LABORATORY INVESTIGATION

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Role of Nitric Oxide in Leukocyte-Endothelial Interaction in Cerebral Venules during Reperfusion after Global Ischemia

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Objective : Reactive oxygen metabolites and polymorphonuclear leukocytes have been implicated in the pathophysiology of reperfusion injury. The mechanisms involved in superoxide-mediated leukocyte adherence remain unclear, however, nitric oxide(NO) may contribute to this response. The present study is undertaken to elucidate mechamisms controlling NO based mechanisms that regulated leukocyte-endothelial interactions in the cerebral vasculature after global cerebral ischemia and reperfusion.

Methods : Pial venular leukocyte adherence of anesthetized newborn piglets was quantified by in situ fluorescence videomicroscopy through closed cranial windows during basal conditions and during 2hours of reperfusion after global ischemia induced by 9minutes of asphyxia. Nitric oxide synthase(NOS) was inhibited by local window superfusion of L-nitroarginine(NA); superfusion of sodium nitroprusside(SNP) was used to donate NO.

Results : The mean number of adherent leukocytes to cerebral venules in the 9minutes asphyxia and 2hours reperfusion group were 161±19 compared with 13±4 in the nonasphyxial group. Superfusion of L-NA through the cranial window for 2hours resulted in leukocyte adherence similar to that observed during the initial 2hours of reperfusion after asphyxia. Leukocyte adherence was not additionally increased in asphyxic animal treated with L-NA. SNP inhibited asphyxia induced leukocyte adherence back to control levels.

Conclusions : Nitric oxide inhibits leukocyte adherence to cerebral venules during the initial hours of reperfusion after asphyxia, and that NO supplementation inhibit asphyxia induced leukocyte adherence back to control levels. These results indicate that NO is an important factor in ischemia-reperfusion induced leukocyte adherence.

KEY WORDS : Reperfusion injury · Leukocyte adherence · Nitric oxide · L-arginine · Sodium nitroprusside · Videomicroscopy imaging.

Introduction

When a tissue is subjected to ischemia, a sequence of chemical reaction is initiated, which may ultimately lead to cellular dysfunction and necrosis. Interventions directed toward rapid restoration of tissue oxygenation are commonly used, and it is undeniable that reestablishing blood flow is necessary in rescuing ischemic tissues.

However it is now clear that reperfusion of ischemic tissues initiates a complex series of reaction that paradoxically injures tissues. Although several mechanisms have been identified as the critical event in ischemia-reperfuson injury, most attention has focused on a role for reactive oxygen metabolites and inflammatory leukocytes[12,33].

Reactive oxygen metabolites are believed to be implicated in ischemia. Reperfusion after ischemia provides oxygen as a substrate for numerous enzyme oxidation reactions that produce free radicals[4]. This oxidative stress results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death[29], and promote the formation of inflammatory agents that recruit and activate polymorphonuclear leukocytes[33].

These leukocyte appear to inflict reperfusion-induced tissue injury. The superoxide initiate the production and release of proinflammatory agents leading to leukocytes adherence and emigration and the adherent leukocytes then mediate injury

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either by release of proteases or by physical disruption of the microvascular barrier. This inflammatory cascade could be an important contributor to brain damage after stroke[1,2].

The role of the neural messenger nitric oxide(NO) in cerebral ischemia has been investigated extensively in the past decade. NO may play either protective or deleterious role during ischemia depending on the nitric oxide synthase(NOS) involved[7,17,30]. Immediately after brain ischaemia, NO release from endothelial NOS(eNOS) is protective mainly by promoting vasodilation, inhibition of platelet aggregation and of leukocytes adhesion[15]. However, after ischaemia develops, NO produced by overactivation of neuronal NOS(nNOS) and later, NO release by de novo expression of inducible NOS (iNOS) contribute to the brain damage[16,30].

Thus, the objective of this study was to determine whether NO plays a role in the leukocyte adherence observed in an model of global cerebral ischemia induced by asphyxia and reperfusion.

