Oxygen-dependent Tension in Vascular Smooth Muscle

Does the Endothelium Play a Role?

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SUMMARY. We investigated a hypothesis that an oxygen sensor involved in hypoxia-induced relaxation of vascular smooth muscle may reside in endothelial cells. We also determined the oxygen dependence of hypoxia-induced decreases in cyclic guanosine 3',5'-monophosphate concentrations in vascular smooth muscle rings. Rings of canine femoral artery, rabbit thoracic aorta, and lamb ductus arteriosus, either with an intact endothelium or with damaged or absent endothelium, were studied using organ baths that allowed changes in Po2 without a change in pH. Hypoxia-induced relaxations of rabbit thoracic aorta, lamb ductus arteriosus, and canine femoral artery were not dependent on an intact endothelium. The magnitude of hypoxia-induced relaxations was unchanged in rings of canine femoral artery without intact endothelium compared to rings with endothelium. Quasi-steady state organ bath Po2-mechanical tension relationships were unchanged in rings of canine femoral artery without endothelium over an organ Po2 range of 200–20 mm Hg. With rabbit thoracic aorta, magnitudes of hypoxia-induced relaxations were significantly smaller in rings without endothelium. Quasi-steady state plots, where mechanical tension was given as percentage of maximal relaxation, were similar in rings either with or without intact endothelium. Cyclic guanosine 3',5'-monophosphate concentrations were shown to be oxygen-sensitive, decreasing during hypoxia-induced relaxations with a threshold Po2 of 80–100 mm Hg with canine femoral artery, and 60–80 mm Hg with rabbit thoracic aorta rings, but this finding seems unrelated to the mechanism of hypoxia-induced relaxation. (Circ Res 58: 341–347, 1986)

THE cellular mechanisms involved in Po2-dependent changes in tension in vascular smooth muscle are not understood. The phenomenon has been studied in a variety of different preparations (Carrier et al., 1964; Detar and Bohr, 1968; Fay, 1971; Nair and Dyer, 1973; Clyman et al., 1975; Coburn, 1977; Coburn et al., 1979; Demey and Vanhoutte, 1983). Studies of the nature of the O2-sensing reaction and transducing mechanisms between O2 sensor and contractile proteins in vascular smooth muscle cells may give information about in vivo mechanisms of vasodilation and the general area of O2 chemoreception. There is no agreement about O2-sensing reactions or transduction mechanisms in vascular smooth muscle. There is evidence that the O2 sensor is cytochrome oxidase (Fay, 1971; Hellstrand, 1977; Scott and Coburn, 1985)—that it is not cytochrome oxidase (Coburn, 1977, 1979). Transduction mechanisms may involve generation of cyclooxygenase by-products (Kalsner, 1976; Rubanyi and Paul, 1984), β-adrenergic mechanisms (Rubanyi and Paul, 1984), alterations in electrical properties of the plasma membrane (Hellstrand, 1977; Roulet and Coburn, 1981), or other mechanisms. There may be multiple mechanisms involved, triggered by O2-sensing reactions which have different O2 affinities (Coburn et al., 1979; Rubanyi, 1984).

We were stimulated to study the role of the endothelium in hypoxia-induced relaxation of vascular smooth muscle by a host of studies showing the importance of endothelium-dependent relaxing factor in various drug-induced relaxations of vascular smooth muscle (Furchgott and Zawadzki, 1980; Furchgott et al., 1981). Busse et al. (1983) have shown that removal of the endothelium in a branch of the canine femoral artery abolishes hypoxia-induced vasodilation caused by lowering intraluminal Po2. However, Singer et al. (1981) found that hypoxia-induced relaxations (Po2 <30 mm Hg) were unchanged in vitro rabbit thoracic aortic rings with intact or absent endothelium contracted with either phenylephrine or angiotensin II.

We have extended the above data using norepinephrine-contracted rabbit thoracic aorta and canine femoral artery, and neonatal lamb ductus arteriosus, and describe the effects of graded changes in organ bath Po2 on mechanical responses of these tissues, either with intact or damaged endothelium. Since it is possible that an endothelium-dependent relaxing factor (EDRF) is involved in hypoxia-induced relaxations, and since this factor is reported to elevate cyclic guanosine 3',5'-monophosphate concentrations ([cGMP]) (Rapoport and Murad, 1983; Diamond
and Chu, 1983), we also studied the oxygen tension-dependence of [cGMP] in vascular rings.

