

## Reoxygenation Stimulates EDRF(s) Release from Endothelial Cells of Rabbit Aorta

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We have reported that hypoxia stimulates EDRF(s) release from endothelial cells and the release may be augmented by previous hypoxia. As a mechanism, it was hypothesized that reoxygenation can stimulate EDRF(s) release from endothelial cells and we tested the hypothesis via bioassay experiment. In the bioassay experiment, rabbit aorta with endothelium was used as EDRF donor vessel and rabbit carotid artery without endothelium as a bioassay test ring. The test ring was contracted by prostaglandin F<sub>2α</sub> ( $3 \times 10^{-6}$  M) which was added to the solution perfusing through the aorta. Hypoxia was evoked by switching the solution aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub> mixed gas to one aerated with 95% N<sub>2</sub>/5% CO<sub>2</sub> mixed gas. Hypoxia/reoxygenation were interexchanged at intervals of 2 minutes (intermittent hypoxia). In some experiments, endothelial cells were exposed to 10-minute hypoxia (continuous hypoxia) and then exposed to reoxygenation and intermittent hypoxia. In other experiments, the duration of reoxygenation was extended from 2 minutes to 5 minutes. When the donor aorta was exposed to intermittent hypoxia, hypoxia stimulated EDRF(s) release from endothelial cells and the hypoxia-induced EDRF(s) release was augmented by previous hypoxia/reoxygenation. When the donor aorta was exposed to continuous hypoxia, there was no increase of hypoxia-induced EDRF(s) release during hypoxia. But, after the donor aorta was exposed to reoxygenation, hypoxia-induced EDRF(s) release was markedly increased. When the donor aorta was pretreated with nitro-L-arginine ( $10^{-5}$  M for 30 minutes), the initial hypoxia-induced EDRF(s) release was almost completely abolished, but the mechanism for EDRF(s) release by the reoxygenation and subsequent hypoxia still remained to be clarified. TEA also blocked incompletely hypoxia-induced and hypoxia/reoxygenation-induced EDRF(s) release. EDRF(s) release by repetitive hypoxia and reoxygenation was completely blocked by the combined treatment with nitro-L-arginine and TEA. Cytochrome P450 blocker, SKF-525A, inhibited the EDRF(s) release reversibly and endothelin antagonists, BQ 123 and BQ 788, had no effect on the release of endothelium-derived vasoactive factors. Superoxide dismutase (SOD) and catalase inhibited the EDRF(s) release from endothelial cells. From these data, it could be concluded that reoxygenation stimulates EDRF(s) release and hypoxia/reoxygenation can release not only NO but also another EDRF from endothelial cells by the production of oxygen free radicals.

Key Words: Endothelium, EDRF(s), Hypoxia, Reoxygenation, Oxygen free radicals

### INTRODUCTION

Endothelial cells release a number of vasorelaxing

and contracting factors in response to a variety of hormonal and physical stimuli. Among the vasorelaxing factors released by endothelial cells nitric oxide (NO) is the most intensively studied, which is produced from L-arginine (Palmer et al, 1988). Hypoxia, which is the most important component of brief ischemia, is one of the stimuli to evoke the release of vasoactive factors from endothelial cells. The

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studies about the effect of hypoxia on EDRF(s) release from endothelial cells have shown conflicting results. Some studies have proposed that hypoxia reduces EDRF(s) 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 EDRF(s), such as EDNO and prostaglandin (Archer et al, 1989; Brown et al, 1993; Michiels et al, 1993; Pearson et al, 1993). These conflicting results might depend on the experimental conditions, such as animal species, the degree of hypoxia, and kinds of blood vessels. None the less, some other findings support that endothelial cells release EDRF(s) in response to hypoxia. Hypoxia induces an increase in  $[Ca^{2+}]_i$  in cultured endothelial cells (Luckhoff et al, 1986; Arnould et al, 1992). The increase of  $[Ca^{2+}]_i$  critically depends on the presence of extracellular  $Ca^{2+}$  and correlates tightly with the release of NO (Busse et al, 1993). Recently, we also demonstrated that hypoxia stimulates the release of EDRF(s) from the aortic endothelium and the release can be augmented by the previous hypoxia (Han et al, 1998).

Although there are many reports about the effect of hypoxia on the release of EDRF(s) from endothelial cells, the effect of reoxygenation on the release is poorly understood. Arnould et al (1992) reported that  $[Ca^{2+}]_i$ , which is increased during hypoxic period, decreased exponentially during following reoxygenation period almost to the basal level of resting cells. The decrease of  $[Ca^{2+}]_i$  by reoxygenation may cause to cease the hypoxia-induced EDRF(s) release from endothelial cells. On the contrary, there are several reports suggesting that reoxygenation might stimulate the synthesis of EDRF(s). Reoxygenation produces oxygen free radicals including hydrogen peroxide and hydroxyl radicals. Hydrogen peroxide and hydroxyl radicals can trigger EDRF(s) release from endothelial cells (Rubanyi & Vanhoutte, 1986a; Rubanyi, 1988). Although oxygen free radicals inactivate NO (Beckman et al, 1990; Beckman, 1991; Stamler, 1994), there is a possibility that oxygen free radicals produced by hypoxia and reoxygenation might stimulate endothelial cells to release EDRF(s). Close et al (1994) reported that reoxygenation induces endothelium-dependent relaxation, but the relaxation was supposed to be induced by another EDRF but NO. In addition, Paller et al (1998) reported that increased generation of NO by hypoxia

and reoxygenation cause cell death in renal epithelial cells.

