAH was attenuated not only by local administration of superoxide dismutase (Steele et al, 1991; Shishido et al, 1994), but also by overexpression of Cu/Zn-superoxide dismutase in the transgenic mice, suggesting the pivotal role of oxygen radicals in the development of cerebral vasospasm (Kamii et al, 1999). In the present experiment, we demonstrated that in the two-hemorrhage model of SAH rats, the time course of the development of vasospasm was similar to that of vasospasm after a subarachnoid hemorrhage under the clinical situation. Minimal narrowing was present for 1 to 2 days and has progressively increased by 5 days following the second injection of autologous blood into cisterna magna. These delayed cerebral vasospasm in SAH rats were significantly attenuated by pretreatment with DPI, an NAD(P)H oxidase inhibitor. Consistent with other reports, our results also showed that NAD(P)H-derived superoxide was closely involved in the pathogenesis of cerebral vasospasm in subarachnoid hemorrhage.

Among various sources of ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide (NO) synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered as a major source of ROS in the vasculature (Zalba et al, 2000), and thus, was implicated in numerous cellular processes and vascular diseases (Griendling et al, 2000). Recently, Wang et al (1998) have demonstrated that NADH/NADPH oxidase localized in the adventitia served as a primary site for superoxide production. Furthermore, our previous report (Choi et al, 2001) clearly showed that periarterial blood application to the aortic segments significantly augmented the NADH/NADPH-dependent production of superoxide. Thus, it was suggested that NAD(P)H oxidase-derived superoxide was involved in the experimental vasospasm induced by periarterial blood, since the generation of superoxide was significantly inhibited by DPI.

With regard to the oxidase activation, the regulation of NAD(P)H oxidase activity in cardiovascular cells occurs at least on 2 levels. First, activation of the oxidase can be mediated by intracellular second messengers, including calcium (Meier, 1996), thus activating cytosolic components to translocate to the plasma membrane, and the functional NAD(P)H oxidase is assembled (Quinn et al, 1993). Secondly, oxidase activity can be also modulated by upregulation of the component mRNAs. In the present experiments, at 24 hrs after the second injection of autologous blood into cisterna magna, both the activity and the membrane translocation of p47phox were markedly increased, however, at 48 hours, these values returned to the normal levels and remained for 14 days after SAH. However, there was no significant changes in the expression of p22phox mRNA throughout the experiments. These findings suggest that the enhanced activity of NAD(P)H oxidase in the early stages of SAH rats was mediated by the enhanced assembly of oxidase through increased membrane translocation of cytosolic components of oxidase, p47phox, instead of upregulation of p22phox mRNA expression.

Production of superoxide in the vascular wall has been

shown to inactivate nitric oxide, leading to the impaired endothelium-dependent vasodilation (Rubbo et al, 1995; Mugge et al, 1991), to oxidize LDL (Aviram et al, 1996), and to increase adhesion molecule expression in endothelial cells which result in monocyte infiltration (Marui et al, 1993) and activate matrix metalloproteinases leading to vascular remodeling (Rajagopalan et al, 1996). With regard to SAH, OFR are involved in cerebral vasospasm not only by protein kinase C-dependent augmentation of contraction, but also by suppression of vasodilation, which is mediated by endothelium-derived relaxing factor/nitric oxide (Asano & Matsui, 1993). Considering these previous reports and our present results that DPI has a preventive effect on the SAH-induced cerebral vasospasm even though there was a significant time discrepancy between vascular NAD(P)H oxidase activation and cerebral vasospasm that was maximized 5 days after second injection of blood, it is apparent that intracellular production of ROS and the consequent activation of specific signaling pathways and induction of redox-sensitive genes coordinate the delayed cerebral vasospasm in SAH rats. However, further studies are required to elucidate the precise afferent pathways involved in the activation of NAD(P)H oxidase and efferent pathways for oxidative stress, and the role of NADPH oxidase in the delayed cerebral vasospasm in SAH rats.

