Hypoxic vasorelaxation inhibition by organ culture correlates with loss of Kv channels but not Ca\(^{2+}\) channels

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Thorne, George D., Laura Conforti, and Richard J. Paul. Hypoxic vasorelaxation inhibition by organ culture correlates with loss of Kv channels but not Ca\(^{2+}\) channels. Am J Physiol Heart Circ Physiol 283: H247–H253, 2002; 10.1152/ajpheart.00569.2001.—We (Thorne GD, Shimizu S, and Paul RJ, Am J Physiol Cell Physiol 281: C24–C32, 2001) have recently shown that organ culture for 24 h specifically inhibits relaxation to acute hypoxia (95% N\(_2\)-5% CO\(_2\)) in the porcine coronary artery. Here we show similar results in the porcine carotid artery and the rat and mouse aorta. In the coronary artery, part of the inability to relax to hypoxia after organ culture is associated with a concomitant loss in ability to reduce intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) during hypoxia (Thorne GD, Shimizu S, and Paul RJ, Am J Physiol Cell Physiol 281: C24–C32, 2001). To elucidate the mechanisms responsible for the loss of relaxation to hypoxia, we investigated changes in K\(^{+}\) and Ca\(^{2+}\) channel activity and gene expression that play key roles in [Ca\(^{2+}\)]\(_i\) regulation in vascular smooth muscle (VSM). Reduced mRNA expression of O2-sensitive K\(^{+}\) channels (Kv1.5 and Kv2.1) was shown by reverse transcriptase-polymerase chain reaction in the rat aorta. In contrast, no change in other expressed voltage-gated K\(^{+}\) channels (Kv1.2 and Kv1.3) or Ca\(^{2+}\) channel subtypes was found. Modified K\(^{+}\) channel expression is supported by functional evidence indicating a reduced response to general K\(^{+}\) channel activation, by pinacidil, and to specific voltage-dependent K\(^{+}\) (Kv) channel blockade by 4-aminopyridine. In conclusion, organ culture decreases expression of specific Kv channels. These changes are consistent with altered mechanisms of VSM contractility that may be involved in Ca\(^{2+}\)-dependent pathways of hypoxia-induced vasodilation.

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channels, such as Kv1.1–1.6, Kv2.1, and certain members of the Kv3 and Kv4 families, as O₂ sensors (19). Our laboratory (24) has demonstrated a reduced magnitude of relaxation to hypoxia in the presence of the Kv channel blocker 4-aminopyridine (4-AP). Prolonged hypoxia changes the expression of many Kv channel subtypes (30) and hypoxia is known to activate K⁺ currents in arterial myocytes (28).

Here we use the loss of O₂ sensing in our organ culture model (29) to elucidate the Ca²⁺-dependent mechanism of hypoxia-induced relaxation. Using molecular, physiological, and pharmacological approaches, we show that 1) organ culture results in a decreased expression of specific O₂-sensitive Kv channels, 2) the effects of K⁺ channel activators and Kv channel inhibitors are reduced after organ culture, and 3) neither Ca²⁺ channel expression nor activity is altered by organ culture for 24 h. These findings provide new insight into the mechanisms of hypoxic relaxation and suggest that Kv channels play a more important role than Ca²⁺ channels in O₂ sensitivity of these tissues.

METHODS

Artery culture conditions. Organ culture was carried out as previously described (29). Briefly, left descending coronary arteries were dissected from adult porcine hearts obtained from the slaughterhouse on the day of death and two rings per artery were cleaned of adhering connective tissue. One ring was cultured in sterile Dulbecco’s modified Eagle’s medium + 1% antibiotic solution at 37°C. A paired ring (control) was stored in the same solution at 4°C. After 24 h, arterial rings were prepared for organ bath experiments. All organ culture preparations were performed under sterile conditions in a culture hood. Porcine carotid arteries were obtained from the slaughterhouse and similarly prepared.

