Role of myocardium and endothelium in coronary vascular smooth muscle responses to hypoxia

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Kerkhof, Cornel J. M., Peter J. W. Van Der Linden, and Pieter Sipkema. Role of myocardium and endothelium in coronary vascular smooth muscle responses to hypoxia. Am J Physiol Heart Circ Physiol 282: H1296–H1303, 2002. First published November 15, 2001; 10.1152/ajpheart.00179.2001.—Hypoxia triggers a mechanism that induces vasodilation in the whole heart but not necessarily in isolated coronary arteries. We therefore studied the role of cardiomyocytes (CM), smooth muscle cells (SMC), and endothelial cells (EC) in coronary responses to hypoxia (P O2 of 5–10 mmHg). In an attempt to determine the factor(s) released in response to hypoxia, we inhibited the contribution of adenosine, ATP-sensitive K+ channels, prostaglandins, and nitric oxide. Isolated rat septal artery segments without (–T) and with a layer of cardiac tissue (+T) were mounted in a double wire myograph, and constriction was induced. Hypoxia induced a decrease in isometric force of 21% and 61% in –T and +T segments, respectively (P < 0.05). EC removal increased the relaxation to hypoxia in –T segments to 33% but had the same effect in +T segments (61%). Only one of the inhibitors, the adenosine antagonist in +T segments, partially affected the relaxation due to hypoxia. The role of adenosine is thus limited and other mechanisms have to contribute. We conclude that hypoxia induces a relaxation of SMC that is augmented by the presence of CM and blunted by the endothelium. A single mediator does not induce those effects.

endothelial cells; smooth muscle cells; cardiomyocytes; ATP-sensitive potassium channels; adenosine

SEVERAL INVESTIGATORS (1, 2, 4, 6, 10, 20, 21, 27) have shown in isolated whole heart preparations that hypoxia (lowering the P O2 in the perfusion solution) induces vasodilation in the whole heart. However, it is not clear whether or not mediators of hypoxia-induced vasodilation originate from vascular or myocardial tissue.

The results obtained from studies on the effect of hypoxia on isolated coronary artery tone are not consistent and range from dilation or decreases in isometric force (3, 8, 12, 34) to constriction or increases in isometric force (9, 18, 22, 26, 30) or no effect at all (7). Efforts to understand oxygen-sensitive mechanisms by means of studying isolated cells complicated things even more. Several investigators have shown in isolated endothelial cells that hypoxia can increase the production of prostaglandins (17, 33) and nitric oxide (NO) (11, 24) or can decrease the production of prostaglandins (31) and NO (33). Gellai et al. (8) provided evidence for a direct effect of oxygen on coronary smooth muscle cells. These authors showed that contractile function was markedly depressed at a P O2 below 5 mmHg. Myocytes are also sensitive to changes in oxygen tension. Mustafa (19) showed that isolated embryonic cardiac cells from chickens release adenosine and its degradation products in response to hypoxia. Furthermore, Kawaguchi et al. (14) showed that isolated heart tissue from neonatal rats released free fatty acid and prostacyclin during hypoxia.

Thus evidence from the literature suggests that different cell types that are involved in local flow regulation are sensitive to changes in oxygen tension. However, based on the studies so far, it is not clear how each cell type (cardiomyocyte, endothelial cell, and smooth muscle cell) is involved in the integrated vascular response to hypoxia (i.e., when all cell types are present, as in an intact heart). Therefore, we aimed at developing a new method to investigate the role of cardiomyocytes, endothelial cells, and smooth muscle cells in coronary vascular responses to hypoxia. With the use of this method, we studied the effect of hypoxia on KCl- and U-46619-induced contraction in coronary artery segments with and without surrounding myocardial tissue and with and without endothelium. In an attempt to determine the factor(s) released in response to hypoxia, we studied the contribution of adenosine, ATP-sensitive K+ (KATP) channels, prostaglandins, and NO in hypoxia-induced relaxation.

MATERIALS AND METHODS

The animal experimental committee of Vrije Universiteit approved all experiments. Male Wistar rats weighing 250–350 g were anesthetized with 50 mg/kg pentobarbital sodium (Nembutal, Sanofi Sante; Maassluis, The Netherlands). The chest was opened, and the heart was quickly removed and placed in ice-cold (4°C) MOPS-buffered physiological salt solution (PSS) containing (in mM) 145 NaCl, 5 KCl, 2 CaCl2, 1 MgSO4, 1 NaH2PO4, 5 d-glucose, 2 pyruvate, 0.02 EDTA, and 3 MOPS; pH was adjusted to 7.35–7.40 with 1 M NaOH.