Previously, we reported that hypoxia-induced EDRF(s) release is augmented by previous hypoxia/reoxygenation and repetitive hypoxia can release EDRF(s) more than continuous hypoxia does (Han et al, 1998). As a mechanism, we hypothesized that reoxygenation stimulates EDRF(s) release from endothelial cells. The additive effect of reoxygenation to hypoxia-induced EDRF(s) release from endothelial cells may cause to increase EDRF(s) release by repetitive hypoxia and reoxygenation, compared with initial hypoxia. Therefore, in this study, we investigated the effect of reoxygenation on the release of EDRF(s) from endothelium.

## 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 thoracic and abdominal aorta and common carotid artery 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) in the preparations precontracted with  $10^{-6}$  M norepinephrine.

### *Bioassay experiment*

A long aortic segment including thoracic and abdominal aorta 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  $36.5^\circ C$  (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, of which the endothelium 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 the aortic segment or polyethylene tubing. One stirrup was connected to an isometric force transducer (Grass FT03) and changes in isometric tension were recorded (Grass physiograph model 7E).

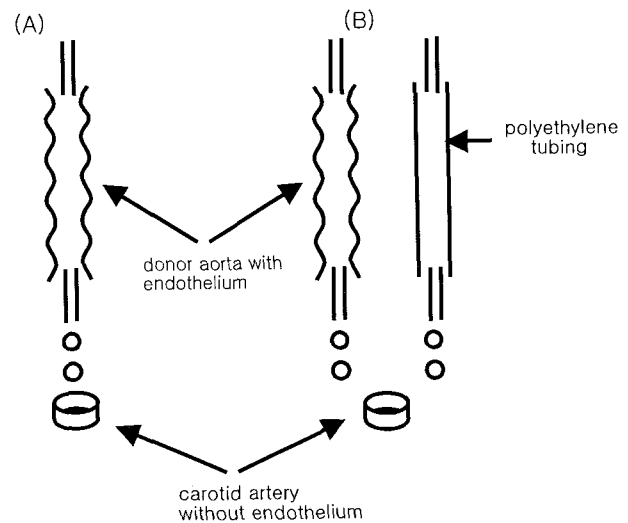
The bioassay test ring was superfused with the solution that had passed through EDRF donor aorta or polyethylene tubing for 60 min, and then it was stretched until the basal tension reached approximately 2 g.

#### Experimental protocol 1

The test ring was superfused with the solution through the donor aorta and contracted by the application of prostaglandin  $F_{2\alpha}$  ( $3 \times 10^{-6}$  M) to the solution perfusing the aortic segment (Fig. 1A). 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). In some experiments, the donor aorta was exposed to 10-minute hypoxia (continuous hypoxia) and then to intermittent hypoxia.

#### Experimental protocol 2

The test ring was superfused with the solution through polyethylene tubing and contracted by the application of prostaglandin  $F_{2\alpha}$  to the solutions perfusing the donor aorta and polyethylene tubing (Fig. 1B). When the contraction of the bioassay ring had reached a steady state, the solution superfusing the bioassay test ring was switched from the perfusate through polyethylene tubing to one through the donor aorta. With the switch, the tension of the bioassay ring was changed and reached a new steady state within 2 minutes in most of the cases. After 2 minutes elapsed, the solution perfusing the donor aorta was switched from a solution aerated with 95%  $O_2/5\%$   $CO_2$  mixed gas to one aerated with 95%  $N_2/5\%$   $CO_2$  mixed gas. After the EDRF(s) donor vessel was exposed to hypoxia for 2 minutes, the solution superfusing the test ring was switched to the



**Fig. 1.** A schematic presentation of bioassay system. A carotid arterial ring without endothelium was mounted to record isometric tension and superfused with Krebs-Ringer bicarbonate solution that had passed through aorta with endothelium or polyethylene tubing at constant flow (2 ml/min) by means of a peristaltic pump. When the donor aorta was exposed to the reoxygenated solution for 2 min, the test ring was superfused with the perfusate through the donor aorta only (A). When the duration of reoxygenation was increased to 5 minutes, the test ring was superfused with the perfusate through polyethylene tubing. After the contraction reached a steady state, the superfusate was switched from the perfusate through polyethylene tubing to that through the donor aorta (B).

perfusate through polyethylene tubing. Just at the same time, the solution perfusing the donor aorta was switched to a solution aerated with 95%  $O_2/5\%$   $CO_2$  mixed gas. After the bioassay ring was superfused with the perfusate through polyethylene tubing for about 3 minutes, the superfusate to the test ring was switched to the perfusate through EDRF(s) donor aorta. After the bioassay ring was superfused by the perfusate through EDRF(s) donor aorta for 2 minutes, the donor aorta was re-exposed to hypoxia by switching the perfusing solution to hypoxic one. These procedures exposing to hypoxia and reoxygenation were repeated 3 or 5 times in each experiment.

In these experimental protocols, the duration of reoxygenation following hypoxia was 2 minutes in the protocol 1 and 5 minutes in the protocol 2.

### Solutions and drugs

The ionic composition of the modified Krebs Ringer solution was as follows (in mM): NaCl 118.3, KCl 4.7, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.22, CaCl<sub>2</sub> 2.5, NaHCO<sub>3</sub> 25.0, CaEDTA 0.016, and glucose 11.1. The solution was aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub> or 95% N<sub>2</sub>/5% CO<sub>2</sub> mixed gas (pH 7.3~7.4). All drugs and chemicals except BQ 123, BQ 788, and SKF-525A (RBI, USA) used in this study were purchased from Sigma Chemical Co. (USA).