Adult male Sprague-Dawley rats and adult male C57 Black Swiss mice were euthanized by CO₂ asphyxiation. Small animal handling was done in accordance with, and with approval of, the local Institutional Animal Care and Use Committee. Aortas were dissected and rinsed with cold sterile culture media. Excess fat and connective tissue were removed and aortas were prepared for organ culture as described above.

Organ bath studies. All tissue used for these experiments were mechanically deendothelialized by gentle rubbing of the luminal surface with a cotton-tipped applicator. Rings were mounted onto two wires; one of which was fixed and the other was connected to a force transducer. One cultured ring and its paired control were placed into a bath containing physiological saline solution of the following composition (in mmol/L): 118.3 NaCl, 25.0 NaHCO₃, 11.1 dextrose, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 0.026 EDTA, and 2.5 CaCl₂. Bath pH was 7.4 when aerated with 95% O₂–5% CO₂ at 37°C. Tissues were allowed to equilibrate for 1 h. Tension was adjusted to 40 mN for coronary and carotid arteries and 30 mN for mouse and rat aortas, which set each tissue length in the range for optimal force generation. At least two precontraction-relaxation cycles to 80 mM KCl (for coronary and carotid arteries) or 60 mM KCl (aortas) were performed until maximum reproducible muscle forces were observed. The absence of the endothelium was confirmed by lack of a response to substance P (10⁻⁸ M). A test contraction was elicited using 40 mM KCl for hog coronary and carotid arteries and 30 mM KCl for rat and mouse aortas. After steady force was obtained, hypoxia was obtained by bubbling the bath with 95% N₂–5% CO₂ for ~20 min. The final O₂ tension of the bath solution, measured polarographically, was ~1–2%. We define these conditions as hypoxia. In some experiments, concentration-response curves to KCl were obtained in coronary artery. Rings were stimulated with increasing concentrations of KCl starting with 5 mM and ending with 55 mM in 5 mM increments.

In experiments with ion channel modulators, coronary rings were first tested for response to hypoxia as described above. Subsequently, arterial rings were stimulated with 1 μM U-46619. After stable forces were obtained, rings were treated with 4 mM pinacidil or 1 μM nifedipine. In separate experiments, rings were stimulated with 40 mM KCl for a reference contraction, rinsed with fresh physiological saline solution, and then treated with 1 mM 4-AP.

The hypoxic response was characterized in terms of the maximum hypoxic relaxation, expressed as a percentage of the initial developed isometric force, as were all other relaxation responses. All organ bath measurements were recorded using a digital data acquisition system (Biopac). Force was normalized to cross-sectional area (F/A = change in force × circumference/2 × wet weight). Concentration force relations are given in terms of percentage of the maximum force of the reference contraction.

Reverse transcriptase-polymerase chain reaction of K⁺ and Ca²⁺ channels. Total RNA from the rat aorta was isolated and used for reverse transcriptase-polymerase chain reaction (RT-PCR) according to the protocol of PE Life Sciences (Boston, MA). Briefly, an oligo-dT primer was used with the murine leukemia virus RT for first-strand synthesis. Amplification of desired K⁺ channel cDNA was performed with sense and antisense primers designed after the rat brain Kv channel cDNA and used for reverse transcriptase-polymerase chain reaction (RT-PCR) according to the protocol of PE Life Sciences (Boston, MA). The following K⁺ channel primers was also used: L-type sense TGC TCT GCC TGA CTC TGA AG, antisense GAG TGC CTT CAC ATC GAA TC, annealed at 46°C for 38 cycles; T-type sense GCC GTG TCA GCC GGC GCC TTT CT, antisense CAA AGG TGA GTT TAT CCT CAG GC, annealed at 50°C for 38 cycles; and P/Q-type sense CCA GTC TGT GGA GAT GAG AAT GGG, antisense TTT GGA GGG CAG CAG ACC CGA TTG, annealed at 52°C for 38 cycles. PCR products were analyzed on 1.2% DNA agarose gels containing ethidium bromide (3 μg/ml) for visualization.

