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Hypoxia-Induced EDNO Release is Further Augmented by Previous Hypoxia and Reoxygenation in Rabbit Aortic Endothelium

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The present study was designed: (1) to determine whether or not hypoxia stimulates the release of endothelium-derived relaxing factors (EDRFs) from endothelial cells, and (2) to examine whether or not the hypoxia-induced EDRFs release is further augmented by previous hypoxia-reoxygenation, using bioassay system. In the bioassay experiment, rabbit aorta with endothelium was used as EDRFs donor vessel and rabbit carotid artery without endothelium as a bioassay test ring. The test ring was contracted by prostaglandin $F_{2\alpha}$ (3×10^{-6} M/L), which was added to the solution perfusing through the aortic segment. Hypoxia was evoked by switching the solution aerated with 95% O_2/5% CO_2 mixed gas to one aerated with 95% N_2/5% CO_2 mixed gas. When the contraction induced by prostaglandin $F_{2\alpha}$ reached a steady state, the solution was exchanged for hypoxic one. And then, hypoxia and reoxygenation were interchanged at intervals of 2 minutes (intermittent hypoxia). The endothelial cells were also exposed to single 10-minute hypoxia (continuous hypoxia). When the bioassay ring was superfused with the perfusate through intact aorta, hypoxia relaxed the precontracted bioassay test ring markedly. Whereas, when bioassay ring was superfused with the perfusate through denuded aorta or polyethylene tubing, hypoxia relaxed the precontracted ring slightly. The relaxation was not inhibited by indomethacin but by nitro-L-arginine or methylene blue. The hypoxia-induced relaxation was further augmented by previous hypoxia-reoxygenation and the magnitude of the relaxation by intermittent hypoxia was significantly greater than that of the relaxation by continuous hypoxia. The results suggest that hypoxia stimulates EDNO release from endothelial cells and that the hypoxia-induced EDNO release is further augmented by previous hypoxia-reoxygenation.

Key Words: Endothelium, Nitric oxide, Hypoxia, Reoxygenation

INTRODUCTION

Ischemia followed by reperfusion (ischemia/reperfusion) occurs in many pathologic conditions such as myocardial infarction, cerebral ischemia, or organ transplantation and results in functional and structural damage to the organs. Previous ischemia/reperfusion (preconditioning ischemia) reduces the injury by the following ischemia/reperfusion (Murky et al, 1986; Yellon et al, 1993) and is suggested to protect vascular endothelium from ischemia/reperfusion injury (Defily & Chilian, 1993). Although there is a good evidence that preconditioning has a protective effect on the myocardial cells through A1 receptor activation (Liu et al, 1991) and adenosine is an initiator and mediator of preconditioning effect in myocardial cells (Thornton et al, 1993), the mechanism of preconditioning ischemia is not yet completely understood.

Endothelial cells are the first target of ischemia/reperfusion due to their localization at the interface between blood and tissue, and have been shown to be very sensitive to such conditions (Suval et al,

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1987; Forman et al, 1989). Endothelial cells can release a number of vasoactive factors in response to a variety of hormonal and physical stimuli. Endothelial cells metabolize L-arginine to nitric oxide (NO) (Palmer et al, 1988), the active component of endothelium-derived relaxing factor (EDRF). Hypoxia, which is the most important component of ischemia, is one of the stimuli to release a number of vasoactive factors from endothelial cells. The studies on the effect of hypoxia on the release of EDRFs from endothelial cells have produced conflicting hypotheses. Some studies have proposed that hypoxia reduces EDRFs release to contribute hypoxic vasoconstriction (Johns et al, 1989; Roadman et al, 1990; Kovitz et al, 1993; Pearson et al, 1993), whereas others have proposed that hypoxia stimulates the synthesis of endothelium-derived autacoids, such as EDRF and prostaglandin (Archer et al, 1989; Brown et al, 1993; Michiels et al, 1993; Pearson et al, 1993).

What are the effects of repetitive episodes of brief hypoxia and reoxygenation, which is similar to preconditioning ischemia, on the release of endothelium-derived autacoids? With the exception of two previous studies (Yang & Mehta, 1994; Yang & Mehta, 1995), the effects of repetitive episodes of brief hypoxia and reoxygenation on the release of endothelium-derived autacoids remain poorly understood. Thus, our objective in the present study was twofold. First, we tried to determine whether or not hypoxia stimulates the release of EDRFs from endothelial cells. Second, we examined whether the hypoxia-induced EDRFs release is further augmented by previous hypoxia and reoxygenation.