### Statistics

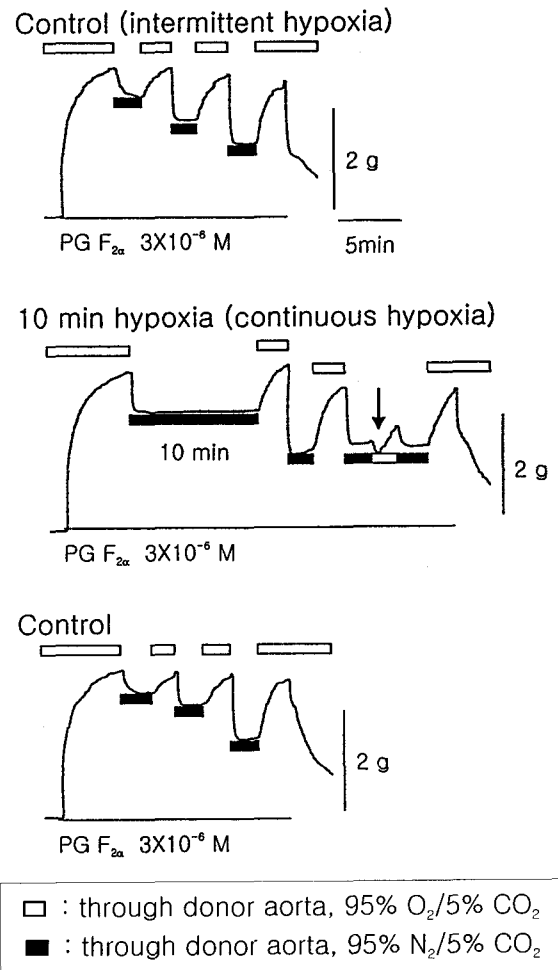
Experimental values were expressed as means  $\pm$  SEM and *n* is the number of the experiments or of animals from which blood vessels were taken. Statistical significance was determined using paired Student's *t*-test, and probabilities of less than 5% (*p* < 0.05) were considered significant.

## RESULTS

### Effect of reoxygenation on the release of EDRF(s) by hypoxia from endothelial cells

Fig. 2 shows a typical response of EDRF(s) release to hypoxia and reoxygenation by the first experimental protocol. When the donor aorta was exposed to hypoxia at a steady state of the contraction by prostaglandin F<sub>2 $\alpha$</sub> , the precontracted test ring was relaxed by the perfusate through the donor aorta and the hypoxia-induced relaxation was increased by the re-exposure to hypoxia. We demonstrated that the hypoxia-induced relaxation was endothelium-dependent and not blocked by indomethacin but by nitro-L-arginine or methylene blue (Han et al, 1988).

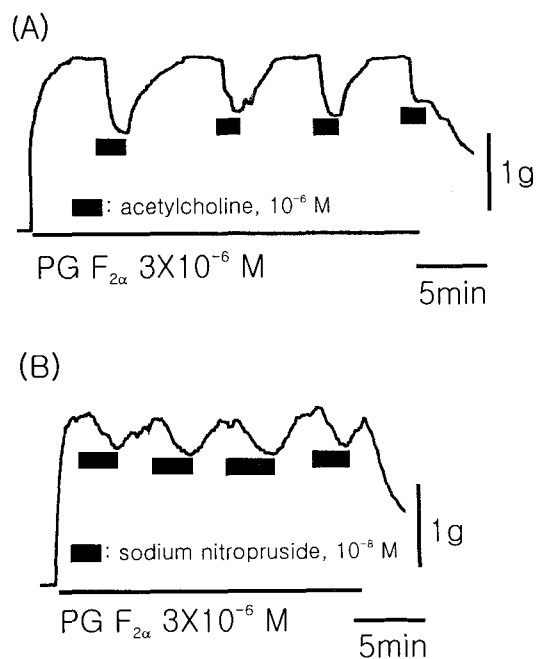
When the donor aorta was exposed to hypoxia for 10 min (continuous hypoxia), the relaxation of test ring was not increased and remained relatively constant. After 10-minute hypoxia, the donor aorta was exposed to reoxygenation and the test ring was recontracted by the exposure. And then the donor aorta was exposed to intermittent hypoxia subsequently. After 2-minutes exposure to reoxygenation, the donor aorta was re-exposed to hypoxia. The re-exposure relaxed the test ring more than the previous continuous hypoxia did. In almost all cases, tensions were recovered by reoxygenation. But, in a case



**Fig. 2.** Representative recordings showing the effect of continuous and intermittent hypoxia on the contraction induced by prostaglandin F<sub>2 $\alpha$</sub> . When the donor aorta and test ring were exposed to hypoxia, the test ring was relaxed by the superfusate. The hypoxia-induced relaxation was augmented step by step on the repetitive application of hypoxia/reoxygenation procedures. When the donor aorta was exposed to continuous hypoxia, the hypoxia-induced relaxation of test ring was much potentiated after reoxygenation.

indicated by arrow, the test ring was initially relaxed by reoxygenation and then contracted. Compared with the previous contractions, the magnitude of the contraction was greatly decreased. These data suggested that reoxygenation plays an important role in hypoxia-induced EDRF(s) release.

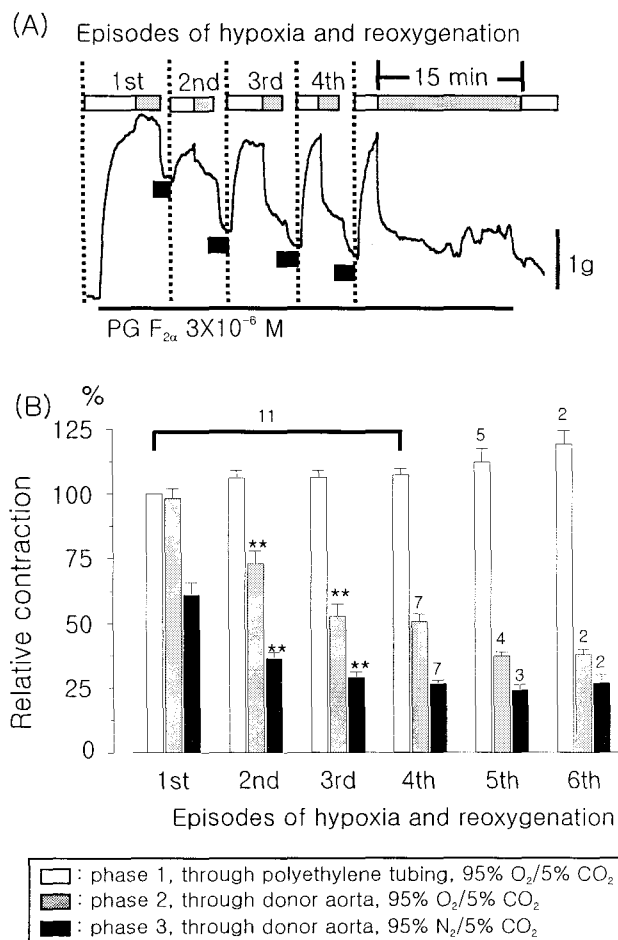
To test whether repeated exposures to a stimulant cause to increase the magnitude of the relaxation of test ring, the effects of repeated application of ACh were examined (Fig. 3A). When the contraction of