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The heart was transferred to a dissecting dish also containing ice-cold MOPS PSS and pinned down to the silicone bottom. The right ventricle was opened, and the right ventricular wall was pinned down to the bottom of the dish, revealing the septum.

Two adjacent segments of the septal artery were dissected with microscissors. One segment was cleared of connective tissue and myocytes (−T), whereas the other segment was left surrounded by a layer of 100–200 μm of cardiac tissue (+T). Both segments were placed in a water-jacketed dual wire myograph setup (made in the technical department of the Laboratory of Physiology, Vrije Universiteit, Amsterdam, The Netherlands) containing a Krebs bicarbonate-buffered PSS [containing (in mM) 110 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 KH₂PO₄, 10 D-glucose, 24 NaHCO₃, and 0.02 EDTA] equilibrated with 95% air-5% CO₂; pH 7.35–7.40. The chamber had a volume of 8 ml. Two tungsten wires were inserted through each vessel lumen. One was connected to a force transducer to measure isometric force, and the other was fixed to a micrometer to stretch the segment.

Experimental Protocol

The temperature of the tissue bath was increased to 37°C, and vessel segments were equilibrated for a period of 30 min. The rings were then stretched stepwise and at each length exposed to 100 mM KCl Krebs until the induced contraction was maximal (Fig. 1). After the optimal length was determined, segments were set at this length, and the response to 100 mM KCl was repeated to test reproducibility. The segments were then allowed to equilibrate for another 15 min. 100 mM KCl was used as an agonist in groups 1 and 2. In group 1, the effect of the nonselective adenosine receptor antagonist 8-phenyltheophylline (8-PT; 10 and 200 μM) was tested. In group 2, the effect of the selective adenosine A₂ receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX; 50 μM) was tested. Chromakalim (1 μM) was used as an agonist in group 3, and the effect of the potassium channel inhibitor glibenclamide (10 μM) was studied. In group 4, arachidonic acid (10 μM) was used as an agonist, and the contribution of prostaglandins in the hypoxic response was investigated using the enzyme blocker indomethacin (10 μM). Finally, in group 5, acetylcholine (1 μM) was used to stimulate NO production, and the effect of the NO inhibitor N⁴-nitro-L-arginine (L-NNa; 100 μM) on the hypoxic response was studied.

Vessels were constricted with 30 mM KCl-Krebs and randomly assigned to one of the five groups, and the relative force level was determined with respect to the force due to 100 mM KCl. The change in force in response to the agonist was recorded, and the agonist was washed out. After the force returned to the preagonist level, the oxygen tension was lowered and the change in force was recorded. Oxygen tension was increased, and the bath was washed with normal Krebs. Coronary artery segments were then incubated for 15 min with the appropriate inhibitor and contracted again with 30 mM KCl-Krebs in the presence of the inhibitor. After 10 min, segments were exposed to 15 min of hypoxia, similar to that as in the absence of the inhibitor, and the change in force in response to hypoxia was quantified. After a return to normoxia and after force reached prehypoxia levels, the response to the agonist was repeated (in the presence of the inhibitor). From these measurements, the preconstriction force levels, the relaxation to the agonist, and the relaxation

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to hypoxia without (control) and with inhibitor were determined.

Hypoxia

Hypoxia was induced by changing the bubbling of the tissue chamber from 95% air-5% CO₂ (PO₂ 150 mmHg) to 95% N₂-5% CO₂ (PO₂ 5–10 mmHg). Oxygen tensions were measured with a fast-responding Clark-type oxygen electrode (15), which was calibrated before and after the experiment. Because this electrode repeatedly yielded the same PO₂ values, we decided not to measure oxygen tension in all experiments. Some oxygen could still diffuse into the organ chamber while bubbling with 95% N₂-5% CO₂ and, therefore, the oxygen tension was between 5 and 10 mmHg. After 15 min of hypoxia, the changes in force generally stabilized and the gas mixture was returned to normoxia.

Viability of the Tissue

To test whether the isolation procedure or the duration of the experiment damaged the vessel segment with surrounding myocardial tissue, two staining methods were used. Three vascular segments were stained before the start of the experiment, and three vascular segments were stained after the experimental protocol.

Segments (length of 1.5–2 mm) were cut in half. One half was used to determine peroxidase activity as an index of myoglobin content to check whether the cell membrane of cardiac cells remained intact. When the cell membrane is damaged, myoglobin leaks out of the cell. The other half was used to determine succinate dehydrogenase (SDH) activity as an index of mitochondrial activity.