Materials and Methods

Animal preparation and drug superfusion

All expreiments were preformed on newborn pigs weighing between 1.8 and 3.2kg that were less than 5days. Animal were anesthetized ketamine hydrochloride(20mg/kg) administered intramuscularly. A tracheostomy was then performed and animals were ventilated with a mix of room air and oxygen and anesthesia was maintained for the remainder of the experiment with isoflurane(1.5%), using a Harvard 683 rodent ventilator. Core body temperature was monitored with a rectal probe and maintained with a heating pad at 39℃±0.5℃. The left femoral artery was cannulated for the measurement of mean arteial blood pressure and the left femoral vein was prepared for central infusion of 5% dextrose solution mixed with pancuronium(0.25mg/kg/hour).

The right femoral artery was cannulated for determination of arterial blood gases and glucose. Intermittent samples of arterial blood were obtained for measurement of gas tensions, glucose concentration, pH, and hematocrit. After an 18mm craniectomy and removal of the dura, a closed cranial window made of Plexiglas was mounted over the right parietal cortex. Through ports at the edge of the window, intracranial pressure was continuously monitored, juxtaposed ports were used to superfuse artificial cerebrospinal fluid(CSF); containing NaCl 132.8mM, KCl 3.0mM, $CaCl_2$ 0.5mM, $MgCl_2$ 0.7mM, $NaHCO_3$ 24.6mM, urea 6.7mM, glucose 3.7mM. Buffer or drug solutions were introduced into the window space by superfusion at 1mL/min for 1 minute, followed by a continuous superfusion rate of 50㎕/min for 2.0 or 2.5hours, with the use of an automated syringe pump.

Fluorescence videomicroscopy

We used an epifluorescence microscope(model BHMJ, Olympus Corp.) mounted on a position flexible boom stand, with a 100W mercury arc light source and a ×3.3 photo eyepiece. Two filter cubes were used. For imaging of rhodamine labled leukocytes, the excitation filter was 535/35nm, the dichroic filter was 565nm, and the emission filter was 610 /75nm. The coupling of a ×10 immersion lens(Olympus Corp.) featuring a 0.4 numerical aperture and 3.1mm working distance with a Newvicon tube camera with contrast and brightness controls provided real time, high resolution images of individual fluorescently labled leukocytes moving through the pial microcirculation on the surface of the brain. Video recordings 30seconds in duration were obtained at regular intervals (30minutes) before and after asphyxia.

Leukocyte imaging

Leukocytes were fluorescently labeled in situ with rhodamine 6G, which stains 100% of circulating leukocytes as assessed by flow cytometry. In brief, 30minutes before baseline measurements, a 2.5㎖/kg loading dose of R6G(60㎍/㎖) was administered at 1.5㎖/min. One to 2minutes before each 60 second imaging period, rhodamine 6G was infused at 800㎕/min to enhance labeling. Leukocyte dynamics in pial venules were recorded in real time with the use of a Newvicon camera mounted on an epifluorescence microscope.

Protocols

After the surgical preparation of the cranium, a 30minutes stabilization period was allowed in all animals before baseline measurements. At that time, animals were rendered asphyxia for 9minutes by turning off the ventilator and clamping the respiratory tubing. A blood gas sample was obtained during the last minute of asphyxia, after which mechanical ventilation was resumed and they were observed for 2hours of reperfusion. Drug superfusion in asphyxic animals was initiated either 0.5 hour before asphyxia or at the start of reperfusion.