**Fig. 3.** Effect of repetitive application of acetylcholine (ACh) or sodium nitroprusside on the contraction induced by prostaglandin  $F_{2\alpha}$ . The magnitude of relaxation by ACh was decreased with the repetitive application (A) and the relaxation by sodium nitroprusside was not altered on the repetitive application (B).

the test ring by prostaglandin  $F_{2\alpha}$  reached a steady state, ACh was applied to the solution perfusing the donor aorta and the test ring was relaxed by the ACh application. We applied ACh repetitively to evoke repetitive relaxation of the test ring, but the magnitude of relaxation was not increased but slightly decreased by the repeated application of ACh. The amplitude of contraction by prostaglandin  $F_{2\alpha}$  was not changed with the repetitive application of ACh. These data suggested that the repeated exposure to a stimulant cannot increase EDRF(s) release.

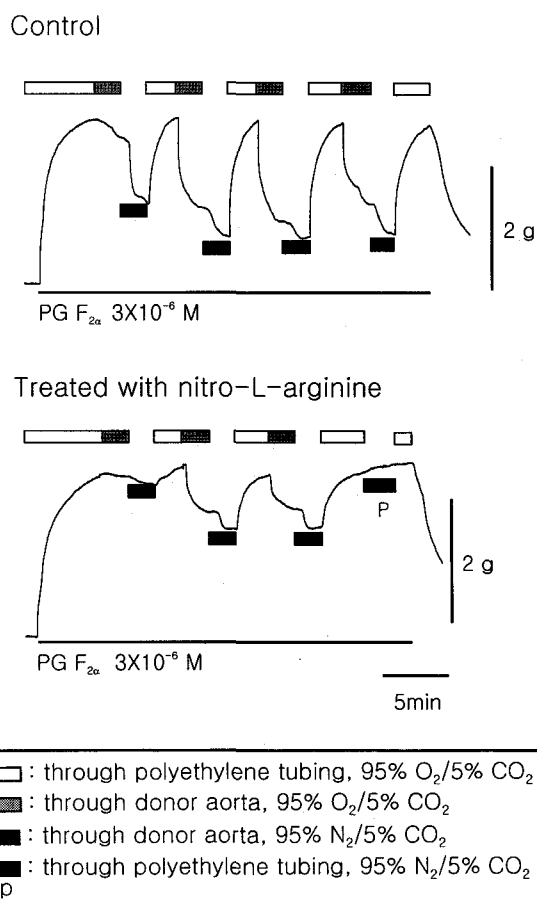
It was also examined whether the repeated exposure to a relaxant can cause to increase the magnitude of relaxation or not (Fig. 3B). When the contraction of the test ring reached a steady state, sodium nitroprusside was applied to the solution perfusing the donor aorta. The test ring was relaxed by the application, but the magnitude of relaxation was not increased by the repeated application. The magnitude of contraction by prostaglandin  $F_{2\alpha}$  was not changed by the repetitive application of nitroprusside. These data suggested that the repeated exposure to a relaxant cannot increase the sensitivity to that relaxant.



**Fig. 4.** Effect of the increase in reoxygenation duration on hypoxia and hypoxia/reoxygenation-induced relaxation of test ring. A; during reoxygenation periods, the test ring was still relaxed. After the donor aorta was exposed to 2-minute hypoxia and reoxygenation four times, the reoxygenated perfusate through the donor aorta relaxed the test ring for more than 15 minutes. We classified the continuous phase 1, phase 2, and phase 3 as one episode of hypoxia and reoxygenation. B; the tension of phase 1 at each episode was represented as percentage to the initial contraction. The tensions in phase 2 and phase 3 were represented as percentage to that of the phase 1 at same episode. Values represent means  $\pm$  SEM and the numbers above the column indicate the number of experiments. Each value of a phase was compared with the value of the previous same phase and \*\* indicates significantly different from each other  $P < 0.01$ .

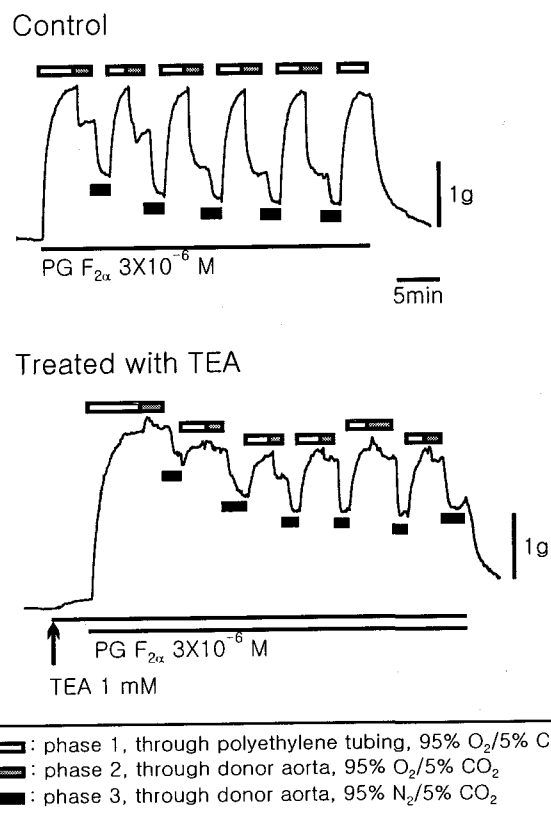
#### *Effect of the increase in reoxygenation duration on EDRF release*