Methods

Experiments were performed on 2- to 2.5-mm-wide rings cut from canine femoral artery (CFA), neonatal lamb ductus arteriosus (LDA), and rabbit thoracic aorta (RA). Femoral arteries were dissected from 13- to 16-kg female mongrel dogs anesthetized with pentobarbital. Neonatal lambs were delivered by Caesarean section, exsanguinated, their chests opened, and the heart and great vessels dissected. Rabbits were killed by cervical dislocation and exsanguination, their chests were opened, and the thoracic aorta was dissected. Care was taken during dissection to avoid touching the endothelial surface of the arteries. Tissues were placed in cool modified Krebs' solution, dissected free of fascia, and cut into rings. Dissection solutions in RA and CFA experiments were gassed with 95% O2/5% CO2. When LDA was studied, solutions were gassed with 95% N2/5% CO2. Krebs-Ringer bicarbonate solution had the following composition (mM): Na+, 137; K+, 5.9; Cl-, 134; HCO3-, 25; Ca++, 2.0; Mg++, 1.2; Na2PO4, 1.2; glucose, 11.5, which when gassed with 5% CO2 produced a pH 7.30 to 7.35. In most experiments, half of the rings were controls [intact endothelium (END+)] and half were rings which were rubbed on their endothelial surface for 20 seconds with a small wooden spatula (END-). We studied RA END- rings using standard techniques (Furchgott, 1980) to assess the degree of removal or damage of endothelium. END- rings, contracted with 10 μM norepinephrine (NOR), did not relax with 20 μM carbachol-induced increases in [cGMP] were blunted, compared to END+ rings (Table 1). Light microscopy showed that most endothelial cells were absent in END- rings and present in END+ rings. As will be described later in the manuscript, END+ and END- rings had markedly different [cGMP]. We assumed that rubbing the endothelial surface of LDA and CFA rings, using the same technique, would also effectively remove the endothelium.

Rings were attached to isometric force transducers, suspended in 6-ml organ baths perfused with Krebs-Ringer bicarbonate solution at 37°C, and gassed with 25-30% O2/5% CO2 in N2 (RA and CFA rings), or (for LDA rings) 95% N2/5% CO2. The gas system was constructed so that gas flowing from "O2" and "N2" tanks via flow meters entered a mixing chamber. The size of the mixing chamber determined the rate of decrease in organ bath PO2 after changing from an O2 to a N2 gas mixture. Gas in the mixing chamber was directed to each of four organ baths utilizing small bore tubing of equal lengths. The size of the mixing chamber was adjusted so that organ bath PO2 decreased from approximately 200 to 20 mm Hg in 20-25 minutes, as shown in Figure 1. It was determined that this slow decrease in organ PO2 produced PO2-mechanical tension relationships which were very near steady state values previously reported (Coburn et al., 1979). We, therefore, consider mechanical tension responses produced in this system to be quasi-steady state and believe that we can study effects of endothelium removal on mechanical tension responses over a wide range of organ bath PO2.

Our general approach was to compare mechanical tension responses of END+ and END- rings exposed to the same or very similar slow decreases in organ bath PO2. We analyzed data in terms of time-mechanical tension relationships, comparing, in paired END+ and END- rings, time of onset of relaxation, time-mechanical tension relationships during the slow decreases in PO2, and the time for complete relaxation. In several experiments there were slow, constant decreases in isometric tension that persisted for long periods of time after hypoxia-induced relaxations were apparently completed. In these experiments, we estimated the time for complete relaxation as the time when the relaxation rate failed to decrease further. Organ bath PO2 could be estimated from quasi-steady state mechanical tension in END+ rings. In a few experiments in

