Korean J. of Pharmacology
Vol. 29, No. 2, pp. 203-212, 1993

Study on Mechanical Responses Induced by Hypoxia in Porcine Isolated Cerebral Artery

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ABSTRACT

This study was designed to observe hypoxia-induced mechanical responses of porcine cerebral artery and to clarify their possible mechanisms. Hypoxia produced a transient vasoconstriction, recovering to the basal tension within 10 min and subsequent reoxygenation produced a biphasic (relaxation-contraction) response in rings with endothelium under resting tension. Hypoxia produced a further contraction in rings precontracted with KCl or $PGF_{2\alpha}$, and following reoxygenation caused only sustained relaxation. Removal of the endothelium and pretreatment with nimodipine or indomethacin markedly attenuated the hypoxia- and reoxygenation-induced contractions. The KCl-induced contraction was not affected in hypoxic state, but contractions induced by $PGF_{2\alpha}$ or endothelin (ET) were inhibited in the hypoxia, the latter being more sensitive to the hypoxia. Upon reoxygenation, the attenuated contraction rapidly recovered to the original tension. Both hypoxia and reoxygenation significantly increased cyclic GMP content in the intact preparations, but not in the endothelium-removed ones. Acetylcholine (ACh) produced concentration-dependent relaxations in the intact endothelial rings precontracted with $PGF_{2\alpha}$ or endothelin, and the ACh-induced relaxation was inhibited by removal of endothelium and by hypoxia. ACh also increased cyclic GMP content in tissues pretreated with $PGF_{2\alpha}$ and the increase of cyclic GMP was abolished in hypoxic state.

These results suggest that hypoxia- and reoxygenation-induced contractions are dependent on endothelium and extracellular calcium, and related to the release of prostaglandin-like substance(s).

Key Words: Porcine cerebral artery, Hypoxia, Hypoxia-induced contraction, Endothelium, Cyclic GMP, Acetylcholine

INTRODUCTION

Since it was known that hypoxia in vascular smooth muscles produces mechanical responses, vasorelaxation and vasoconstriction, numerous research efforts have been focused on the mechanisms underlying the responses, but no consensus has been reached thus far. Some have shown that mechanical tension in some vascular smooth muscles is sensitive to oxygen tension in the bath (Carrier *et al.*, 1964; Gellai *et al.*, 1973; Shepherd and Vanhoutte, 1979; Singer *et al.*, 1981) and suggested that oxygen directly affects vascular tone by changing ion conductance across the cell membrane(Coburn *et al.*, 1979; Wei *et al.*, 1980; Marshall and Marshall, 1988). Others reported that the hypoxia limits oxidative energy generation, which in turn inhibits the mechanical responses (Pittman and Duling, 1973; Boddeke *et al.*, 1989). On the other hand, hypoxic mechanical responses are attributed to endothelium-derived relaxing factor (EDRF) and contracting factor (EDCF) and/or to arachidonic acid metabolites in peripheral arteries (Busse *et al.*, 1983; Jackson, 1988; O'Brien *et al.*, 1987).

Cerebral vasospasm is the leading cause of mor-

bidity and motality in patients with intracranial hemorrhage (Nakagomi *et al.*, 1987). When bleeding takes place in the brain, the cerebral artery is exposed to lysed blood and is also ischemic and becomes very sensitive to vasoconstrictor effects of blood products (Duckles *et al.*, 1977; Svendgaard *et al.*, 1977). Katusic and Vanhoutte (1986) found that in dog isolated basilar artery, removal of the endothelium abolished the hypoxic contraction under basal tension and reduced the contraction in precontracted rings. Klaas and Wadsworth (1989) reported that hypoxia caused contraction and reoxygenation caused a transient further relaxation in sheep middle cerebral artery precontracted with 5-hydroxytryptamine, and the hypoxic contraction was abolished by removal of the endothelium. Others, however, have found that hypoxia produces vasodilatation in cerebral arteries and the vasodilatation is not dependent upon the endothelium (Ment *et al.*, 1983; Norins and Madden, 1990; Sakabe and Siesjo, 1979; Wei *et al.*, 1980). Thus, discrepancies have been found among the species employed as well as among the vessels studied.

In the porcine cerebral artery, the hypoxic response has not yet been extensively investigated. Therefore, this work was undertaken to investigate the effects of hypoxia on the resting tension, on the contraction induced by vasoconstrictors, and on the endothelium-dependent relaxation of acetylcholine (ACh) in porcine isolated cerebral arteries.