The peroxidase activity of myoglobin in fixed tissue was determined as described by Lee-de Groot et al. (16). After being fixed, embedded, and frozen, the air-dried sections were incubated, and myoglobin staining of the sections was detected qualitatively with a light microscope.

SDH activity was determined in segments that were directly embedded in gelatin, frozen in liquid nitrogen, cut in sections of 10 μm thick, and stained as described by Pool et al. (25). The precipitation of colored formazan, which is formed by the action of SDH on its substrate in the presence of a tetrazolium salt, was measured qualitatively with a light microscope. Staining quality and intensity were assessed blindly by an independent expert in the field of the used methods (16).

Chemicals

Salts, acetylcholine, arachidonic acid, indomethacin, cromakalim, glibenclamide, and 8-PT were obtained from Sigma (St. Louis, MO). Adenosine was obtained from Boehringer-Mannheim. L-NNA was obtained from Bachem (Bubendorf, Switzerland), and DMPX was obtained from ICN. U-46619 was obtained from from Fluka Biochemika. Acetylcholine, adenosine, and L-NNA were dissolved in distilled water. Arachidonic acid was dissolved in ethanol under nitrogen. Indomethacin was dissolved in 0.2 M Na₂CO₃. Cromakalim, glibenclamide, and DMPX were dissolved in DMSO. 8-PT was dissolved in ethanol and 1 M NaOH (75:25% vol/vol). All the drugs used were added to the superfusion solution and reported as final concentrations.

Calculations and Statistical Analysis

Active force (F₁₀₀) development is defined as the force induced by 100 mM KCl minus the passive force. The force level induced by 30 mM KCl is referred to as the precontraction level (F₃₀). Precontraction level and changes in isometric force induced by agonists or hypoxia are expressed as the percentage of the force induced by 100 mM KCl only to correct for differences in segment length and/or differences in the number of smooth muscle cell layers. Values are reported as means ± SE. We compared the data from segments with and without surrounding tissue or endothelium using a factorial ANOVA. The preconstriction levels of these segments were similar. The effect of inhibitors on precontraction level was assessed by a paired t-test. A general linear model was used to assess the effect of the inhibitor on the response to the agonist or to hypoxia. A Bonferroni correction for multiple comparisons was applied when necessary. A probability value of P < 0.05 was considered significant for all tests.

RESULTS

A force-length relationship from a typical experiment is shown in Fig. 1. The vessel characteristics (part I) are summarized in Table 1. The acetylcholine-induced decreases in isometric force were significantly larger (P < 0.05) in −T than in +T segments, both for 1 and 10 μM. After endothelial removal, acetylcholine (1 and 10 μM) had no significant effect on isometric force in both −T and +T segments. In part 2 of the study, 31 vessel pairs were used. F₁₀₀ and F₃₀ were not different between −T and +T segments. F₃₀ was 91 ± 2% and 91 ± 3% of F₁₀₀ in −T and +T segments, respectively. All vessels responded to acetylcholine, and decreases in force were significantly larger (P < 0.05) in −T than in +T segments, both for 1 and 10 μM. The overall decreases in force due to hypoxia were −17 ± 3% and −56 ± 4% of F₁₀₀ in −T and +T segments, respectively, and were significantly larger in +T than in −T segments.

<table>
<thead>
<tr>
<th>Table 1. General characteristics</th>
<th>−T</th>
<th>−EC</th>
<th>+T</th>
<th>+T</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>6</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Optimal length, μm</td>
<td>387 ± 23</td>
<td>375 ± 19</td>
<td>292 ± 29†</td>
<td>271 ± 28†</td>
</tr>
<tr>
<td>F₁₀₀, mg/mm</td>
<td>450 ± 14</td>
<td>417 ± 25</td>
<td>368 ± 31</td>
<td>334 ± 26†</td>
</tr>
<tr>
<td>ΔF with 1 μM ACh, % normalized to F₁₀₀</td>
<td>−43 ± 7</td>
<td>−24 ± 7†</td>
<td>5 ± 4†</td>
<td>2 ± 1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of vessel pairs. +EC and −EC, with and without endothelial cells, respectively; +T and −T, with and without tissue, respectively; F₁₀₀, force due to 100 mM KCl at optimum length; ΔF, change in force. Note that ACh-mediated dilation is not present under −EC segments. *P < 0.05 vs. −T segments; †P < 0.05 vs. +EC segments.