| Table 1 Effect of Nitroglycerin and Carbamylcholine on [cGMP] in END+ and END- Rabbit Aorta |
|---------------------------------|-----------------|-----------------|
|                                | [cGMP] (pmol/g) | % Relaxation    |
| **END+ rings**                 |                |                 |
| Control (5)                    | 6.8 ± 1.5      | 69.4 ± 11.8     |
| Nitroglycerin (0.9 mM) (5)     | 50.8 ± 18.7*   | 69.4 ± 11.8     |
| Control (9)                    | 8.5 ± 1.8      |                 |
| Carbamylcholine (20 μM) (9)   | 23.9 ± 10.6*   | 15.4 ± 6.1      |
| **END- rings**                 |                |                 |
| Control (5)                    | 2.48 ± 1.5     |                 |
| Carbamylcholine (20 μM) (5)   | 7.1 ± 3.6      | -1.3            |

Results are expressed as mean ± SEM. n values are given in parentheses. Aortas were rubbed (END-) or left intact (END+), as described in Methods. Rings were studied at a PO2 of 200-300 mm Hg in the presence of 20 μM NOR.

* Statistically significant at P < 0.05, compared to control.
which there were differences in time-mechanical tension data between END+ and END− rings, we confirmed "threshold" organ bath Po2 using a Po2 electrode. We tested END+ rings with 95% O2/5% CO2 in LDA experiments, since large, tonic contractions developed previously (DeMey and Vanhoutte, 1983), and the resultant relaxation was determined. After maximal relaxation had been achieved, gas Po2 was increased back to control level. When tension was constant, NOR was washed out of the organ bath, and the ring was allowed to "recover" for 60 minutes. The second run was identical, except that rings were freeze-clamped in the control state or at various times during development of hypoxia-induced relaxations. With LDA rings, initial runs were obtained, as above, by changing Po2, and rings were freeze-clamped just before, or during, the second hypoxia-induced relaxation. Frozen-clamped rings were stored at −40°C prior to analysis for [cGMP].

[cGMP] Analysis

We used the method of Gruetter et al. (1981) to measured [cGMP]. Antibody was obtained from New England Nuclear Corp. Cold perchloric acid was added to the frozen tissue which then was ground to a powder in a dental amalgamator with steel ball bearings. Analyses from the same tissue performed in duplicate had a SD of ±4.9%. Duplicate tissues varied ±12.4%. Known samples taken through the extraction procedure gave average recoveries of 40–60%. Analyses with [3H]cGMP showed similar yields, and indicated that the major loss occurred during the ether extractions. We did not measure "recoveries" in every sample and have correct data for loss during extraction assuming a 50% recovery.

Drugs

The drugs used were carbamylcholine chloride (Sigma), l-norepinephrine bitartrate (Breon Labs). Stock solutions were prepared in distilled water. We injected 20 µl of the stock solution into organ bath fluid to give the final concentration.

Statistical Analysis

Mean values ± SEM were calculated from paired strips from the same artery. Data were analyzed by paired t-test. Differences were considered significant at P < 0.05.

Results

Oxygen Sensitivity in END+ and END− Rings

Figure 2 shows typical examples of O2 sensitivity of the various preparations studied. We did not see initial hypoxia-induced contractions in RA, as reported previously (Singer et al., 1981). In CFA, the hypoxia-induced response was biphasic, as described previously (DeMey and Vanhoutte, 1983), with a small initial contraction, followed by a large prolonged relaxation. In eight END+ rings, the con-
tractile phase of the CFA biphasic response varied between 5% and 15% of active tension.

We could detect no difference in NOR-induced contractions between END+ and END- rings (Table 2). All RA, CFA, and LDA END- rings relaxed during hypoxia, indicating that these relaxations are not entirely dependent on an intact endothelium. Figure 3 illustrates typical data obtained with CFA rings. The magnitudes of hypoxia-induced relaxations were not significantly different in END- and END+ CFA rings; however with RA, END- rings had significantly smaller maximal relaxations (Table 2). In both RA and CFA, maximal hypoxia-induced relaxations varied considerably (Table 2) in different rings. The reason for this is not known.