METHODS

Preparations and tension experiments

Heads of pigs, which were obtained from a local slaughterhouse, were cooled immediately after decapitation by infusing cold saline into foramen magnum, kept in an ice-box, and transferred to the laboratory. The entire brain was rapidly excised from the skull, and basilar and circle of Willis arteries were carefully dissected out. The isolated arteries were placed in cold (~4℃) physiological salt solution (PSS) and cleaned of connective and adipose tissues under stereoscope. Then, the arteries were cut into rings of 3~4 mm in width. In the denuded preparations, the endothelium was removed by gentle rubbing 2 to 3 times with a metal rod inserted into the lumen of the rings. Ring segments of arteries were mounted in an organ bath by sliding the ring over two parallel stainless-steel hooks. The lower hook was fixed on the bottom of the bath and the upper was connected to isometric transducer (Grass FT03) with thread, and the changes of tension were recorded on polygraph (Grass 7D). The double-jacketed organ bath was connected to a circulator and filled with 4 ml PSS saturated with 95% O_2 and 5% CO_2 at 37℃ (pH 7.4). All arterial rings were equilibrated for 2 hours and maintained under the resting tension of 1 g. The ring was tested for viability challenging with 50 mM KCl 2~3 times. Hypoxia was induced for the indicated period by substituting 95% N_2+5% CO_2 (0% O_2) for 95 % O_2+5% CO_2. Po_2 of bath fluid was measured with gas analyzer (Ciba-Corning 228 Blood Gas System). The Po_2 was 486±23.9 mmHg (n=4) during normal oxygenation and it decreased to 175± 9.3, 157±5.4, 74±3.0 and 49±2.1 mmHg, respectively, at 1, 2, 5 and 10 min after changing to 0% O_2.

Radioimmunosaasy for cyclic GMP

The experimental protocols for assaying cyclic GMP were desingned to parallel the condition used in the tension experiments. After 2-hour equilibration, each ring was exposed to the indicated agent and 0% O_2 gas. At the end of experiment the preparations were instantly frozen by placing them between bronze-plate clamps that had been pre-cooled in liquid nitrogen and kept at −80℃. Frozen tissues were homogenized in 0.5 ml of 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 2500×g for 30 min at 4℃. The pellet was used for protein assay (Lowry *et al.*, 1952) and the supernatant fraction was extracted 4 times with 3 ml water-saturated ether and a portion of aqueous solution was acetylated and radioimmunoassayed for cyclic GMP.

Drugs

Composition of PSS was NaCl 115, $NaHCO_3$ 35, KH_2PO_4 1.2, KCl 4.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, EDTA 0.03, glucose 11.1 mM. Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$), indomethacin, endothelin I (ET), acetylcholine (ACh), and atrial natriuretic peptide (ANP) were obtained from Sigma, and nimodipine from Bayer.

Nimodipine was dissolved and diluted to 10^{-3} M with ethanol, and further dilutions were made with distilled water. Other drugs were dissolved and diluted with distilled water. Statistical significances were examined by Student's unpaired t-test.

RESULTS

Effects of hypoxia on resting tenslon

In the circle of Willis artery with intact endothelium, switching to 0% O_2 produced transient contraction of 0.44±0.06 g (n=21) 3~5 min later, followed by a relaxation below the basal level within 5~7 min. And the reoxygenation elicited biphasic responses: a slight relaxation (−0.08 ± 0.02 g) preceding a contraction (0.10±0.04 g), which was far smaller than that induced by the hypoxia. In the basilar artery with intact endothelium, hypoxia also produced transient contraction of 0.46±0.09 g (n=17) and reoxygenation caused biphasic responses in the same fashion as those in the circle of Willis artery. The patterns and magnitudes of responses of both arteries did not significantly differ in all the experiments. On the other hand, the hypoxia-induced contraction in the endothelium-removed preparations were 0.17 ±0.054 g (n=19), and relaxation and contraction induced by reoxygenation were −0.02±0.013 and 0.12±0.037 g, respectively. The magnitudes of the both responses were significantly reduced by removing the endothelium (Fig. 1 and 2).

