Nitric oxide protects blood-brain barrier in vitro from hypoxia/reoxygenation-mediated injury

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Abstract A cell culture model of blood-brain barrier (BBB, coculture of rat brain endothelial cells with rat astrocytes) was used to investigate the effect of nitric oxide (•NO) on the damage of the BBB induced by hypoxia/reoxygenation (H/R). Permeability coefficient of fluorescein across the endothelium was used as a marker of BBB tightness. The permeability coefficient increased 5.2 times after H/R indicating strong disruption of the BBB. The presence of the •NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 30 μM), authentic •NO (6 μM) or superoxide dismutase (50 units/ml) during H/R attenuated H/R-induced increase in permeability. 30 μM SNAP or 6 μM •NO did not influence the function of BBB during normoxia, however, severe disruption was observed using 150 μM of SNAP and more than 24 μM of •NO. After H/R of endothelial cells, the content of malondialdehyde (MDA) increased 2.3 times indicating radical-induced peroxidation of membrane lipids. 30 μM SNAP or 6 μM authentic •NO completely prevented MDA formation. The results show that •NO may effectively scavenge reactive oxygen species formed during H/R of brain capillary endothelial cells, affording protection of BBB at the molecular and functional level.

Key words: Nitric oxide; Blood-brain barrier; Hypoxia; Endothelial cell; Lipid peroxidation; Oxygen radical

1. Introduction

Nitric oxide (•NO) is known to serve as a messenger, maintaining different physiological and biochemical processes, especially by modulating the cellular content of cyclic GMP [1]. •NO may also cause cell and tissue injury [2], in particular by inhibiting the mitochondrial respiration chain leading to an increase in the rate of superoxide radical generation [3]. On the other hand, it has been reported that •NO directly protects membranes in various pathological situations, especially under conditions of increased rates of radical generation, such as Cu^{2+}- or lipoxygenase-induced lipid peroxidation [4,5]. •NO has been reported to participate in the detoxification of peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) [6,7]. Thus, •NO exerts both damaging and protective effects. Therefore, more information is required to define the physiological and pathophysiological conditions that determine whether •NO acts in a protective or a toxic manner [8].

The penetration of hydrophilic substances into and out of the brain is strictly regulated by the blood-brain barrier (BBB). The restricted permeability of this barrier, formed by brain capillary endothelial cells, is a decisive condition for maintaining the integrity of the brain. The BBB is disturbed under various pathological conditions including hypoxia/reoxygenation (H/R) [9]. Oxygen free radicals are suggested to play a crucial role in the disturbance of the endothelial barrier function under H/R [10]. Nitric oxide production increases in ischemic brain [11]. Recently, the destructive role of endogenous •NO has been demonstrated in the damage of the BBB during acute hypertension [12] or after excessive glutamate administration [13]. However, little is known about the mechanisms of •NO affecting BBB.

In this study, the effect of exogenous •NO was investigated using a cell culture model of BBB injured by H/R. The data obtained support the view that, under conditions of increased radical production, excess of •NO may effectively detoxify reactive oxygen species. This detoxification may prevent membrane lipid peroxidation and disruption of BBB caused by H/R.

2. Materials and methods

2.1. Reagents

S-nitroso penicillamine (SNAP) was purchased from Calbiochem. Immediately after dilution, aliquots of 8 mM solution were frozen in liquid nitrogen. Solutions were thawed immediately before use. Superoxide dismutase (SOD) was obtained from Sigma. Nitric oxide solutions were obtained and purified as described previously [14].

2.2. Cell culture

Cloned immortalized rat brain endothelial cells (RBE4 [15], kindly supplied by Prof. P.O. Couraud, Paris, France), were cultivated in HAM's F10/α-medium 1:1, 10% fetal bovine serum (FBS), 2 mM glutamine, 1 ng/ml bovine fibroblast growth factor (bFGF), 300 μg/ml geneticin (Boehringer Mannheim). Primary rat brain capillary endothelial cells were isolated and cultivated as described [16]. Astrocytes from neonatal rats were cultivated in DMEM with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Biochrom) [9]. To establish an in vitro BBB model, RBE4 were cultivated on filter inserts (polyethylene terephthalate, 0.45 μm pore size, 1.6×10^6 pores/cm^2, Falcon; coated with rat tail collagen I); astrocytes were seeded in 6-well plates containing filter inserts with RBE4.