Animals were randomly divided into the following 5groups : group 1(n=6) served as a normoxic control groups. In these animals, artificial CSF buffer was superfused through the window for 2hours after baseline measurements were obtained. Group 2(n=6) animals were rendered asphyxic and artificial CSF buffer was superfused through the window starting 0.5hour before asphyxia. In group 3 animals(n=8), L-nitroarginine(NA, 100㎛ol/L), an NOS inhibitor, was superfusion for 2hours after baseline measurements. In group 4(n=8) animals, superfusion of L-NA was initiated 0.5hour before asphyxia and continued throughout 2hours of post-asphyxic reperfusion. In group 5 animals(n=8), sodium nitroprusside(SNP, 40㎛ol/L) was superfused immediately on

Table 1. Number of adherent leukocytes to the cerebral venules

Experimental group	Number	Reperfusion Time (hour)	
		1	2
Control	6	8± 2	13± 4
Asphyxia	6	80±11	161±19*
L-NA	8	84± 7	145±20*
Asphyxia + L-NA	8	82± 8	153±23
Asphyxia + SNP	8	17± 5	27± 6**

*p<0.05 vs control group at same time; **p<0.05 vs asphyxia group at same time.
L-NA : L-nitroarginine / SNP : Sodium nitroprusside

reperfusion after asphyxia.

Video images were obtained in all animal groups at 1 and 2 hours of drug superfusion or postasphyxic reperfusion for quantification of adherent leukocytes.

Quantification of leukocyte-endothelial adherence

Leukocyte adherence to the endothelium of the pial venular wall was quantified in 2 preselected venular networks that included several secondary and tertiary postcapillary branches and 1 or 2 larger venules into which they drained. Adherence was quantified manually by counting the number of leukocytes that remained stationary anywhere within each venular network under observation for 10 consecutive seconds. The adherence values reported indicate the mean number of leukocytes per square millimeter of total endothelial vessel surface examined as determined by image analysis software (two dimensional surface area times π).

Statistical analyses

Difference in the physiological, hemodynamic, and leukocyte adherence parameters within and between groups were assessed by repeated measures ANOVA or nonparametric Kruskal Wallis with Dunn's or Dunnett's multiple range test applied when appropriate. $P < 0.05$ was considered significant.

Results

Physiological and Hematological parameters

There were no significant differences in the monitored physiological variables(mean arterial pressure=67±4mmHg, blood glucose=105±8mg/dl, arterial pH=7.4±0.02, $PaCO_2$ =34±1mmHg, PaO_2=102±7mmHg) among the 5 animal group during baseline conditions or at 1 and 2hours of reperfusion.

Asphyxial animals became severe hypoxic(PaO_2=14±2mmHg), hypotensive(mean arterial pressure=22±5mmHg), acidotic (pH=7.10±0.04), hypercapnic($PaCO_2$ =76±5mmHg) by the end of 9minute asphyxial insult : these parameters recovered to preasphyxial levels by 30minutes of reperfusion. There were no significant differences in hematocrit between the