Effects of nimodipine and indomethacin on hypoxia-induced vasoconstriction and reoxygenation-induced vasodilatation

Nimodipine produced a transient vasoconstriction by itself in the rings with intact endothelium. The contractile responses to hypoxia and reoxygenation were concentration-dependently inhibited by the pretreatment with nimodipine and indomethacin in cerebral arterial rings with the intact endothelium (Fig. 1 and 3).

In contrast to hypoxia-induced vasoconstriction, the magnitudes of reoxygenation-induced

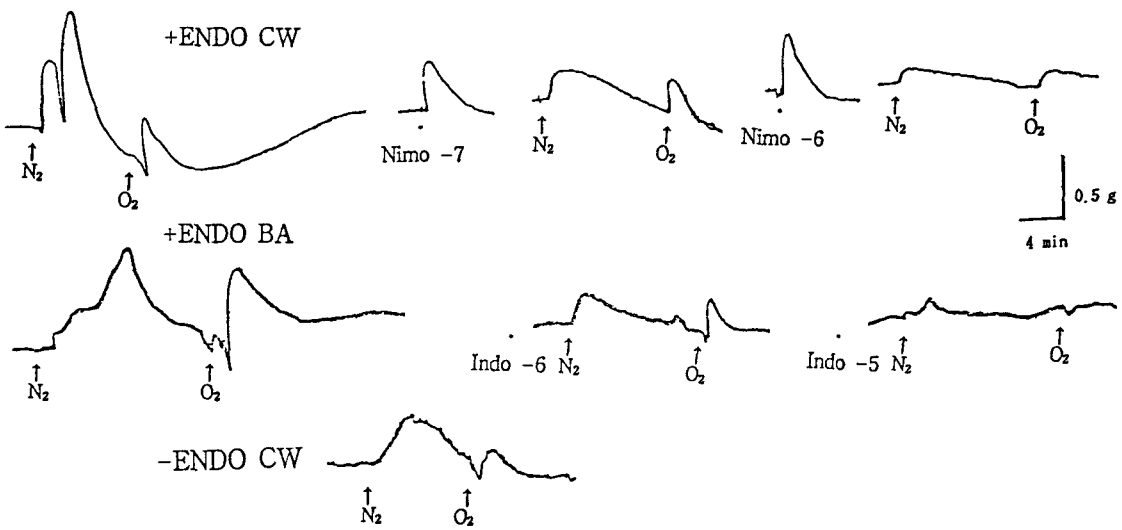

Fig. 1. Typical tracings showing effects of nimodipine (Nimo) and indomethacin (Indo) on the contractions induced by hypoxia (N_2) and reoxygenation (O_2) in isolated porcine basilar (BA) and circle of Willis (CW) artery. Hypoxia was induced by bubbling with 95% N_2+5% CO_2 at N_2 and reoxygenation by returning to 95% O_2+5% CO_2 at O_2. The preparations were pretreated with the indicated drugs for 10~30 min at dots. +ENDO and −ENDO show arterial rings with and without endothelium, respectively. Numerals (−×) show log molar concentration of the indicated drugs.

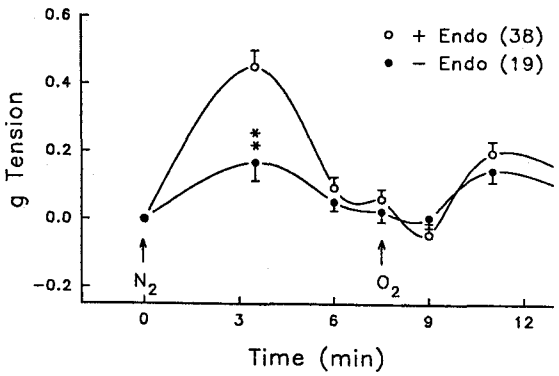

Fig. 2. The effects of hypoxia and reoxygenation on the resting tension of porcine cerebral artery. Each dot represents mean±SEM. Numerals in parentheses are the number of preparations.