AJP-Heart Circ Physiol • VOL 282 • APRIL 2002 • www.ajpheart.org
There was no damage of the myocytes close to the coronary artery, and no differences among the three segments that were stained after the experiment compared with segments stained directly after the preparation for both myoglobin and SDH.

Effect of Hypoxia

After equilibration, vessels were precontracted with 30 mM KCl (resulting isometric force is F₁₀₀) in the buffer solution. Hypoxia induced a decrease in isometric force in both −T and +T segments, which could be repeated at least four times over a time period of 2.5 h, indicating that the preparation was stable. A typical recording (marked with F₃₀) of the effect of hypoxia on −T and +T segments is shown in Fig. 2. Just before N₂ (hypoxia) is applied, the preconstriction force level is determined, and just before O₂ (reoxygenation) is applied, the decrease in force due to hypoxia is determined. F₁₀₀ is the force due to 100 mM KCl at the optimal length, as explained in Fig. 1. In both −T and +T segments, hypoxia induced a reduction in force and significantly more in +T than in −T segments; the average results are shown in Fig. 3 (identified as KCl). Hypoxia reduced isometric force from 90 ± 2% to 69 ± 5% of F₁₀₀ in −T segments and from 90 ± 2% to 29 ± 5% of F₁₀₀ in +T segments (P < 0.05 vs. −T segments, n = 11). There was no relation between the wet or dry weights of +T segments and the effect of hypoxia.

Effect of Hypoxia and Type of Preconstrictor

In Fig. 3, the effect of hypoxia in U-46619 (0.003–1 μM)-preconstricted vessels is also shown. The force levels normalized to F₁₀₀ are 88 ± 3% and 83 ± 5% for −T and +T segments, respectively, and are similar to the 30 mM KCl-induced force levels. Hypoxia reduced force significantly to 72 ± 5% and to 34 ± 5% for −T and +T segments, respectively (n = 11, P < 0.05). The reduction in isometric force induced by hypoxia during U-46619-induced contraction was not significantly different from the reduction in KCl-induced contraction in both −T and +T segments but significantly larger in +T than in −T segments (P < 0.05) for both constrictors.

Effect of Hypoxia After Endothelial Removal

After endothelial removal (Fig. 4), hypoxia reduced force in −T segments from 89 ± 2% to 56 ± 8% (n = 6, P < 0.05). In +T segments, hypoxia reduced isometric force from 90 ± 6% to 29 ± 8% (n = 6, P < 0.05), significantly more than in −T segments (P < 0.05). ANOVA revealed a significant effect of both tissue and endothelium. The relaxation to hypoxia is significantly larger in −T segments without endothelium than in −T segments with endothelium (post hoc t-test, P < 0.05). However, endothelium removal had no effect on the response to hypoxia in +T segments (post hoc t-test, P > 0.05).

Mediators in the Response to Hypoxia

For the second part of the study, 31 pairs of vessels were used. In all cases, F₁₀₀ was determined and used to correct (force levels as a percentage of F₁₀₀) for differences in segment length and/or the number of smooth muscle cell layers. In group 1, the effect of the
nonselective adenosine receptor antagonist 8-PT on the response to hypoxia was tested (Fig. 5). In the absence of 8-PT, 30 mM KCl induced a precontraction level of 78\% and 95\% in T and +T segments, respectively. After the addition of 8-PT, these force levels were 73\% (P = 0.27 vs. control) and 65\% (P < 0.01). The response to exogenous adenosine on the precontracted segments in the absence and presence of 8-PT is shown in Fig. 5B. In the presence of 8-PT, the relaxation due to adenosine was significantly reduced, implying that the inhibitor is effective in our preparation. Finally, the response to hypoxia is shown in Fig. 5C. The response to hypoxia was larger in both the absence and presence of 8-PT in +T than in −T segments (P < 0.01 and P = 0.02 for control and 8-PT, respectively). The response to hypoxia in +T segments was significantly reduced by 8-PT.

The results of the other inhibitors (groups 2–5) are summarized in Table 2.

Table 2 is organized similarly to Fig. 5. The preconstriction force due to 30 mM KCl, the relaxation of the mediator, and finally the relaxation due to hypoxia was always without (control) and with inhibitor. An effect of DMPX on preconstriction force was found in −T and +T segments and an effect of L-NNA was found in −T segments. All relaxations due to the used mediators were blocked with the appropriate inhibitor, indicating that each of the inhibitors is functional in our setup. None of the inhibitors affected the relaxation due to hypoxia.