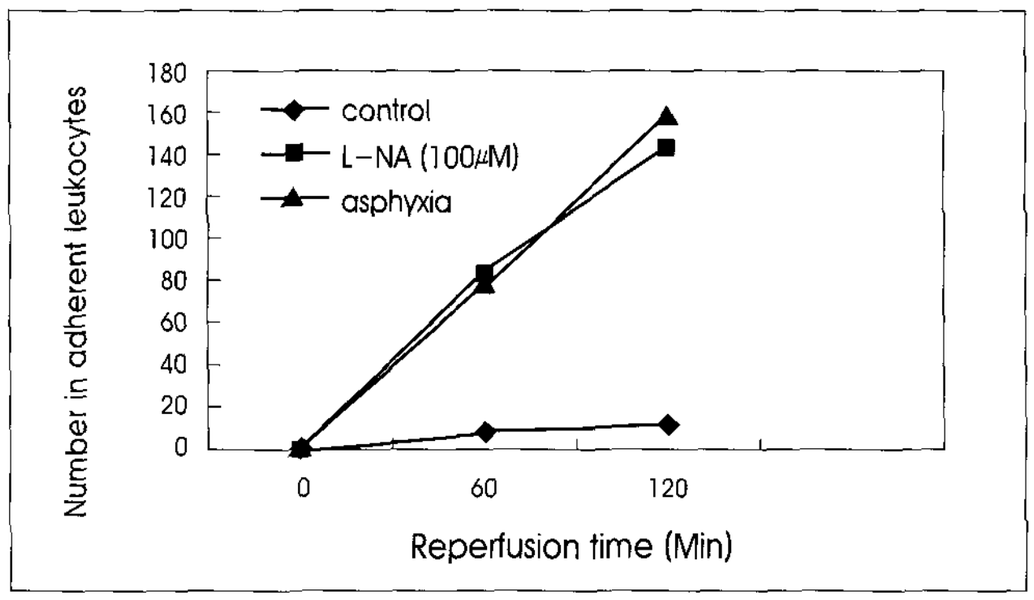

Fig. 1. Effects of local nitric oxide synthase inhibition with L-nitroarginine (L-NA) and asphyxia-reperfusion on leukocyte adherence in pial venules. Superfusion of L-NA(100μ mol/L) on nonasphyxial animal through the cranial window for two hours resulting in leukocyte adherence simililar to that observed during the initial two hours of reperfusion after asphyxia.

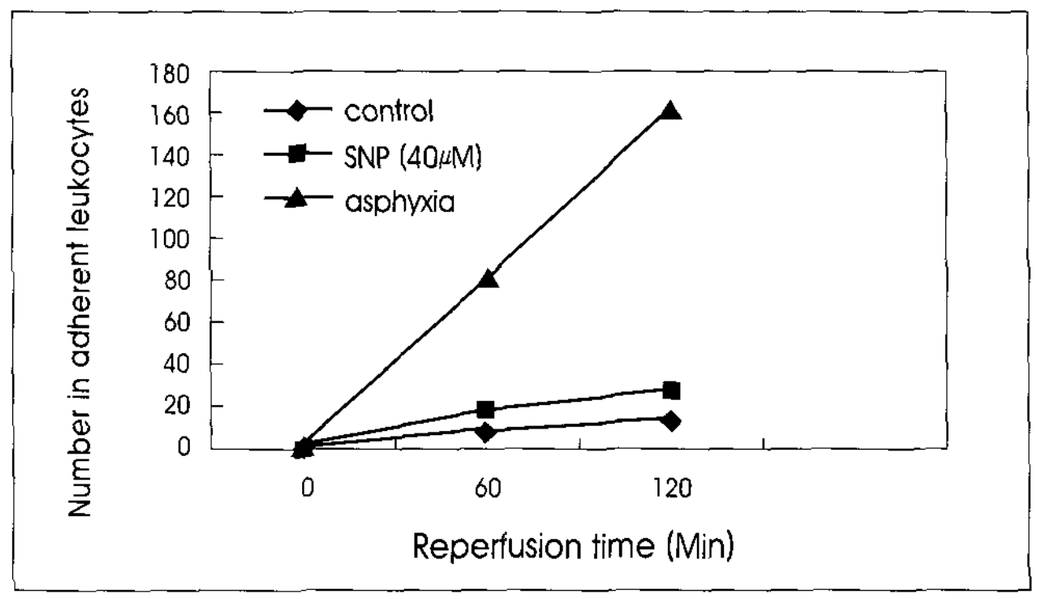

Fig. 2. Effects of sodium nitroprussideon(SNP) asphyxia induced leukocyte adherence in pial venules. SNP treatment(40μmol/L) significantly reducing leukocyte adherence induced by asphyxia alone.

level measured at baseline and that measured at 2hours of reperfusion, within and between groups.

Leukocyte-endothelial adherence

1) In normoxic control group, the mean number of leukocyte adherent to cerebral venules were 8±2(1hour) and 13±4(2hour). A slight increase in leukocyte adherence occurred over the 2hour observation period relative to that measured during baseline conditions(Table 1).

2) In asphyxia/reperfusion group, the mean number of leukocyte adhrent to cerebral venules were 80±11(1hour) and 161±19(2hour). A global ischemia resulted in a much more robust and significantly greater increase in the number of leukocytes adherent to the venular endothelium during the initial 2hours of reperfusion(Fig. 1).