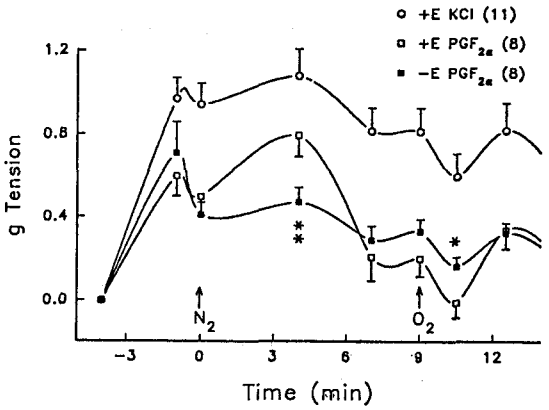

Fig. 4. Effects of hypoxia and reoxygenation on the preparations precontracted with 50 mM KCl or 10^{-6} M $PGF_{2\alpha}$. +E and −E represent the rings with and without endothelium, respectively. Asterisks indicate significant differences between both values of +E and −E $PGF_{2\alpha}$ (*p<0.05; **p<0.01). Other legends are the same as in Fig. 2.

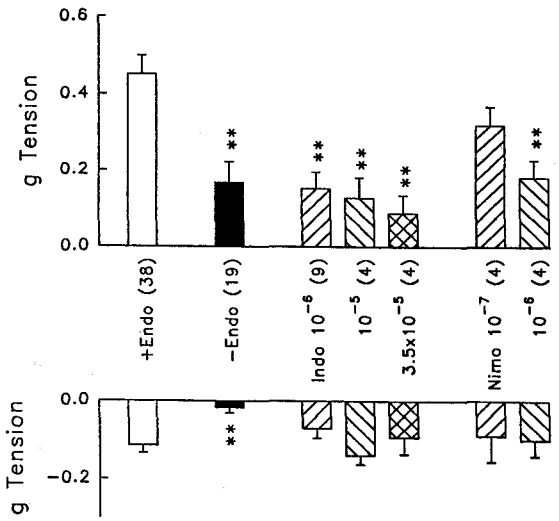

Fig. 3. The influences of removing endothelium and of pretreatment with indomethacin or nimodipine on hypoxia-induced vasoconstriction (upper panel) and on reoxygenation-induced vasodilatation (lower panel). In parentheses are the number of experments. Each column represents the mean, and the horizontal bar are SEM. Asterisks indicate significant difference from the control (+Endo, **p<0.01).

vasodilatation were not altered by pretreatment with either indomethacin or nimodipine, but removing endothelium reduced the magnitudes of vasodilation (Fig. 3).

Effects of hypoxia and reoxygenation on the preparations precontracted by KCl and $PGF_{2\alpha}$

In normal oxygenation, both 50 mM KCl and 10^{-6} M $PGF_{2\alpha}$ produced sustained contractile responses, and the subsequent hypoxia caused additional contractions of 0.14±0.03 g (n=11) and 0.30±0.14 g (n=8, Fig. 4). Reoxygenation relaxed the rings precontracted by KCl and $PGF_{2\alpha}$ to −0.22±0.05 g and −0.20±0.07 g, respectively, but it did not produce contraction as in the resting tension. The reduced tension induced by reoxygenation quickly recovered to the original levels in KCl-pretreated rings, but slowly in $PGF_{2\alpha}$-pretreated ones.

Both hypoxia-induced vasoconstriction and reoxygenation-induced vasodilation were markedly reduced by removing endothelium in the rings precontracted by 10^{-6} M $PGF_{2\alpha}$(Fig. 4).