The duration of reoxygenation was increased from 2 minutes to 15 minutes to test whether EDRF(s) is



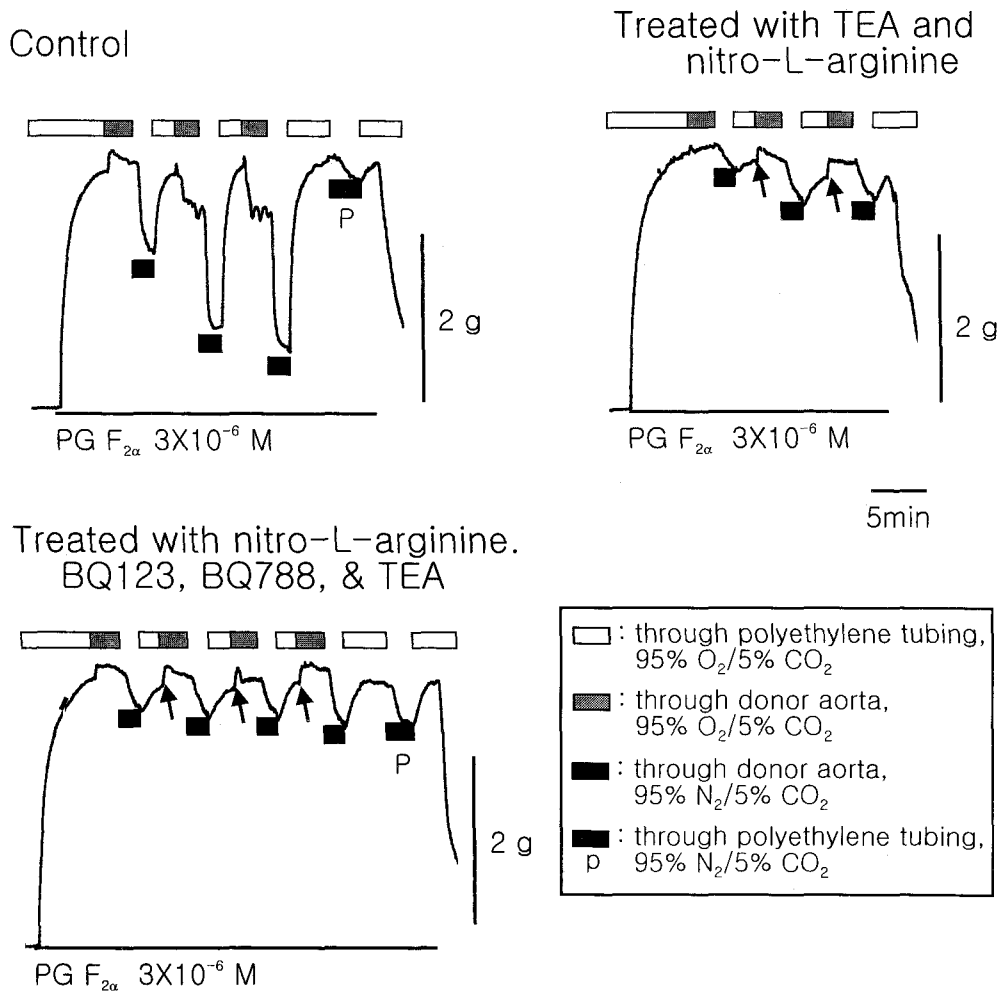
**Fig. 5.** Representative recordings showing the effect of nitro-L-arginine ( $10^{-5}$  M for 30 min) on the hypoxia or hypoxia/reoxygenation-induced EDRF(s) release. When the donor aorta was pretreated with nitro-L-arginine, the initial exposure to hypoxia relaxed the test ring slightly. But, the test ring was relaxed markedly by the exposure to reoxygenation.

released during the reoxygenated period or not (Fig. 4). At first, the test ring was superfused with the perfusate through polyethylene tubing and contracted by the application of prostaglandin  $F_{2\alpha}$  (Phase 1). When the contraction reached a steady state, the solution superfusing the test ring was switched from the solution through polyethylene tubing to one through EDRF donor aorta and the tension of the test ring was slightly changed by the switch (Phase 2). The change in tension reflected the amount of basal EDRF(s) release from the donor aorta. When the donor aorta was exposed to hypoxia, the test ring was relaxed (Phase 3). After the donor aorta was exposed to hypoxia for 2 minutes and the test ring was superfused by the perfusate through the donor aorta,



**Fig. 6.** Representative recordings showing the effect of TEA ( $10^{-3}$  M for 5 min) on the hypoxia or hypoxia/reoxygenation-induced EDRF(s) release. The EDRF(s) release was inhibited by the pretreatment, but not completely and the relaxation at phase 2 was markedly inhibited.

the donor aorta was reoxygenated and the solution superfusing the test ring was switched to the solution through polyethylene tubing (Phase 1). When the test ring was superfused with the perfusate, the relaxed test ring was recontracted almost to the initial contraction level. This means that the responsiveness of test ring to prostaglandin  $F_{2\alpha}$  was not altered by the exposure to hypoxia and reoxygenation. When the solution superfusing the test ring was switched from the solution through polyethylene tubing to one through the donor aorta, the test ring was relaxed (Phase 2). And the test ring was further relaxed by the exposure to hypoxia. The tension in phase 1 was not changed with the repetition of hypoxia and reoxygenation, which means the sensitivity to prostaglandin  $F_{2\alpha}$  was not changed by the repetition. Compared with the initial hypoxia-induced relaxation, the reoxygenation and repeated hypoxia evoked greater