3) In nonasphyxial animal group, superfused of L-NA through the cranial window for 2hours, the mean number of leukocyte adhrent to cerebral venules were 84±7(1hour) and 145±20(2hour)(Fig. 1, $p<0.05$). These findings were similar to that observed during the initial 2hours of reperfusion after asphyxia.

4) In asphyxial animal superfused with L-NA, no further increase in leukocyte adherence was observed at any time point relative to animal subjected to asphyxia alone(Table 1).
5) In animals group treated postasphyxically with SNP, the mean number of leukocyte adhrent to cerebral venules were 17±5(1hour) and 27±6(2hour)(Fig 2, $p<0.05$). The SNP treatment significantly reduced leukocyte adhrence induced by asphyxia relative to asphyxia alone.

Discussion

Early reperfusion has been believed to be beneficial to reduce infarct extension and minimize neurological damage. However recent evidence indicates that reperfusion itself may be detrimental to the ischemic tissue. A major pathway leading toward tissue injury involves elevation of extracellular glutamate and activation of glutamate receptors, with a subsequent increase in intracellular calcium and generation of reactive oxygen species and nitric oxide[28]. And that leukocytes play an important role in the development of ischemia-reperfusion injury by releasing various chemical mediators such as proteases, free radicals, and lipid-derived mediators[12,13,24].

Leukocyte doesn't adhere to venules by physical forces under normal perfusion conditions. However, as physical force reaches the zero point under ischemic conditions, leukocyte inclines to adhere to venule which it originally had, and adheres to endothelium. It causes the blocking of capillaries and the stoppage of capillary circulation[3,8,26]. Several factors, in addition to shear rate, may contribute to the modulation of leukocyte-endothelial cell adhesion in postcapillary venules exposed to ischemia-reperfusion. These include electrostatic cell surface charges, intercellular adhesion molecule-1(ICBM-1) and a group of CD11/CD18 gylcoproteins[12,31]. And the reactive oxygen metabolites released by activated leukocytes and endothelial cells has also been implicated in ischemia-reperfusion induced leukocyte accumulation[10]. Then NO may contribute to this response[12,21].

NO plays important physiological roles as well as pathophysiological roles in a wide range of disease. NO is synthesized by NO synthase(NOS). Three major types of NOS have been characterized : constitutive calcium-calmodulin dependent enzymes in endothelial cells(eNOS) and neurones(nNOS) and an inducible calcium independent enzyme(iNOS) in macrophage and many other cells. Meanwhile, nNOS and eNOS are both called constitutive NOS(cNOS) because they don't need new protein composition in the activation. On the other hand, iNOS must be compounded before producing NO. This procedure takes some time to compound protein affected by DNA transcription[5,9,18,27].

Under aqueous aerobic conditions NO is spontaneously oxidized to its inactive stable end product nitrite in a few seconds[19]. On the cellular level, NO has several targets of action. The low concentrations of NO activate the enzyme guanylate cyclase and thus increase the synthesis of cGMP leading to relaxation of vascular smooth muscle, inhibition of platelet aggregation and adhesion as well as to signal transduction in central and peripheral nervous system. NO has direct effects on various other enzymes. NO is known to inhibit cytochrome P-450. Ribonucleotide reductase in DNA synthesis are inactivated by NO. NO has direct effects on the synthesis of inflammatory mediators as it inhibits the activity of enzymes 5-lipoxygenase and NADPH oxidase which produce leucotrienes and superoxide anion in activated neutrophils. NO can act as a radical itself[23].