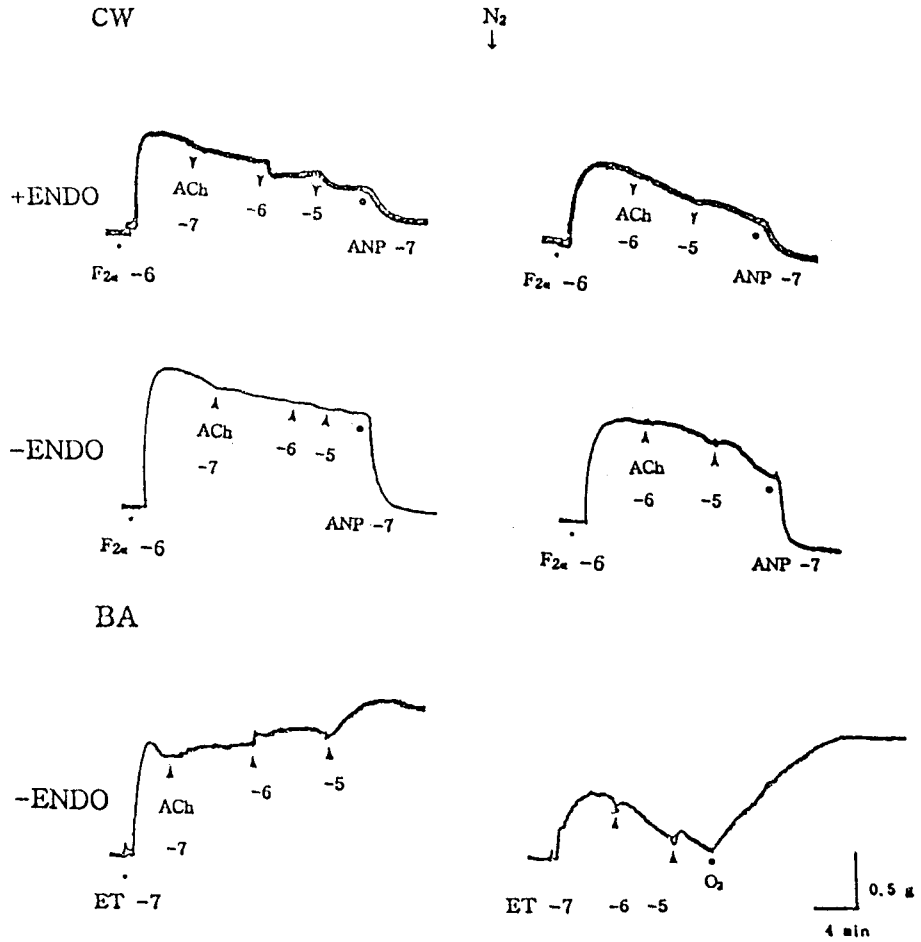

Fig. 5. Effects of hypoxia (N_2 ↓) on vasorelaxation induced by acetylcholine (ACh) and atrial natriuretic peptide (ANP) in circle of Willis (CW) or basilar (BA) arterial rings. The preparations were precontracted with 10^{-6} M $PGF_{2\alpha}$($F_{2\alpha}$ −6 M) or with endothelin 10^{-7} M (ET). Other legends are as in Fig. 1.

Effects of hypoxia on contractile responses Induced by KCl, $PGF_{2\alpha}$ and endothelin

In normal oxygenation, addition of 50 mM KCl, 10^{-6} M $PGF_{2\alpha}$ and 10^{-7} M endothelin (ET) produced sustained contractions of 1.72±0.39 g (n=20), 0.62±0.06 g (n=21) and 0.66±0.10 g (n=8), and 3~5 min after switching to 0% O_2, they produced tensions of 1.98±0.41 g (n=6), 0.38±0.08 g (n=7), 0.23±0.08 g (n=4), respectively. In contrast to the KCl contraction, which was not altered by hypoxia, the contractions induced by $PGF_{2\alpha}$ and ET were significantly reduced under hypoxia and not sustained as in the normal oxygenation. The ET-induced contraction was the most sensitive to the hypoxia condition (Fig. 5 and 6).

Effects of hypoxia on vasorelaxation induced by ACh and ANP

In the preparations with intact endothelium, ACh, under normal oxygenation, relaxed the tension induced by 10^{-6} M $PGF_{2\alpha}$ and 10^{-7} M ET in a dose-dependent fashion. But the ACh-induced vasorelaxation was abolished by removing the en-

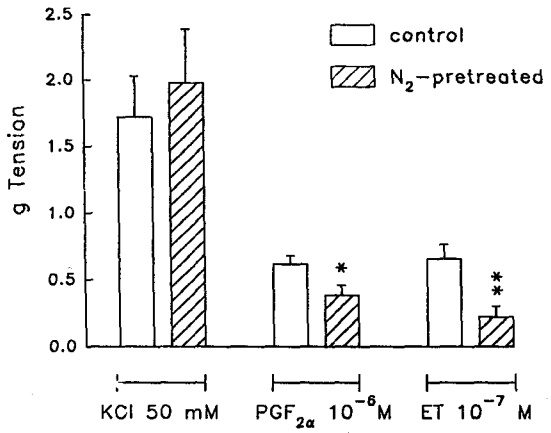

Fig. 6. Effects of hypoxia on the contractions induced by KCl, $PGF_{2\alpha}$ and endothelin (ET). Hatched bars represent the contractions in hypoxic condition, and open bars the normal oxygenation. Each column represents mean from 7～21 experiments. Asterisks represent significant differences between both groups (*p< 0.05; **p<0.01).