2.3. Assay of sGC mRNA

The expression of soluble guanylate cyclase (sGC) mRNA (subunits α1, β1, α2, β2) was investigated by means of reverse transcription polymerase chain reaction (RT-PCR) in primary rat brain capillary endothelial cells, rat astrocytes and RBE4. Total cellular RNA was isolated using TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. Total RNA concentration was estimated by UV absorbance at 260 nm. The first-strand cDNA was synthesized by reverse transcription with 200 U MMLV-RT (Gibco-BRL) per μg RNA and 5 μM random hexamers. Control PCR assays were performed using specific primers to rat β-actin [17] as an external stand-

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ard. The presence of sGC subunits was examined by specific primers to α1 subunit and β1 subunit [18] and to α2 subunit and β2 subunit [19]. cDNA was amplified (25–35 cycles) at 95°C for 30 s, 60°C for 1 min, 72°C for 30 s for β-actin and 95°C for 30 s, 58°C for 1.5 min, 72°C for 1 min for sGC (melting, annealing and extension temperatures, respectively). Phage φX174 RF DNA digested with *Hae*III was used as molecular weight standard.

2.4. Hypoxia/reoxygenation experiments

To study the effect of H/R on malondialdehyde (MDA) content confluent monolayers of RBE4 cells cultivated on 6-well plates (Falcon) were washed twice with phosphate buffered solution (PBS, with Ca^{2+}, Mg^{2+}, glucose-free, pH 7.2, Biochrom), then 1 ml of PBS saturated with Nelson gas (95% N_2/5% CO_2) was added to each well. Cells were gassed for 120 min in an incubator at 37°C with Nelson gas to produce hypoxia. Reoxygenation was started by gassing the cells with Carbogen (95% O_2/5% CO_2) for 60 min. Since SNAP solutions have a short half-life, 15 or 30 μl of a 1 mM SNAP solution was added twice during reoxygenation time (at 0 and 30 min) to each well to afford the presence of •NO during reoxygenation. Similarly, 10 μl of 100 μM of authentic •NO solution was added six times every 10 min, starting from 0 min of reoxygenation.

To study the effect of H/R on permeability, filters of RBE4 cells cocultivated with primary astrocytes (BBB model) were put into the 6-well plates containing 1 ml PBS saturated with Nelson gas, then 1 ml of PBS was added into each filter. Duration of hypoxia and reoxygenation was 60 min and 30 min, respectively. 10 μl of 1 mM SNAP solution was added every 30 min (at 0 and 30 min of hypoxia and at 0 min of reoxygenation) to each well to afford the presence of •NO during both hypoxia and reoxygenation. Similarly, 10 μl of 100 μM of authentic •NO solution was added three times (every 20 min) during hypoxia and three times during reoxygenation (every 10 min). Oxygen tension under conditions of hypoxia was 25–30 mm Hg. pH of PBS saturated with Nelson gas was corrected to 7.2 by addition of KOH.

To study the effect of different concentrations of •NO and SNAP on BBB permeability under normoxic conditions, aliquots of SNAP and •NO were added to the filter inserts in a manner similar to H/R experiments.

2.5. Measurement of MDA and protein content

After H/R of RBE4, PBS was removed, and 0.5 ml of fresh PBS was added to each well. Cells were scraped off and frozen immediately in liquid nitrogen. MDA content of the samples was determined according to [20] using HPLC (Shimadzu LC-10A chromatograph with RF-10A fluorescence detector). Protein content was measured by a modified Lowry procedure [21] using a Beckman spectrophotometer DU 640.

2.6. Determination of permeability coefficient of Na-fluorescein

After incubation of cocultures PBS of filter inserts was replaced by 2 ml Na-fluorescein (50 μM, Molecular Probes) in PBS. Filters were put into a 6-well plate containing 2 ml of PBS. After 10 min of incubation, fluorescence ($\lambda_{exc} = 488$ nm, $\lambda_{em} = 512$ nm) was measured in the donor and receptor chambers. The permeability coefficient was calculated according to [22].