**DISCUSSION**

This study shows that two segments of the isolated rat coronary artery, one with and one without a layer of myocardial tissue, mounted in a double wire myograph is a suitable setup for studying the effect of hypoxia on myocardial tissue and the embedded coronary artery.
Our results clearly show that the relaxation in response to hypoxia is not caused by a dilatory factor released by the endothelium, because endothelium removal did not abolish the decrease in force induced by hypoxia. However, the relaxation induced by hypoxia is larger in endothelium-denuded vessels. This might be caused by a hypoxia-induced constriction mechanism present in the endothelium. Indeed, several studies have shown that hypoxia can induce contractions in endothelial cells, possibly due to changes in intracellular calcium or other signaling pathways. These findings highlight the complex interplay between the endothelium and smooth muscle cells in response to hypoxia, and underscore the importance of considering the role of mediators in this process.
(30, 34) on coronary preparations have shown that a hypoxic contraction was abolished by endothelial removal. In +T segments, removal of the endothelium had no significant effect on the response to hypoxia. This could be caused by the effect of a mediator released from the endothelium that has an effect on the contractile state of the surrounding cardiac myocytes (28) that counteracts the effect of endothelin on the contractile state of the vessel. In addition, the myocyte itself responds to hypoxia and releases substances that regulate the secretion of endothelin and a relaxant by the endothelial cells (32). Thus a complex cross-talk between myocytes and endothelium exists.

**Smooth Muscle Cells**

The observation that endothelial removal in the segment without surrounding tissue does not inhibit the response to hypoxia suggests that changes in oxygen tension have a direct effect on the smooth muscle cells. According to Paul (23), the total phosphagen pool in smooth muscle cells is small compared with its utilization; energy supply for contraction relies for the greater part on continuous intermediary metabolism. In this process, oxygen is required, and a reduction in oxygen availability will impair intermediary metabolism and contraction.

The effect of hypoxia on vascular relaxation appears independent of the type of constrictor used. Smooth muscle cells were activated by depolarization with a 30 mM KCl PSS, increasing the open probability of the voltage-sensitive Ca\(^{2+}\) channels (13). U-46619, a thromboxane mimetic, was used to stimulate smooth muscle cells in a receptor-dependent manner, activating the inositol (1,4,5)-trisphosphate/diacetylglycerol pathway (5). Thus the effect of hypoxia is independent on the intracellular pathway of contraction, and without both tissue and endothelium, there is a significant relaxation, indicating a role for the smooth muscle as a sensing element for oxygen.

**Myocardial Tissue**

The decrease in isometric force due to hypoxia (Figs. 2 and 3) is more in segments with a layer of cardiac myocytes. This strongly supports that the myocardial tissue releases a factor in response to hypoxia that reduces isometric force. Figure 3 shows that the additional relaxation (difference in relaxation between +T and −T segments) due to the surrounding myocardial tissue is larger than the relaxation of the vessel alone (−T segments). It should be noted that the energy need of the cardiac tissue in our preparation is primarily determined by basal metabolism, whereas in actively contracting tissue, the energy need is also determined by excitation-contraction coupling metabolism and mechanical activity associated with cross-bridge turnover (29). Therefore, the contribution of the cardiac myocytes in the beating heart is expected to be stronger. It seems that in +T with low metabolic activity, the large relaxation in response to hypoxia leaves little capacity to relax even more should the myocytes contract. However, it should be noted that the change in the oxygen tension used in this study is quite dramatic (from 150 to 5–10 mmHg). In the working heart, changes in oxygen tension induced by changes in activity are smaller.

**Inhibitors**

Prostaglandins and NO are not the mediators of the relaxation in both arteries without tissue and with tissue. Furthermore, the mechanism of relaxation is not through opening of K\(_{ATP}\) channels. Adenosine contributes to the hypoxic response via activation of A\(_1\) receptors, but its role is limited. More experiments, such as those using other inhibitors or combinations of inhibitors, may shed more light on the mechanism. Although isolated small coronary arteries were used in this study, these were considering the rat large coronary vessels. The lack of response of these vessels, for example, to K\(_{ATP}\) channel blockers, may therefore not be representative for the resistance vessels, as shown in isolated perfused guinea pig hearts by Daut et al. (4).

In conclusion, we developed a method to study the interaction between myocardial tissue and the vessel wall. We have also shown that the response to hypoxia is independent of the type of constrictor used. The vasoactive effects of hypoxia are not mediated by a single uniform mechanism but are probably interplay of several mechanisms that are activated under different conditions. The surrounding myocardial tissue is the dominant regulator of vascular tonus changes under conditions where the oxygen supply is impaired.

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