METHODS

Animal preparation

Rabbits of either sex, weighing about 2.5 kg, were killed by exsanguination from the femoral artery under sodium pentobarbital (40 mg/kg) anesthesia. The common carotid artery and a long aortic segment including thoracic and abdominal aorta were excised and immersed in the Krebs Ringer bicarbonate solution at room temperature and cleaned by removing connective tissues surrounding the vessels. The endothelial cells of the carotid artery were removed by gentle rubbing with a moistened cotton ball. Successful removal of functional endothelial cells was assumed from the absence of any detectable relaxation by acetylcholine (10^{-6} M/L) in the preparations contracted with 10^{-6} M/L norepinephrine.

Bioassay experiment

A aortic segment with intact endothelium (about 5 cm in length) was cannulated with polyethylene tubing and placed in an organ chamber filled with Krebs Ringer bicarbonate solution which was aerated with 95% O_2/5% CO_2 mixed gas and kept at 36.5°C (Fig. 1). The aortic segment was perfused at a constant flow (2 ml/min) by means of roller pump (Pharmacia Fine Chemicals) with modified Krebs Ringer solution (Rubanyi et al, 1985). A ring of carotid artery, the endothelium of which had been removed (bioassay test ring), was suspended directly below the organ chamber by means of stainless steel stirrups and superfused with the perfusate that had passed through

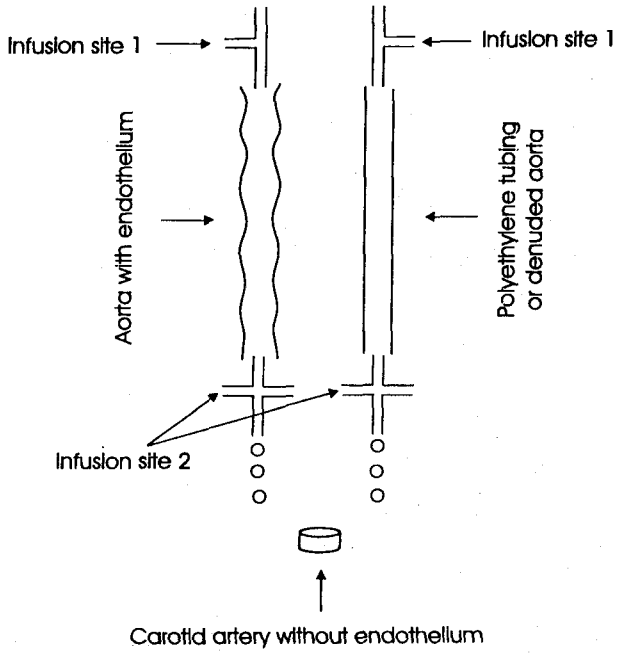

Fig. 1. A schematic presentation of bioassay system. A carotid arterial ring without endothelium was mounted for isometric tension recording and superfused with Krebs-Ringer bicarbonate solution that had passed through polyethylene tubing or through aortic segments with or without endothelium. Drugs could be infused separately into perfusion line either above (infusion site 1) or below the aortic segment (infusion site 2). Methylene blue was infused at the site 2 and indomethacin and nitro-L-arginine was infused at the site 1.

the aortic segment. One stirrup was connected to an isometric force transducer (Grass FT03) and changes in isometric tension were recorded (Grass physiograph model 7E).

The bioassay ring was superfused with the solution that had passed through the aortic segment for 60 min, and then it was stretched until the basal tension reached approximately 2 g. One hour later, prostaglandin $F_{2\alpha}$ was added to the solution perfusing the aortic segment. When the contraction of the bioassay ring had reached a steady state, the solution perfusing the aortic segment was switched to a solution aerated with 95% N_2/5% CO_2 mixed gas (hypoxia) and the concentration of prostaglandin $F_{2\alpha}$ was not changed. After the donor aorta was exposed to hypoxia for 2 minutes, the solution perfusing the aortic segment was switched to a solution aerated with 95% O_2/5% CO_2 mixed gas (reoxygenation). Hypoxia and reoxygenation were interchanged at intervals of 2 minutes (intermittent hypoxia). The endothelial cells were also exposed to 10 minute hypoxia and 2 minute reoxygenation (continuous hypoxia). When endothelial cells and vascular smooth muscles are exposed to hypoxia/reoxygenation, they may be injured by hypoxia and/or reoxygenation. As intermittent hypoxia and continous hypoxia are different experimental conditions, there may be a difference in the severities of the injuries caused by them. Therefore, in the experiments to compare the effect of intermittent hypoxia on endothelial cells with that of continuous hypoxia, EDRF donor aorta and the bioassay ring were exposed to intermittent hypoxia at first and then to continuous hypoxia in half of the experiments. In the rest of the experiments, the vessels were exposed to continuous hypoxia at first and then to intermittent hypoxia.