2.7. Measurement of nitric oxide concentrations

•NO concentrations were measured by ozone-mediated chemiluminescence using a Sievers 270B analyzer (purge vessel mode). The analyzer was calibrated by aliquots of sodium nitrite solutions added to acidified potassium iodide [23]. 100 μl aliquots of •NO solution were injected into the purge vessel. In order to estimate steady-state concentrations of •NO during H/R experiments using SNAP, 100 μl aliquots were taken from filter inserts after 10 min of hypoxia and after 60 min of hypoxia followed by 10 min of reoxygenation and injected into the purge vessel.

3. Results

The permeability coefficient of fluorescein was $0.8–2 \times 10^{-3}$ cm/min before any treatment. The permeability increased dramatically after 60 min hypoxia and 30 min reoxygenation, indicating strong disruption of the barrier; normoxic control did not show considerable increase in permeability. Addition of SNAP, authentic •NO and SOD during H/R strongly attenuated the changes in the permeability of fluorescein (Fig. 1). One day old SNAP solutions were not protective (data not shown) supporting the assumption that SNAP protects BBB via the release of nitric oxide. Steady-state concentrations of •NO in experiments with SNAP (final concentration 30 μM), measured after 10 min of hypoxia and after 60 min of hypoxia followed by 10 min of reoxygenation, were approximately 300 nM and 200 nM, respectively.

Addition of SNAP or •NO to normoxic controls did not cause disruption of the BBB at the concentrations used in H/R experiments, however, severe disruption was observed at 150 μM of SNAP (Fig. 2A) and at more than 24 μM of •NO solution (Fig. 2B).

RBE4 monolayers were used to investigate the effect of •NO or SNAP on the formation of MDA induced by H/R. No significant change in the MDA level was observed after 60 min of hypoxia and 30 min of reoxygenation (data not shown). MDA content was approximately doubled (as compared to the normoxic control) after longer-lasting hypoxia and reoxygenation (120 and 60 min). Addition of authentic •NO (6 μM) or SNAP (30 and 60 μM) to the incubation solution during reoxygenation completely prevented the increase in MDA content (Fig. 3). As shown in Fig. 3, neither authentic •NO nor SNAP alone had any effect on the MDA content in the normoxic control.

In order to investigate the involvement of •NO/sGC signal transduction pathway in effects of •NO on BBB, the presence of sGC mRNA was investigated in cells of BBB by RT-PCR. While there is a clear expression of α1/β1 subunits of sGC in rat astrocytes, no expression of sGC mRNA subunits was found in RBE4 (Fig. 4). However, expression of α1/β1 sGC subunits occurs in primary cultures of rat brain capillary en-

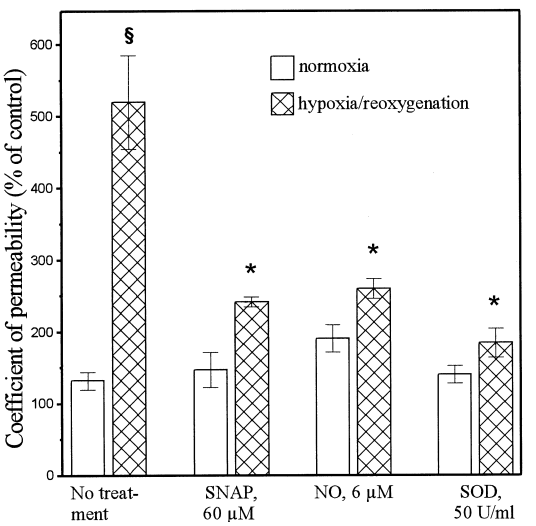

Fig. 1. Attenuation of hypoxia/reoxygenation-induced increase in permeability of fluorescein through a cell culture model of BBB by SNAP (30 μM), authentic •NO (6 μM) and SOD (50 U/ml). The BBB model was subjected to 60 min hypoxia and 30 min reoxygenation. SNAP was added at 0 and 30 min of hypoxia and at 0 min of reoxygenation. •NO was added at 0, 20 and 40 min of hypoxia and at 0, 10 and 20 min of reoxygenation. 100% refers to permeability coefficients obtained immediately after exchange of cell culture medium by PBS. Cumulative concentrations are shown (mean ± S.E.M., $n = 5–7$; $^{§}P < 0.05$ versus normoxia; $^{*}P < 0.05$ versus hypoxia).