**Fig. 7.** Representative recordings showing the effect of nitro-L-arginine ( $10^{-5}$  M for 30 min), TEA ( $10^{-3}$  M for 5 min), BQ 123 ( $10^{-6}$  M for 5 min), and BQ 788 ( $10^{-6}$  M for 5 min) on the hypoxia or hypoxia/reoxygenation-induced EDRF(s) release. The EDRF(s) release was almost completely inhibited by the pretreatment.

relaxation of the test ring. In Fig. 4A, the first reoxygenation relaxed the test ring a little, but the tension at phase 2 was greatly decreased with the repetition of hypoxia and reoxygenation. After the donor aorta was exposed to hypoxia and reoxygenation four times, the tension at phase 2 remained at a very decreased level for more than 15 minutes.

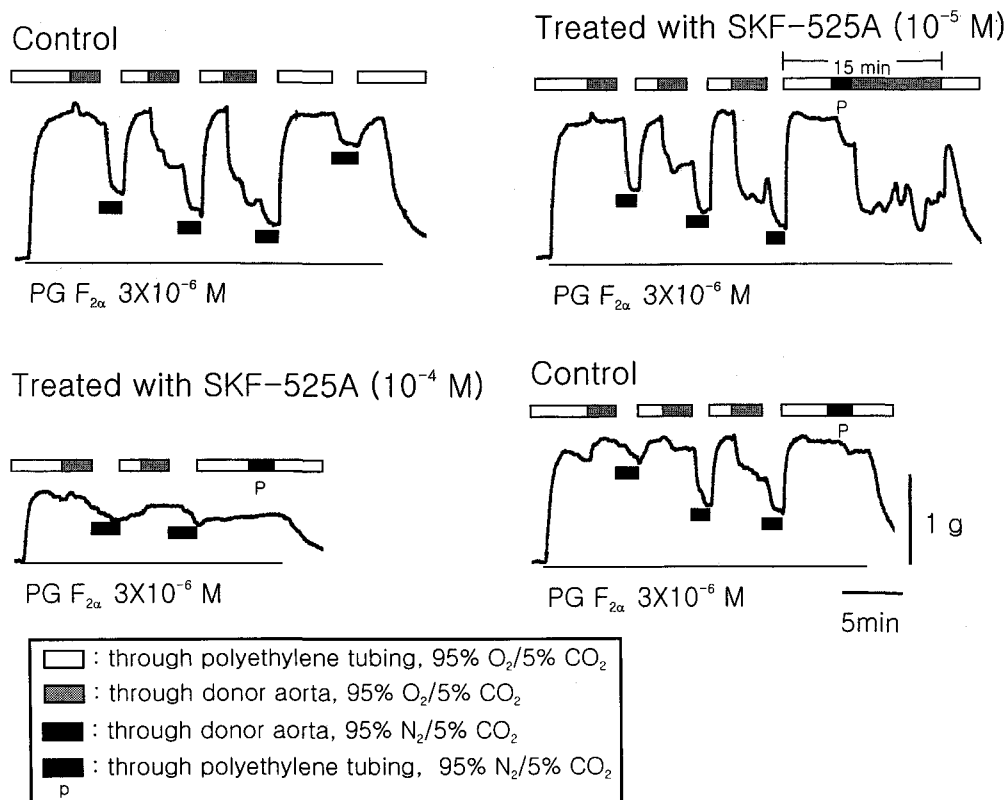
Nitro-L-arginine was used to inhibit EDNO production by hypoxia and reoxygenation (Fig. 5). Without the pretreatment with nitro-L-arginine, hypoxia and reoxygenation/hypoxia relaxed the test ring markedly. When the donor aorta was pretreated with nitro-L-arginine ( $10^{-5}$  M) for 30 minutes, the initial hypoxia-induced relaxation was almost completely inhibited. But, when the donor aorta was exposed to reoxygenation, the reoxygenation produced a greater

relaxation than the initial hypoxia did. The following hypoxia evoked a further relaxation to the test ring. When the test ring was exposed to hypoxia by the solution through polyethylene tubing, there was no change in the contraction of the test ring, which means the relaxation by hypoxia and reoxygenation was endothelium-dependent response.

All of these data indicated that reoxygenation stimulates EDRF(s) release from endothelial cells.

*How many kinds of endothelium-derived vasoactive factors can be released from endothelial cells by hypoxia and hypoxia/reoxygenation?*

Although nitro-L-arginine blocked almost completely the initial hypoxia-induced relaxation, reoxy-



**Fig. 8.** Representative recordings showing the effect of SKF-525A on the hypoxia or hypoxia/reoxygenation-induced EDRF(s) release. The EDRF(s) release was almost completely inhibited by the pretreatment with SKF-525A at high concentration.

genation and repeated hypoxia-induced relaxation was not completely blocked by the pretreatment (Fig. 5). When TEA was applied to the solution perfusing the donor aorta and test ring, the relaxation in phase 2 was selectively blocked and the relaxation in phase 3 still remained (Fig. 6). When the donor aorta was pretreated with TEA and nitro-L-arginine or TEA, nitro-L-arginine, BQ 123 and BQ 788, hypoxia and reoxygenation/hypoxia slightly relaxed the test ring (Fig. 7). But, a similar magnitude of relaxation was evoked by the hypoxic superfusate through polyethylene tubing. These data suggest that the small relaxation was due to a direct effect of hypoxia on smooth muscle, and that hypoxia and reoxygenation might release another EDRF(s) except NO.