Methylene blue was infused at infusion site 2 below the donor aorta at a constant speed (0.02 ml/min) by infusion pump (SRI) (Fig. 1) and nitro-L-arginine and indomethacin was applied to the solution above the donor aorta (infusion site 1).

Solutions and drugs

The ionic composition of the modified Krebs Ringer solution was as follows (in mM/L) : NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.22, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, CaEDTA 0.016, and glucose 11.1. The solution was aerated with 95% O_2/5% CO_2 or 95% N_2/5% CO_2 (pH 7.3~7.4). Drugs used were indomethacin, methylene blue, prostaglandin $F_{2\alpha}$ (all from Sigma, U.S.A.), and nitro-L-arginine (Aldrich).

Statistics

Experimental values were expressed as means± SEM and n is the number of the experiments and of animals from which blood vessels were taken. Statistical significance was determined using paired Student's t-test, and probabilities of less than 5% ($p<0.05$) were considered significant.

RESULTS

The effect of hypoxia on the release of relaxing factors from endothelial cells

Fig. 2 shows the effect of hypoxia on the release of vasoactive factors from endothelial cells. When the solution perfusing EDRFs donor aorta was switched to hypoxic one, the perfusate through EDRFs donor vessel with intact endothelium relaxed the precontracted bioassay ring. The tension of the bioassay ring in hypoxia was gradually decreased with the repetition of hypoxia and reoxygenation. On return to reoxygenation from hypoxia, the relaxed rings were contracted in most recordings. In a few cases (3 among 32 cases), the bioassay ring was further

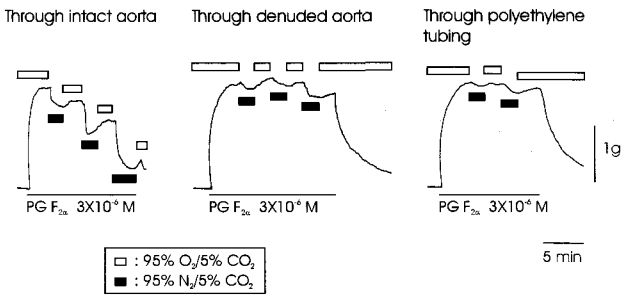

Fig. 2. Representative recordings showing the effect of hypoxia on the contraction induced by prostaglandin $F_{2\alpha}$ (3×10^{-6} M/L). When the solution perfusing the donor aorta was switched to hypoxia, the perfusate through the aorta with intact endothelium relaxed bioassay ring markedly. Subsequent hypoxia followed by hypoxia-reoxygenation induced a further decrease in the tension of bioassay ring. The bioassay rings were slightly relaxed by the perfusate through denuded aorta and polyethylene tubing.

relaxed by reoxygenation but in most cases (the rest among 32 cases), the bioassay ring was contracted by reoxygenation. In these cases, the amounts of the recontractions were variable. In most cases, the contractions in the reoxygenation status were lesser than the initial contractions. But, in a few cases, the recontractions in the reoxygenation status were greater than the initial contractions (Fig. 4B)

When the EDRFs donor aorta was exposed to hypoxia for the second time, the tension of the ring was further decreased, compared with the decreased tension induced by the first exposure to hypoxia. The third exposure to hypoxia reduced the tension of the ring further to a much lower level than the second exposure did. Whereas, when bioassay ring was superfused with the perfusate through denuded aorta or polyethylene tubing, the precontracted bioassay ring was slightly relaxed by the hypoxic perfusate and the relaxation of the bioassay ring in hypoxia was not gradually increased with the repetition of hypoxia and reoxygenation. As the hypoxic superfusate through denuded aorta or polyethylene tubing did not contact with endothelial cells, the slight relaxation induced by