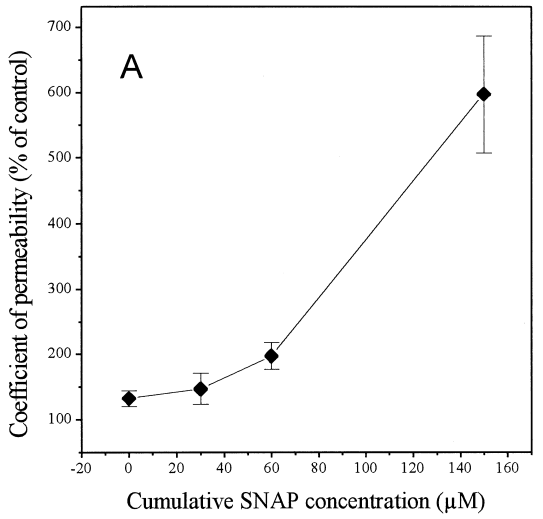

Fig. 2. Influence of different concentrations of SNAP (A) or authentic •NO (B) on the permeability of the BBB model under normoxic conditions (filters with rat brain endothelial cells were put in PBS for 90 min). Solutions of SNAP or •NO were added as described for Fig. 1. Cumulative concentrations are shown (mean ± S.E.M., $n = 4$).

dothelial cells. Expression of α2/β2 subunits was not observed in any cell type investigated (data not shown).

4. Discussion

Disruption of the BBB occurs under various pathological conditions, such as ischemia/reperfusion [24] leading to an increased cerebrovascular permeability with subsequent development of tissue edema [25]. Cell culture models of BBB have been applied to study the mechanisms of pathological processes, such as H/R [10], and specific aspects of barrier function, such as transport of substances through BBB [26].

The BBB model (using RBE4) provides characteristic features of the BBB, such as low permeability coefficient for fluorescein which is known to be not permeable into the brain. H/R results in a strong increase in fluorescein permeability indicating opening of BBB. Interestingly, there is almost no disruption of BBB by H/R in the presence of SOD. This is consistent with the observation of Terada [27] that the release of superoxide is the major source of membrane damage in bovine arterial endothelial cell under H/R. As SOD does not penetrate into cells, one may assume that superoxide released from the cells accounts for the BBB disruption under H/R, or that extracellular SOD may effectively decrease the intracellular superoxide concentration.

The mechanism of superoxide-mediated opening of BBB under H/R is not known. Very recently, the decisive role of superoxide radical has been shown in the post-hypoxic actin rearrangement in macrovascular endothelial cells [28]. The potential role of cytoskeleton has been reported in the disturbance of the endothelial barrier function induced by hypoxia [29] and by thrombin administration [30]. Therefore, it is probable that superoxide produced by H/R influences the state of the cytoskeleton and, consequently, cellular shape, opening paracellular pathways and increasing permeability.

There are complex mechanisms conceivable for the observed protection of the post-hypoxic BBB by •NO. •NO regulates many cellular processes via activation of sGC [1]. However, in contrast to astrocytes and primary rat brain endothelial cells, sGC mRNA has not been found in RBE4 in this study, suggesting that a •NO-induced cGMP pathway cannot be activated. The lack of sGC activity in RBE4 was also observed by other authors [31]. •NO may influence the transmembrane ion current in a cellular model of BBB [32]. Roussin's black salt (•NO donor compound) has been found to disturb a macrovascular endothelial barrier in a reversible manner [33]. The data presented here also suggest that relatively high doses of •NO open the barrier. In addition, there is no effect of SNAP or •NO on the tightness of BBB at con-