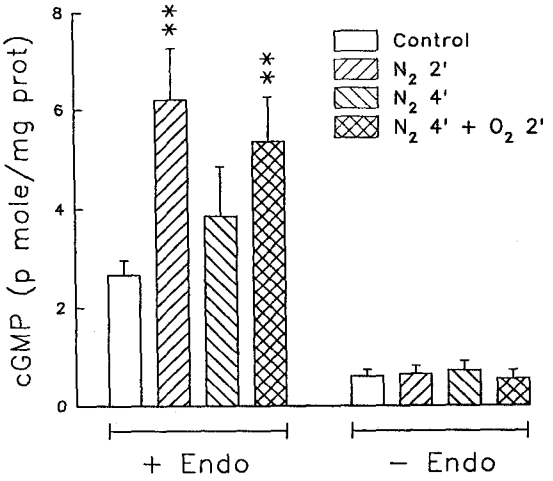

Fig. 8. Effects of hypoxia (N_2) and reoxygenation (O_2) on cyclic GMP contents in cerebral arterial tissues with and without endothelium. Each column shows cyclic GMP content of mean± SEM from 6 rings. Control columns were obtained under normal oxygenation. Primed numbers represent the incubation time with the indicated gas. Other details are given in the text.

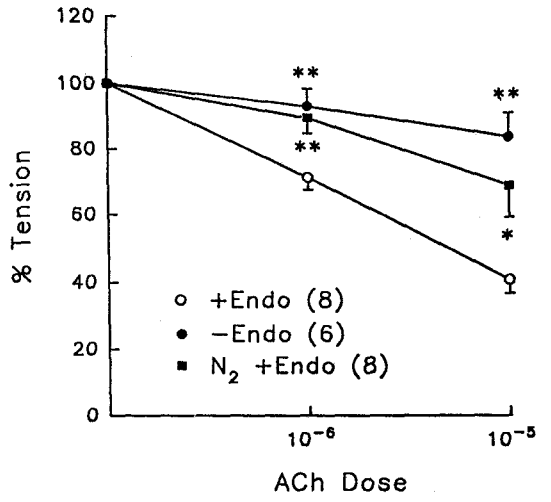

Fig. 7. Effects of hypoxia and removal of endothelium on ACh-induced vasodilatation. The preparations were precontracted with 10^{-6} M $PGF_{2\alpha}$. The $PGF_{2\alpha}$-induced tension before addition of ACh was calculated as 100%. Other legends are as in Fig. 3 and 6.

dothelium. Under hypoxic condition, the endothelium-dependent relaxation of ACh was markedly reduced. However, ANP-induced vasorelaxation was not affected by hypoxia (Fig. 5 and 7). Both ET-induced vasoconstriction and ACh-induced vasodilation inhibited in hypoxic condition were rapidly recovered to the control level by reoxygenation.

Effects of hypoxia on cyclic GMP content

In the preparations with intact endothelium, cyclic GMP content was 2.7±0.3 pmol/mg protein (n =6) in normal oxygenation, and treatment with hypoxia for 2 min, which produced transient contraction in tension experiment, significantly increased the cyclic GMP content to 6.2±1.1 pmol/mg protein (n=6, $p<0.01$). The cyclic GMP content of the preparations under hypoxic condition for 4 min, when the hypoxia-induced tension was recovered to basal levels, did not differ from those of control. However, reoxygenation markedly increased the cyclic GMP content to 5.4±0.9 pmol/