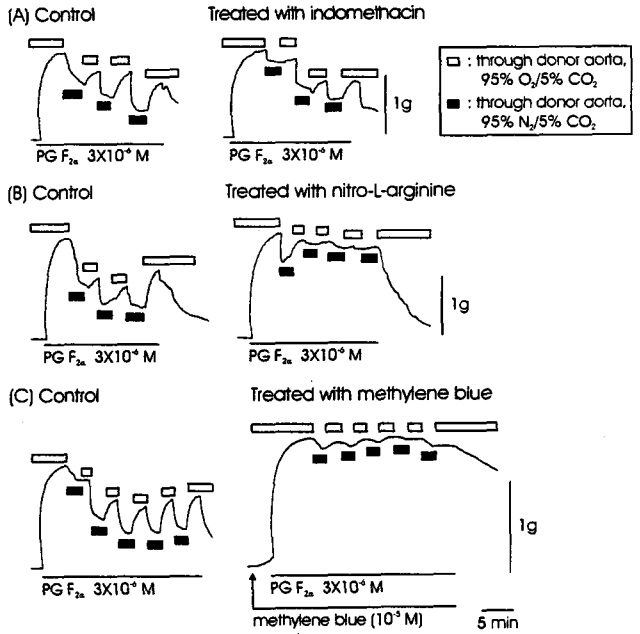

Fig. 3. Representative recordings showing the effects of indomethacin (10^{-5} M/L for 30 min) (A), nitro-L-arginine (10^{-5} M/L for 30 minutes) (B), and methylene blue (10^{-5} M/L for 5 minutes) (C) on hypoxia-induced relaxation. The hypoxia- induced relaxation was not attenuated by the treatment with indomethacin, while the relaxation was inhibited by nitro-L-arginine or methylene blue.

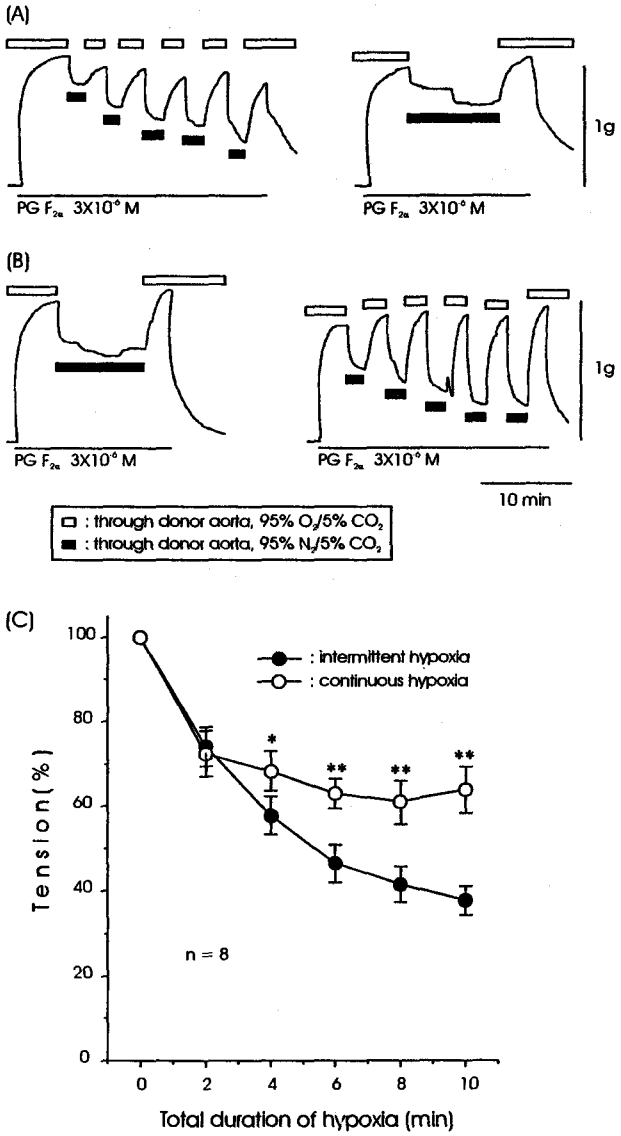

Fig. 4. Effect of intermittent hypoxia and continuous hypoxia on the release of EDNO. In upper traces (A), endothelial cells were exposed to intermittent hypoxia at first and then continuous hypoxia. In lower traces (B), endothelial cells were exposed to continuous hypoxia at first. The responses shown in A and B were recorded from the different tissues. The magnitude of hypoxia-induced relaxation was significantly greater in the groups exposed to intermittent hypoxia than that of the relaxation in the group exposed to continuous hypoxia (C). In half of the cases, endothelial cells were exposed to intermittent hypoxia at first and then continuous hypoxia. In the remaining half, endothelial cells were exposed to continuous hypoxia at first. Values are means ± SEM and expressed as percent of initial contraction to prostaglandin $F_{2\alpha}$. *$p<0.05$, **$p<0.01$ between intermittent hypoxia and continuous hypoxia.

hypoxia was not endothelium-dependent one but direct vascular smooth muscle relaxation by hypoxia (Fig. 2).