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REFERENCES

Asano T, Matsui T. Cerebral vasospasm: a disorder of the local mechanotransduction system in cerebral arteries. *Crit Rev Neurosurg* 45: 825−838, 1993

Aviram M, Rosenblat M, Etzioni A, Levy R. Activation of NADPH oxidase required for macrophage-mediated oxidation of low-density lipoprotein. *Metabolism* 45: 1069−1079, 1996

Chakraborti S, Michael JR. Role of protein kinase C in oxidant-mediated activation of phospholipase A2 in rabbit pulmonary arterial smooth muscle cells. *Mol Cell Biochem* 122: 9−15, 1993

Chanock SJ, el Benna J, Smith RM, Babior BM. The respiratory burst oxidase. *J Biol Chem* 269: 24519−24522, 1994

Choi JM, Kim CD, Hong KW. Involvement of NADH/NADPH oxidase-derived superoxide in experimental vasospasm induced by periarterial blood in rat femoral artery. *Life Sci* 69: 1753−1763, 2001

Cross AR, Erickson RW, Curnutte JT. The mechanism of activation of NADPH oxidase in the cell-free system: the activation process is primarily catalytic and not through the formation of a stoichiometric complex. *Biochem J* 341: 251−255, 1999

Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74: 1141−1148, 1994

Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494−501, 2000

Fukui T, Lassegue B, Kai H, Alexander RW, Griendling KK. Cytochrome b-558 alpha-subunit cloning and expression in rat aortic smooth muscle cells. *Biochim Biophys Acta* 1231: 215−219, 1995

Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 320: 454−456, 1986

Kamii H, Kato I, Kinouchi H, Chan PH, Epstein CJ, Akabane A, Okamoto H, Yoshomoto T. Amelioration of vasospasm after subarachnoid hemorrhage in transgenic mice overexpressing CuZn-superoxide dismutase. *Stroke* 30: 867−872, 1999

Li PF, Dietz R, von Harsdorf R. Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *FEBS Lett* 404: 249−252, 1997

Macdonald RL, Weir BK. A review of hemoglobin and the pathogenesis of cerebral vasospasm. *Stroke* 22: 971−982, 1991

Maron BJ, Nishimura RA, McKenna WJ, Rakowski H, Josephson ME, Kieval RS. Assessment of permanent dual-chamber pacing as a treatment for drug-refractory symptomatic patients with obstructive hypertrophic cardiomyopathy. *Circulation* 99: 2927−2933, 1999

Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 92: 1866−1874, 1993

Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. *J Immunol* 137: 3295−3298, 1986

Meier B. Regulation of the superoxide releasing system in human fibroblasts. *Adv Exp Med Biol* 387: 113−116, 1996

Mugge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG. Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res* 69: 1293−1300, 1991

Pagano PJ, Chanock SJ, Siwik DA, Colucci WS, Clark JK. Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. *Hypertension* 32: 331−337, 1998

Quinn MT, Evans T, Loetterle LR, Jesaitis AJ, Bokoch GM. Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. *J Biol Chem* 268: 20983−20987, 1993

Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 98: 2572−2579, 1996

Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res* 70: 593−599, 1992

Rubbo H, Tarpey M, Freeman BA. Nitric oxide and reactive oxygen species in vascular injury. *Biochem Soc Symp* 61: 33−45, 1995

Sano K, Asano T, Tanishima T, Sasaki T. Lipid peroxidation as a cause of cerebral vasospasm. *Neurol Res* 2: 253−272, 1980

Sasaki T, Wakai S, Asano T, Watanabe T, Kirino T, Sano K. The effect of a lipid hydroperoxide of arachidonic acid on the canine basilar artery. An experimental study on cerebral vasospasm. *J Neurosurg* 54: 357−365, 1981

Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J* 10: 709−720, 1996

Shishido T, Suzuki R, Qian L, Hirakawa K. The role of superoxide anions in the pathogenesis of cerebral vasospasm. *Stroke* 25: 864−868, 1994

Steele JA, Stockbridge N, Maljkovic G, Weir B. Free radicals mediate actions of oxyhemoglobin on cerebrovascular smooth muscle cells. *Circ Res* 68: 416−423, 1991

Wang HD, Pagano PJ, Du Y, Cayatte AJ, Quinn MT, Brecher P, Cohen RA. Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. *Circ Res* 82: 810−818, 1998

Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR, Griendling KK. Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. *Hypertension* 32: 488−495, 1998

Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etayo JC, Diez J. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* 35: 1055−1061, 2000