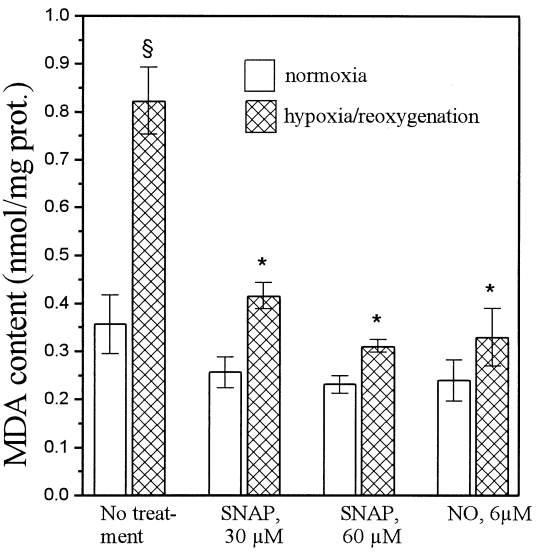

Fig. 3. Effect of SNAP or authentic •NO on the accumulation of MDA induced by hypoxia/reoxygenation in RBE4 cells. RBE4 monolayers were subjected to 120 min of hypoxia followed by 60 min reoxygenation. Solutions of SNAP were added at 0, 20 and 40 min of reoxygenation. Solutions of •NO were added every 10 min of reoxygenation starting from 0 min. Cumulative concentrations are shown (mean ± S.E.M., $n = 4$; $^{\S}P < 0.001$ versus normoxia; $^{*}P < 0.001$ versus hypoxia).

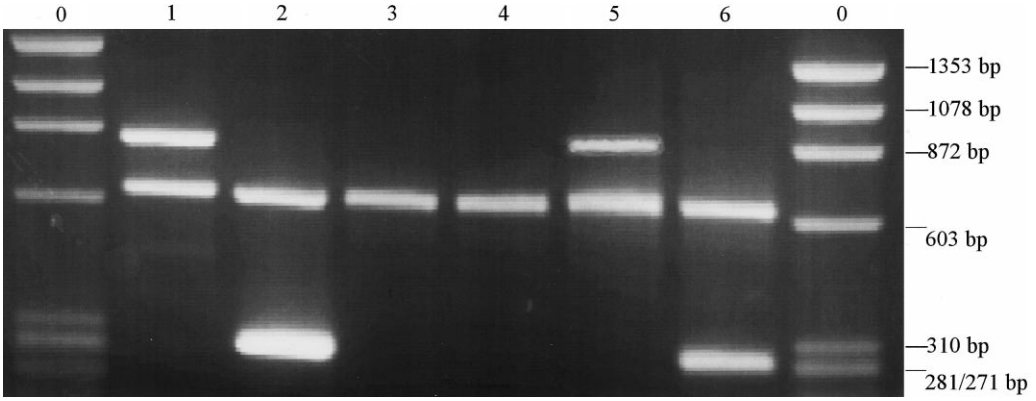

Fig. 4. RT-PCR detection of the α1 (826 bp, lanes 1, 3, 5) and β1 (284 bp, lanes 2, 4, 6) subunits of sGC mRNA in primary rat brain capillary endothelial cells (lanes 1, 2), RBE4 cells (lanes 3, 4) and astrocytes (lanes 5, 6). Lanes 0 are DNA size markers. β-Actin (630 bp) was amplified in all samples as an external standard.

centrations used for hypoxia/reoxygenation experiments in the normoxic control.

The protective effect of •NO may also be due to a scavenging of superoxide and/or peroxyl radicals since the corresponding rate constants of the reactions between these radicals are diffusion-controlled [34]. One possible mechanism of cellular protection against reactive oxygen species by •NO is the termination of the chain reaction of lipid peroxidation by scavenging peroxyl radicals [6]. •NO has been reported to prevent both Cu^{2+}-induced lipid peroxidation in low-density lipoproteins [5] and radical-induced accumulation of lipid hydroperoxides in phosphatidylcholine liposomes [35]. The appearance of MDA is a late event in lipid peroxidation [36] since two double bonds must be broken in the same fatty acid chain to release MDA. This is consistent with the data presented here since MDA accumulation occurs later than disruption of BBB. Nevertheless, we also observed a strong protective effect of •NO on H/R-induced MDA accumulation. As it is known that addition of SOD inhibits lipid peroxidation [37] and barrier opening [10] in post-hypoxic endothelial cells, •NO exerts protective properties similar to SOD under H/R of endothelial cells.

In conclusion, this study demonstrates the protective effect of nitric oxide on H/R-induced increase in blood-brain barrier permeability and lipid peroxidation. We suggest that the observed protection at the molecular and functional level is due to scavenging of oxygen free radicals by •NO.

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