These observations suggest that endothelial cells play an important role in the hypoxia-induced relaxation and that a relaxing agent released from endothelial cells of EDRFs donor vessel produces the relaxation. Endothelial cells release a variety of EDRFs in response to various stimuli. The hypoxia-induced relaxation was not inhibited by cyclooxygenase inhibitor, indomethacin but by nitro-L-arginine or methylene blue. As nitro-L-arginine is a competitive inhibitor of NO synthase and methylene blue is an inhibitor of soluble guanylate cyclase, these data suggest that the hypoxia-induced relaxation is mediated by endothelium-derived nitric oxide (EDNO) (Fig. 3).

Repetitive hypoxia vs. continuous hypoxia

Subsequent hypoxia followed by hypoxia-reoxygenation induced a further decrease in the tension of the bioassay ring. When the vessels were exposed to intermittent hypoxia, the relaxation induced by the second hypoxic exposure was greatly increased, compared with the initial hypoxic relaxation ($p<0.01$). When the vessels were exposed to the third hypoxic episode, the relaxation induced by the third hypoxic exposure was also greatly increased, compared with the second hypoxic relaxation ($p<0.01$). However, there was no significant increase in the magnitude of the relaxation induced by the fourth or the fifth episode of hypoxia, compared with that of the relaxation induced by the previous hypoxia ($p>0.05$) (Fig. 4).

Hypoxia-induced relaxation was also observed by single exposure to continuous hypoxia for 10 minutes. When the vessels were exposed to the continuous hypoxia, the tension of the bioassay ring was decreased in a initial fast phase and reached a steady-state within 2 minutes of hypoxia. After reached a steady state, the tension of the ring was decreased very slowly, and then increased.

The total hypoxic duration exposed to intermittent hypoxia was increased with the repetition of hypoxia and reoxygenation. However, the magnitude of hypoxia-induced relaxation was greater in the groups exposed to intermittent hypoxia than that of the relaxation in the group exposed to continuous hypoxia. When total duration of hypoxia was 2 minutes, there was no difference in the hypoxia-induced relaxation between intermittent hypoxia and continuous hypoxia. In the case of intermittent hypoxia, total duration of hypoxia was 4 minutes at the end of the second episode of hypoxia. When total duration of hypoxia was 4 minutes, there was a significant difference in the hypoxia-induced relaxation between intermittent hypoxia and continuous hypoxia and the difference was gradually increased with the increase of total hypoxic duration.

DISCUSSION

This study shows that vascular endothelial cells release EDRFs by the exposure to acute hypoxia and that the hypoxia-induced release of EDRFs is further augmented by previous hypoxia and reoxygenation.

In response to hypoxia, the perfusate that had passed through intact donor aorta relaxed the bioassay ring and the hypoxia-induced relaxation was not reduced by indomethacin but was abolished by nitro-L-arginine or methylene blue. Therefore, it could be concluded that the hypoxia-induced relaxation of the bioassay ring is mediated by EDNO released from endothelial cells and that hypoxia stimulates the release of EDNO from endothelial cells.

The effect of reoxygenation on the release of EDRFs from endothelial cells is poorly understood. Arnould et al (1992) reported that $[Ca^{2+}]_i$, which was increased during hypoxic period, decreased exponentially during following reoxygenation period almostly to the basal level of resting cells. However, there are some reports suggesting that reoxygenation may stimulate the synthesis of EDRFs. Close et al (1994) reported that reoxygenation induced endothelium-dependent relaxation, but the relaxation was supposed to be induced by another EDRF but NO. In this experiment, the effect of reoxygenation was not always the same. When the bioassay ring was exposed to reoxygenation, the effect of reoxygenation on the tension of the bioassay ring was variable from relaxation to contraction. Although we did not investigate further about the causes of this conflicting results, it could be interpreted as follows. As a burst of free radicals was reported to occur immediately after reperfusion (Arroyo et al, 1987; Bolli et al, 1988), reoxygenation could produce oxygen free radicals, involving hydrogen peroxide and hydroxyl radicals. Hydrogen peroxide and hydroxyl radicals have been reported to trigger the release of EDRFs

from endothelial cells (Rubanyi & Vanhoutte, 1986a). Therefore, reoxygenation could produce oxygen free radicals to stimulate the release of EDRFs from endothelial cells. As oxygen free radicals inactivate EDNO via a direct interaction, whether the bioassay ring contracts or relaxes by the exposure to reoxygenation might depend on the balance of the amounts of EDNO and oxygen free radicals produced by reoxygenation. If additional EDNO production by reoxygenation overcomes the free radicals production, the bioassay ring relaxes additionally. If superoxide radicals production by reoxygenation overcomes the additional EDNO production by reoxygenation, the bioassay ring contracts.