We estimated relative Po2 thresholds for onset of relaxations by comparing times of onset of decreases in tension in paired END+ and END- rings. With RA, END- rings showed onset of relaxation 0.12 ± 1.2 minutes prior to END- rings; with CFA, END+ rings showed initial relaxations 1.0 ± 1.2 minutes prior to END- rings. These differences were not statistically significant. There also were no differences in the time duration from onset to complete relaxation (relaxation times in RA END+ rings were 1.0 ± 1.5 minutes longer than in RA END- rings; for CFA, relaxation times with END+ rings averaged 0.7 ± 2.0 minutes longer than with END- rings).

Figure 4 compares decreases in quasi-steady state mechanical tension (as percent of maximal relaxation) in END+ and END- rings in CFA and RA during decreases in organ bath Po2. For CFA, END+ and END- data are practically superimposed; the same was true when mechanical tension was plotted as percent total active tension. For RA, where END- rings had significantly smaller maximal hypoxia-induced relaxations, mean data from END- rings were similar to data obtained with the paired END+ rings, when mechanical tension is plotted as a percent of maximal relaxation. When these data were plotted in terms of percent of active tension, END- and END+ data superimpose for the first 15 minutes of the relaxation; at the lowest organ bath Po2, END- rings showed smaller rates of relaxation.

In CFA, END+ rings exhibited an initial contraction preceding hypoxia-induced relaxation which was absent in END- rings (Fig. 3). Because of these initial contractions, we had difficulty in determining the time for onset of relaxation, and arbitrarily chose the time when tension decreased below control. It is possible that END+ CFA plots shown in Figure 4 should be displaced slightly to the left, which would result in even closer agreement of END+ and END- data. Since the initial hypoxia-induced contraction of CFA was absent in END- rings, there was no...
difficulty in determining the onset of relaxation in these rings.

We did not obtain sufficient data to make a detailed comparison of quasi-steady state organ bath PO2-mechanical tension relationships in END+ and END− LDA.

cGMP Data

Mean [cGMP] for each of the three tissues freeze-clamped under control normoxic conditions (END+) are shown in Figure 5A. There were markedly different controls [cGMP] in the three different tissues, CFA>LDA>RA. This finding seems consistent with the differences in [cGMP] reported for different smooth muscles (Diamond and Janis, 1978; Gruetter et al., 1981) END− rings (studied only with RA and CFA) had much smaller [cGMP] under normoxic conditions. This was particularly impressive in CFA rings (Fig. 5B).

Effects of hypoxia on [cGMP] in the various preparations are given in Figures 5 and 6. With CFA, hypoxia-evoked decreases in [cGMP] were seen early during relaxation when organ bath PO2 was in the range 80-100 mm Hg. [cGMP] reached a minimum when relaxation was 25-50% of maximal relaxation. As organ bath PO2 continued to decrease and relaxation progressed, [cGMP] returned toward control value. With RA rings, significant decreases in [cGMP] occurred only at 40% relaxation at organ bath PO2 80-90 mm Hg.

Discussion

The experimental approach used is dependent on the effective removal of most endothelial cells in our preparations and the presence of healthy endothelium in control rings. The evidence of intact endothelium in control rings includes histological studies, and the presence of carbamylcholine-induced relaxations and [cGMP] increases. Carbamylcholine-induced relaxations were about 50% of those reported in rabbit thoracic aorta contracted with 0.1 µM phenylephrine (Singer, 1983). It is likely that the smaller effect is related to the use of a higher relative concentration of α-adrenergic agonist. In our hands, carbamylcholine-induced relaxations, given as percent of active tension, are greater with 0.1 µM NOR than with 10-20 µM NOR. We also saw smaller increases in [cGMP] with carbamylcholine and with nitroglycerin than seen by Rapoport and Murad (1983), who studied rat thoracic aorta, and this may also be related to the higher NOR concentrations used in our study. We cannot exclude the possibility that there was endothelial damage in our "control"
was statistically significant at P < 0.05. Numbers in parentheses regarding carbamylcholine relaxations and [cGMP] rings showed marked differences from END+ rings preparations. However, as indicated above, END- rings showed marked differences from END+ rings regarding carbachol relaxations and [cGMP] increases. In addition, the finding of markedly different [cGMP] in END+ and END- rings under normoxic conditions appears to be strong evidence of a functioning endothelium in END+ rings and loss of a functioning endothelium in END- rings under normoxic conditions appears to be strong evidence of a functioning endothelium in END+ rings and normoxic conditions appears to be strong evidence of a functioning endothelium in END+ rings and nonmuscle cells. The evidence that [cGMP] decreases seen during hypoxia in END+ rings occur in smooth muscle cells, rather than in the endothelium, is the very small fraction of total cells in vascular tissue contributed by the endothelium, and the finding that removal of the endothelium did not cause a decrease in [cGMP] if measured immediately after endothelium removal (Rapoport and Murad, 1983).