When the donor aorta was pretreated with TEA and nitro-L-arginine, the tension of the test ring was increased by switching the superfusate of test ring from the perfusate through polyethylene tubing to the perfusate through the donor aorta (indicated by arrows). These data suggest that vasoconstricting fac-

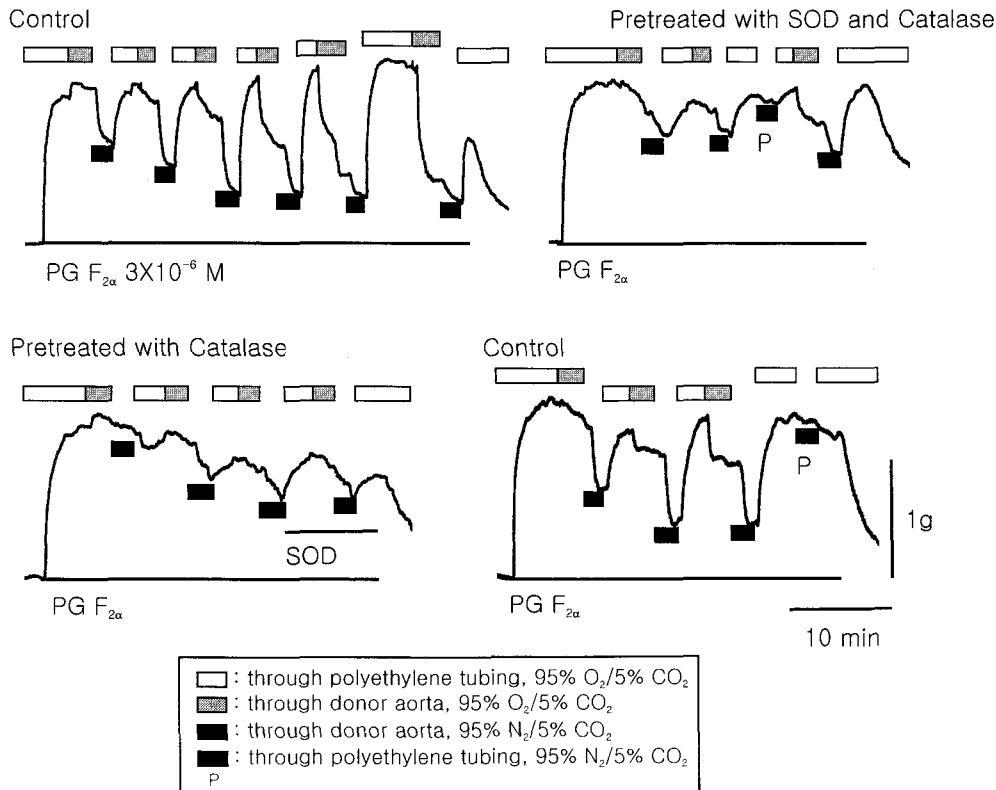
tors, such as endothelins, might be released from the donor aorta. But, the endothelin blockers, BQ 123 and BQ 788 did not block the increase of the tension.

Endothelial cells can release various kinds of EDRFs and one of the candidates is the metabolite of cytochrome P450, which can be blocked by SKF-525A. When the donor aorta was pretreated with  $10^{-5}$  M SKF-525A, there was no change in the relaxation and contraction (Fig. 8). With the increase of the concentration to  $10^{-4}$  M, the relaxation by hypoxia and hypoxia/reoxygenation was markedly decreased and the contraction by prostaglandin  $F_{2\alpha}$  was also markedly decreased. The effect of SKF-525A was reversible, and the hypoxia- and hypoxia/reoxygenation-induced relaxation was recovered with time.

#### *Effect of SOD and catalase on EDRF(s) release by hypoxia and hypoxia/reoxygenation*

As hypoxia and reoxygenation can produce oxygen





**Fig. 9.** Representative recordings showing the effect of superoxide dismutase (SOD) (50 units/ml) and catalase (500 units/ml) on the hypoxia or hypoxia/reoxygenation-induced EDRF(s) release. The donor aorta was pretreated for 5 minutes with SOD or catalase and the EDRF(s) release was inhibited by the pretreatment.

free radicals, we tested the effect of radical scavengers, SOD and catalase, on hypoxia and hypoxia/reoxygenation-induced EDRF(s) release (Fig. 9). Hypoxia and hypoxia/reoxygenation-induced EDRF(s) release was markedly inhibited by the application of catalase and SOD. Pretreatment with catalase also inhibited the EDRF(s) release. But, the treatment with catalase and SOD did not inhibit EDRF(s) release completely.

## DISCUSSION

In this study, we first demonstrated that reoxygenation stimulates EDRF(s) release from endothelial cells. As the EDRF(s) release was partially blocked by the application of SOD and catalase, it could be suggested that the EDRF(s) release might be, at least in part, due to oxygen free radicals generated by hypoxia and reoxygenation. Previously, we reported

that hypoxia stimulates EDRF(s) release from endothelial cells, and previous hypoxia and reoxygenation augment hypoxia-induced EDRF(s) release (Han et al, 1998). As EDRF(s) can be released by reoxygenation from endothelial cells and the release persist for a long times after exposure to repetitive hypoxia and reoxygenation, it could be suggested that the augmented release of EDRF(s) by previous hypoxia and reoxygenation might be due to reoxygenation-induced EDRF(s) release.