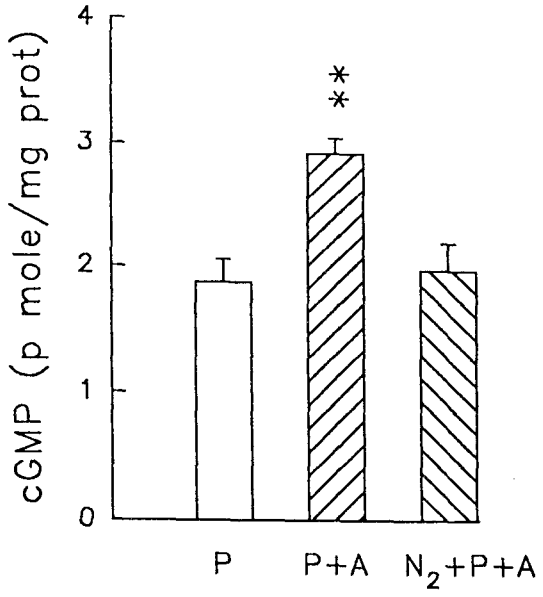

Fig. 9. ACh-induced changes of the cyclic GMP contents in rings precontracted with $PGF_{2\alpha}$ (P) under normal and hypoxic condition. Hypoxia were induced for 3 min before adding $PGF_{2\alpha}$ In hatched columns, 10^{-6}M ACh (A) was added to the bath 3 min after pretreatment with 10^{-6} M $PGF_{2\alpha}$ Each column represents mean from 6 experiments. Other legends are as in Fig. 3.

mg protein (n=6, $p<0.01$).

In the denuded preparations, the cyclic GMP content was significantly reduced to 0.6 ± 0.17 pmol/mg protein ($p<0.01$), and hypoxia and reoxygenation did not alter the cyclic GMP content.

DISCUSSION

In isolated porcine cerebral arteries hypoxia produced an abrupt and transient contraction, followed by a relaxation below the basal level. And reoxygenation elicited biphasic responses, a slight relaxation preceding a marked contraction, which then slowly recovered to the basal tension. Both responses induced by hypoxia and reoxygenation were inhibited by removing the endothelium. The protocol inducing hypoxia in this study is the same as that described by Klass and Wadsworth (1989), who employed middle cerebral artery of sheep. They did not observe the effects of hypoxia on the resting tension, but described that hypoxia caused further contraction and reoxygenation elicited transient relaxation in intact endothelial rings precontracted with 10 μM 5-hydroxytryptamine (5-HT), whereas it elicited only relaxant effect in the denuded rings. The present findings that hypoxia and reoxygenation produced contraction and relaxation in intact endothelial rings precontracted with KCl and $PGF_{2\alpha}$, which were attenuated under hypoxia, are similar to those of Klaas and Wadsworth (1989).

On the other hand, Mai *et al.* (1991) showed in canine basilar artery that under basal tension hypoxia produced a gentle and sustained contraction, which was reversed to basal level by reoxygenation. Their results differ from the present findings in the phases of the responses induced by hypoxia and reoxygenation. The discrepancies may have resulted from the species difference of animals used.

In the present study, it was observed that transient contractile responses induced by hypoxia and reoxygenation were attenuated by removing the endothelium, and were inhibited dose-dependently by nimodipine, a calcium channel inhibitor selective to cerebral artery (Opie, 1984; Haws and Heistad, 1984) and by indomethacin, a cyclooxygenase inhibitor (Rubanyi and Paul, 1984). These suggest that the contractions were endothelium-dependent and related to cellular calcium mobilization and to prostaglandin-like substance(s). These hypotheses are supported by the findings of other investigators that hypoxia contracts the KCl-pretreated sheep coronary artery with intact endothelium but the contraction is abolished by removing endothelium (Kwan *et al.*, 1989), and that hypoxia affects intracellular calcium moblization (Detar, 1980; Ebeigbe, 1982; Karaki and Weiss, 1987), and that leukotrienes may be involved in hypoxia-induced contraction of canine basilar artery (Mai *et al.*, 1991).

In the present study, the attenuated contractile responses to $PGF_{2\alpha}$ and ET by hypoxia recovered to the original contraction level, while the KCl-induced contraction was not affected by hypoxia. Although the endothelium-dependent vasorelaxation of ACh was abolished in hypoxic state, the

vasorelaxation of ANP was not affected. These results suggest that hypoxia does not directly impair the vascular smooth muscle but selectively suppresses the endothelial function in porcine cerebral arteries. KCl contracts vascular smooth muscles by increasing calcium influx through voltage-dependent calcium channels (VDCs), whereas $PGF_{2\alpha}$ and ET cause vasoconstriction by increasing Ca^{2+} influx through receptor-operated calcium channels (ROCs) and by releasing of Ca^{2+} from intracellular storage (Schwartz and Tiggle, 1984; Toda and Miyazaki, 1984). Thus, it is implicated that VDCs are resistant to hypoxia, but ROCs and Ca^{2+}-release mechanisms are sensitive to the hypoxia. Also, the ET-induced contraction seems to be more sensitive to hypoxia than the $PGF_{2\alpha}$-induced one. ET produces vasoconstriction through the stimulation of hydrolysis of phosphatidylinostiol (PI) by activation of protein kinase C (Ohlstein *et al.*, 1989), and hypoxia suppresses the cellular ATP generation (Boddeke *et al.*, 1989). These findings lead us to the postulation that the ET-induced contraction sensitive to hypoxia may be related to the inhibition of PI turnover caused by the ATP depletion.