Various results on roles of NO under brain ischemia-reperfusion condition are reported, which seem to be related to NOS activating time[6,16,27,32]. NO produced by eNOS appears to protect the brain by enhancing cerebral blood flow in ischemic areas and perhaps by its inhibitory effect on platelet and leukocyte adhesion[15]. Enhanced NO production from eNOS may also promote angiogenesis in damaged tissue[34]. Inhibition of leukocyte adhesion to vascular endothelium by NO seems to be of major importance in situations relevant to vascular ischemic disease, because impairment of NO synthesis in ischemia-reperfusion injury result in an increased accumulation of leukocytes in the target tissues[22]. However, after ischemia develops, NO produced by overactivation of nNOS and, later, NO release by de novo expression of iNOS contribute to the brain damage[16,30]. Thus NO exerts both harmful and protective effects depending on its source of production[7,16,30].

There are multifactorial mechamisms of NO, serving to inhibit leukocyte endothelial interactions at the early stage in cerebral ischemia. Change in vessle shear rate, interactions to reactive oxygen metabolite formed by activated leukocyte or vascular endotheliums, alterations of adhesive glycoprotein on the surface of activated leukocyte and vascular endotheliums, constraints on producing inflammatory chemoattractants are likely candidates[24].

Shear rate of artery is closely related to vasodilatative effect of NO. When NO production is diminished, shear rate decreases, then accordingly, blood flow also decreases while adhesion of leukocyte is promoted[25].

NO, which reacts avidly with superoxide, is normally produced by vascular endothelium. Inhibition of NO product with L-arginine results in an intense leukocytes adherence response in venules, which suggest that NO is an endogenous inhibitor of leukocyte endothelial cell adhesion[21]. Consequently one would predict that conditions associated with an enhanced formation of superoxide should lead to increased leukocyte adherence by virtue of superoxide ability to render nitric oxide

biologically inactive[12]. Thus loss of NO after NOS inhibition could lead to increase in th levels of superoxide radical, a well established proadhherent molecule in a varity of microcirculatory beds[10].

NO may regulate in a direct fashion the expression of endothelial and leukocyte adhesion molecules. NO inhibits the endothelial, cyclic GMP dependent expression of P-selectin, which in turn promotes rolling of leukocytes on the endothelium at sites of inflammation before their firm adherence[11]. NO may also tonically prevent leukocyte adherence in a more indirect way by inhibiting the production of proinflammatory chemoattractants.

There is now considerable evidence indicating that an acute inflammatory response occurs after cerebral ischemia, characterized by a progressive increase in leukocyte adherence and infiltration over the initial hours to days after the insult[14]. Our study demonstrated that 9minutes asphyxia groups elicits significant leukocyte adherence during the initial 2hours of reperfusion. Under normal conditions, we confirmed that local inhibition of NOS with L-NA elicited a progressive increase in leukocyte adherence to the pial venular microcirculation over 2hours. Superfusion of L-NA through the cranial window for 2hours resulted in leukocyte adherence similar to that observed during the initial 2hours of reperfusion after asphyxia. Our findings indicate that NO also acts to inhibit the adherence of circulating leukocytes to cerebrovascular endothelium. And then the fact that no additional increase in leukocyte adherence occurred during the early postischemic reperfusion period after NOS inhibition by L-NA suggests that asphyxia-reperfusion resulted in a depletion of endogenous basal levels of NO.

Since our studies only examined the initial 2hours of reperfusion, the effect of large increases in NO production from inducible NOS on postischemic leukocyte adherence remains undefined.

The leukocyte accumulation and tissue destruction can be reversed by infusing NO donors which suggests that NO is an endogenous inhibitor of leukocyte endothelial cell adhesion[11,20]. Postischemic leukocyte adherence was dramatically reduced in our model when the organic nitrate NO donor SNP was superfused across the cortical surface at the start of reperfusion.

Conclusion

Nitric oxide inhibits leukocyte adherence to cerebral venules at early stage in cerebral ischemia, and that NO supplementation can reverse ischemia-reperfusion induced leukocyte adherence. It is now clear that NO plays major roles in modulating brain injury after ischemia-reperfusion events. These studies emphasize the necessity of developing a specific inhibitor of isoform NOS to adequately protect the brain from ischemia-reperfusion injury.

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