Repeated exposure to hypoxia (intermittent hypoxia) resulted in an increase in the magnitude of hypoxia-induced relaxation (Fig. 4A, C). When the vessels were exposed to continuous hypoxia, the bioassay ring was relaxed by the hypoxic perfusate initially and then the hypoxia-induced relaxation was reached a steady-state in spite of the increase of hypoxic duration (Fig. 4B, C). As it can be seen clearly in Fig. 4, when the accumulated hypoxic durations exposed to endothelial cells are equal between continuous hypoxia and intermittent hypoxia, the magnitude of the relaxation by intermittent hypoxia was greater than that of the relaxation by continuous hypoxia. Therefore, it could be inferred from these data that hypoxia-induced EDNO release is further augmented by previous hypoxia and reoxygenation.

Arnould et al (1992) have shown that severe hypoxia leads to an increase in $[Ca^{2+}]_i$, which is not enough to account for loss of viability but is within the range of concentrations observed during stimulation of endothelial cells. As the elevated $[Ca^{2+}]_i$ is an effective stimulus to release EDNO from endothelial cells (Furchgott, 1983; Cannel & Sage, 1989), hypoxia may act as a stimulus to produce EDNO. In addition, there is an evidence that NO directly activates Ca^{2+}-dependent potassium channel (Bolotina et al, 1994). In endothelial cells, Ca^{2+} influx is modulated by membrane potential. Depolarizing endothelial cells decreases Ca^{2+} influx. This in turn reduces the production and release of EDRFs (Nilius et al, 1997). Hyperpolarization of endothelial cells increases Ca^{2+} influx through endothelial cell membrane (Groschner et al, 1992; Demirel et al, 1993) and the increased $[Ca^{2+}]_i$ stimulates to produce NO (Furchgott, 1983; Cannel & Sage, 1989; Groschner et al, 1992; Demirel et al, 1993). All of these findings explain how hypoxia stimulates endothelial cells to release EDNO. However, the mechanisms responsible for hypoxia-induced Ca^{2+} influx and the augmentation of EDNO release by previous hypoxia-reoxygenation still remain under further investigations.

Ischemia and hypoxia are not synonymous and the consequences of hypoxia are not necessarily the same as the consequences of ischemia. Nevertheless, hypoxia may be the most important component of ischemia, especially in brief ischemia. Therefore it could be suggested that ischemia stimulates EDNO release from endothelial cells and that ischemia-induced EDNO release is further augmented by previous ischemia and reperfusion (preconditioning).

NO formation may be protective or toxic (Stamler, 1994). In systems where toxicity is incurred predominantly from oxygen free radicals, NO may be protective and thus limit damage. In systems where toxicity originates from NO biosynthesis, the reactions of NO with oxygen free radicals generate peroxynitrite (Beckman et al, 1990), which is directly cytotoxic (Beckman, 1991). As oxygen free radicals are produced by ischemia-reperfusion (Arroyo et al, 1987; Bolli et al, 1988) and oxygen free radicals act an important role in causing ischemia-reperfusion injury (Granger, 1988; Forman et al, 1990), NO formation in endothelial cells may be protective against ischemia-reperfusion injury. In addition, endothelial cells secrete a variety of compounds that act to maintain microcirculatory flow (Nees et al, 1985). EDRF, the precursor of NO, enhances collateral circulation (Randall & Griffith, 1992) and inhibits platelet aggregation. Therefore it could be suggested that the augmented NO formation contributes to maintain and improve blood flow. Increased blood flow can easily wash out oxygen free radicals from ischemic region. Through these effects, NO may play an important role to lessen ischemia-reperfusion injury and to preserve the integrity of endothelial cells from ischemia-reperfusion injury.

In conclusion, we have demonstrated at first that hypoxia induces EDNO release from endothelial cells and that the hypoxia-induced release of EDNO is further augmented by previous hypoxia-reoxygenation. We need to investigate further the mechanisms responsible for hypoxia-induced Ca^{2+} influx and the augmentation of EDNO release by previous hypoxia-reoxygenation.

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