The cyclic GMP content of the preparation with intact endothelium was increased by hypoxia and by reoxygenation in parellel with contractile responses in tension experiments. And ACh also increased the cyclic GMP content in $PGF_{2\alpha}$-pretreated preparations with intact endothelium. It is well known that in peripheral vessels, vascular endothelium releases EDRF causing activation of guanylate cyclase, which in turn elevates cyclic GMP content (Furchgott, 1983; Diamond and Chu, 1983). Cyclic GMP content in denuded preparations was one fifth of those in intact endothelium and the content was not affected by hypoxia and reoxygenation.

The increase in cyclic GMP content by ACh indicates that cyclic GMP is involved in the ACh-induced vasorelaxation. But the unexpected findings in the present study that the cyclic GMP content was increased in the contractile phase induced by hypoxia and following reoxygenation contradicts the established findings the cyclic GMP produces vasodilation and cannot be easily accounted for. Bigaud *et al.*, (1984), however, observed that clonidine and methoxamine increased the cyclic GMP content in isolated thoracic aortae of rats, although these agents contracted the preparations. They showed that these agents also release EDRF causing increased cyclic GMP formation, possibly to counteract the vasoconstriction produced by the agents. The increased cyclic GMP in hypoxia and reoxygenation may also be a homeostatic phenomenon to ameliorate the vasoconstriction, although the mechanism involved remains to be explored.

In conclusion, the present data suggest that contractions induced by hypoxia and reoxygenation in isolated porcine cerebral artery are dependent on endothelium, and related to cellular calcium mobilization and to prostaglandin-like substance(s). Also, function of endothelium is very sensitive to hypoxia, and the mechanism remains to be clarified.

ACKNOWLEDGEMENT

The author is most grateful to Prof. Yung Hong Baik, Department of Pharmacology, Chonnam University Medical School, Kwangju, Korea and to Prof. Toshimitsu Uchiyama, Department of Pharmacology, Toho University School of Medicine, Tokyo, Japan, for their invaluable advice and assistance during the course of this work.

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= 국문초록 =

돼지 적출뇌혈관의 저산소 유발 수축반응에 관하여

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돼지의 적출뇌동맥에서 저산소 유발 수축반응을 관찰하고 그 기전을 구명하며 저산소가 혈관수축제의 수축반응과 acetylcholine (ACh)의 내피세포 의존성 이완반응에 미치는 영향을 검토코자하여 본 실험을 시행하였다. 내피세포가 존재하는 표본에서는 저산소는 일과성 혈관수축을 일으켰고 산소 재공급시에 일과성 이완 후 수축이 나타나는 이상성 (biphasic) 반응이 관찰되었다. KCl 및 $PGF_{2\alpha}$전처치로 수축된 표본에서 저산소 유발은 추가적 수축반응을 일으켰고, 산소재공급으로 이완반응이 관찰되었다. 내피세포 제거 후 및 nimodipine 또는 indomethacin처리 후 저산소와 산소 재공급에 의한 수축반응은 현저히 감약되었다. 저산소하에서 KCl의 수축반응은 영향받지 않았으나, $PGF_{2\alpha}$와 endothelin (ET)의 수축반응은 현저히 억제되었고 ET수축반응이 저산소에 가장 예민하였다. $PGF_{2\alpha}$와 ET로 수축된 내피세포 존재표본에서 ACh은 용량의존성 이완반응을 일으켰고 이 이완반응은 저산소 하에서 소실되었으며 내피세포 제거표본에서는 ACh의 이완반응이 관찰되지 않았다. $PGF_{2\alpha}$로 처리한 내피세포 존재표본에서 ACh은 cyclic GMP 농도를 증가시켰고 이 증가는 저산소하에서 소실되었다. 이상의 성적으로 저산소와 산소 재공급 수축반응은 내피세포 및 calcium의존성이고, prostaglandin계 물질의 유리에 기인한다고 추론하였다.