Our experiments produced two principal findings: (1) that hypoxia-induced relaxations in RA and CFA, and in LDA, are not dependent on an intact endothelium under the conditions of our experiments, and (2) that [cGMP] decreased during hypoxia-induced relaxations in both CFA and RA and that the threshold organ bath PO2 for this effect was in the range 80–100 mm Hg.

The approach of comparing paired END- and END+ rings exposed to the same decrease in organ bath PO2 allowed us to look at hypoxia-induced relaxations over different ranges of organ bath PO2. A postulate that there might be oxygen-sensing reactions with different O2 affinities, in endothelial and in smooth muscle cells, which can transduce mechanical changes in smooth muscle cells, is not supported by data obtained with CFA rings. This follows from the nearly identical quasi-steady state organ bath PO2-mechanical tension relationships seen with END+ and END- rings. Data obtained with RA END+ and END- rings were also very similar, but the finding that END- preparations failed to relax as completely as END+ strips over very low organ bath PO2 ranges is consistent with the presence of an endothelial oxygen sensor operating over low PO2.

Our findings conflict with those previously described by Busse et al. (1983), who studied a branch of the canine femoral artery. However, these experiments were performed under conditions where only intraluminal PO2 was decreased. We made no attempt to duplicate this approach. It seems possible that there are different mechanisms for hypoxia-induced relaxations in different vascular tissue, even in adjacent vessels.

Endothelial cells are superficial and should have a mean PO2 only slightly below that in organ bath fluid. Therefore, our data suggest that the threshold endothelial PO2 for decrease in formation of a factor causing increase in [cGMP] may be as high as 80–100 mm Hg. The finding that this threshold PO2 is so near physiological levels suggests that O2 limitation of an EDRF might be important during hypoxia. However, our data indicate that for CFA, this does not influence hypoxia-induced relaxations, and it is unlikely that hypoxia-induced decreases in EDRF influence RA hypoxia-induced relaxations. It is possible there are other effects of decreases in [cGMP] on smooth muscle cells. In particular, the hypoxia-induced initial contraction of CFA could be a response to decrease in [cGMP] (Ignarro et al., 1985). The finding that [cGMP] decreases markedly with hypoxia is consistent with previous data which show that acetylcholine-evoked relaxations of vascular smooth muscle are inhibited in the absence of O2 (Furchgott and Zawadzki, 1980; Singer et al., 1981).

It is still possible that [cGMP] measured in vascular rings is markedly influenced by [cGMP] in nonmuscle cells. The evidence that [cGMP] decreases seen during hypoxia in END+ rings occur in smooth muscle cells, rather than in the endothelium, is the very small fraction of total cells in vascular tissue contributed by the endothelium, and the finding that removal of the endothelium did not cause a decrease in [cGMP] if measured immediately after endothelium removal (Rapoport and Murad, 1983).

The finding of large decreases in [cGMP] in END- vascular rings may have significance in terms of conclusions derived using endothelium-damaged vascular tissues, since the preparation is altered in two ways: removal of the endothelium and decrease in smooth muscle [cGMP]. Thus, altered responses of this preparation may reflect changes in smooth muscle as well as the absence of endothelium. We do not know if the altered muscle [cGMP] in our END- rings could influence our data and conclusions. It is very likely that cGMP-dependent kinases play a significant role in control of tension in vascular smooth muscle. Since the mechanisms are not worked out, the significance of the large decrease in [cGMP] on smooth muscle function is not known.
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References


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