Arnould et al (1992) have shown that severe hypoxia increases  $[Ca^{2+}]_i$  within the range of concentrations observed during stimulation of endothelial cells. As the elevated  $[Ca^{2+}]_i$  could lead to an activation of endothelial cells to release EDRF(s) (Furchgott, 1983; Cannell & Sage, 1993), hypoxia may act as a stimulus to produce EDRF(s). However, the possible mechanism that reoxygenation stimulates EDRF(s) release is not elucidated yet. One of the possible mechanisms is that oxygen free radicals

generated by hypoxia and reoxygenation stimulate endothelial cells to release EDRF(s). This suggestion was supported by the finding that hypoxia- and hypoxia/reoxygenation-induced EDRF(s) release was blocked by the application of SOD and catalase. Bolli et al (1988) reported that the production of free radicals occurred during ischemia and most of it took place within seconds of reperfusion. The production of radicals abated but did not cease, persisting up to 3 hours after reperfusion. As oxygen free radical can stimulate EDRF(s) release, it could be suggested that the generation of EDRF(s) last for a long time. This suggestion is consistent with the finding that the relaxing activity of the perfusate of donor aorta still remained even though 17 minutes had passed after reoxygenation. Koliwad et al (1996) reported that oxidant stress activates a nonselective cation channel in calf vascular endothelial cells and elevates cytosolic free  $\text{Ca}^{2+}$ . This report suggests that reoxygenation might activate a nonselective cation current and elevate cytosolic free  $\text{Ca}^{2+}$  to release EDRF(s).

There is an evidence that NO directly activates  $\text{Ca}^{2+}$ -dependent potassium channel (Bolotina et al, 1994). In endothelial cells, receptor-operated  $\text{Ca}^{2+}$  channels are considered to play an important role in  $\text{Ca}^{2+}$  influx, while available evidence argues against a significant role for voltage-operated  $\text{Ca}^{2+}$  channel (Hallam et al, 1986; Demirel et al, 1993). Thus, hyperpolarization of endothelial cells increases  $\text{Ca}^{2+}$  influx through endothelial cell membrane (Groschner et al, 1992; Demirel et al, 1993) and the increased  $[\text{Ca}^{2+}]_i$  stimulates to produce NO (Furchgott, 1983; Cannell & Sage, 1993; Groschner et al, 1992; Demirel et al, 1993). This suggestion can be supported by the finding that hypoxia-induced and hypoxia/reoxygenation-induced EDRF(s) release was inhibited by the pretreatment with TEA that could block  $\text{K}^+$  channel. Therefore, it could be suggested that  $\text{K}^+$  channels play a key role in hypoxia- and hypoxia/reoxygenation-induced EDRF(s) release.

There are several reports suggesting that  $\text{K}^+$  channels play a key role in the mechanisms by which  $\text{O}_2$ -sensitive arterial myocytes respond to hypoxia (Iizuka et al, 1985; Lopez-Barneo et al, 1988; Delpiano & Hescheler, 1989; Ganfornina & Lopez-Barneo, 1991; Peers & Green, 1991; Yuan et al, 1993; Lopez-Barneo, 1994; Yuan et al, 1994; Yuan et al, 1995; Hatton & Peers, 1996) and it was reported that cytochrome *P-450* might be involved in the regulation of  $\text{K}^+$  channel activity of arterial myo-

cytes by hypoxia (Yuan et al, 1995; Hatton & Peers, 1996). These findings suggest an important role for cytochrome *P-450* in  $\text{O}_2$  sensing in these arterial cells. Therefore, the release of EDRF(s) by hypoxia or hypoxia/reoxygenation may be the result of the changes of ion channel activities, which is regulated by  $\text{PO}_2$  and cytochrome *P-450*. Cytochrome *P-450* is also known to be involved in the release of another EDRF, EDHF (Bauersachs et al, 1994; Hecker et al, 1994; Campbell et al, 1996; Popp et al, 1996). In addition, cytochrome *P-450* was reported to be involved in the  $\text{Ca}^{2+}$  entry and EDRF(s) release in human and bovine endothelial cells (Graier WF et al, 1995). All of these data suggest that cytochrome *P-450* may be involved in hypoxia and hypoxia/reoxygenation-induced EDRF(s) release. This suggestion was confirmed by the finding that SKF-525A, cytochrome *P-450* blocker, inhibited the hypoxia- and hypoxia/reoxygenation-induced EDRF(s) release. The contraction by prostaglandin  $\text{F}_{2\alpha}$  was also affected by the pretreatment at high concentration of SKF-525A. These data suggest that cytochrome *P-450* might inhibit EDRF(s) release and the contraction by blocking  $\text{Ca}^{2+}$  entry.

Hypoxia and hypoxia/reoxygenation-induced EDRF(s) release was not blocked completely by the treatment with nitro-L-arginine or TEA and an almost complete blockade was accomplished by the combined treatment with nitro-L-arginine and TEA. These data suggest that hypoxia or hypoxia/reoxygenation may release at least two different EDRFs. As shown in Fig. 6, TEA can inhibit both the release of EDRF(s) and the relaxing activity of EDRF(s). Endothelium-dependent hyperpolarization by EDHF can be blocked by the pretreatment with TEA. Hypoxia and hypoxia/reoxygenation-induced EDRF(s) release was blocked by the treatment with cytochrome *P-450* blocker and EDHF was reported to be the metabolite of cytochrome *P-450* (Popp et al, 1996). These data suggest that EDHF might be released from endothelial cell by hypoxia and hypoxia/reoxygenation.

In conclusion, our data presented that hypoxia or reoxygenation stimulate EDRF(s) release from endothelial cells. The release of EDRF(s) might be, at least in part, due to the oxygen radicals generated by hypoxia or hypoxia/reoxygenation. This finding may explain the mechanism for the previous report (Han et al, 1998) that hypoxia-induced EDRF(s) release can be augmented by previous hypoxia and reoxygenation. In addition, EDRFs released by hypoxia and

hypoxia/reoxygenation may be at least two, NO and EDHF. The mechanisms by which hypoxia and hypoxia/reoxygenation can stimulate EDRF(s) release from endothelial cells are still unknown and remained to be further investigated.

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