RT-PCR using all K⁺ and Ca²⁺ channel primers was also attempted in porcine coronary artery. None of these primers recognized porcine coronary artery cDNA. Therefore, the rat aorta was used for these experiments and subsequent Western blot analysis.

Western blot analysis. Control and organ cultured rat aortic rings were frozen in liquid N₂ and pulverized vigorously in a dental amalgamator. The resulting powder was dissolved in ice-cold homogenization buffer (0.15 M NaCl, 5 mM EDTA, 1 mM dithiothreitol, 20 mM sodium metabisulphite, 20 mM imidazole, and 1 mM phenylmethylsulphonyl fluoride). Homogenates were incubated on ice for 1 h and then centrifuged at 16,000 g and 4°C for 45 min. The supernatant was saved and total protein concentration was determined from this sample by using the Bradford assay. Samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 1.5 h. Proteins were transferred to nitrocellulose membranes for 1 h using the Semi-Dry Transfer apparatus (Bio-
Rad; Hercules, CA). After transfer, membranes were blocked with 3% nonfat dry milk for 1 h and then incubated with primary antibody at 4°C for 16–18 h. Membranes were then washed four times for 20 min each with phosphate-buffered saline, followed by 1-h incubation at room temperature in secondary antibody (horseradish peroxidase-conjugated anti-rabbit). Membranes were washed again four times in phosphate-buffered saline, subjected to enhanced chemiluminescence detection for 1 min, and then exposed to film.

Statistical analysis. Data were analyzed using the t-test for paired two-sample means or two-way repeated-measures analysis of variance with one-factor balance design. Statistical significance was accepted for P < 0.05. Values are expressed as means ± SE. n values represent the number of hearts from which arteries were isolated.

Chemicals. U-46619, pinacidil, 4-AP, and nifedipine were from Sigma-Aldrich (St. Louis, MO). RT-PCR reagents were from Applied Biosystems (Atlanta, GA). Antibodies for K+ channels (Kv1.5 and Kv2.1) were from Upstate Biotechnology (Lake Placid, NY) and for Kv1.3, Kv1.2, and L-type Ca2+ channels (Kv1.5 and Kv2.1) were from Upstate Biotechnology (Lake Placid, NY) and for Kv1.3, Kv1.2, and L-type Ca2+ from Alomone Labs (Jerusalem, Israel). Antibodies to calponin were from Sigma-Aldrich.

RESULTS

Effects of organ culture on hypoxic relaxation in porcine coronary and carotid artery and rat and mouse aorta. We (29) investigated whether the marked reduction in hypoxia-induced relaxation after organ culture was specific to coronary arteries. Figure 1 shows relaxation to hypoxia for four different control and organ-cultured systemic vessels. The reduced relaxation to hypoxia in the cultured porcine coronary artery (29) is similar to that of the rat and mouse aorta. Both show a 50–60% reduction in hypoxic relaxation. Porcine carotid artery exhibited a greater level of inhibition at ~80%. Whereas control carotids had a significantly higher magnitude of hypoxic relaxation compared with the other tissues, cultured carotid arteries relaxed even less compared with the controls. These results indicate that inhibition of hypoxic relaxation after organ culture occurs in many vascular tissue types.

Effects of organ culture on depolarization-contraction coupling, K+ and Ca2+ channel function. We reported that organ culture causes specific inhibition of hypoxic relaxation in porcine coronary artery without significantly affecting maximum force development or relaxation via A- or G-kinase pathways (29). Intracellular Ca2+ handling during hypoxia was also altered after organ culture. One possible explanation for these changes in contractility and Ca2+ handling is a change in depolarization-contraction coupling. Figure 2 shows KCl concentration-force relations for both control and organ-cultured coronary arteries. There is a significant rightward shift in the developed force to KCl after organ culture. The ED50 for control and cultured arteries are 14.8 ± 0.5 and 22.3 ± 0.7 mM KCl, respectively. These data suggest a decrease in sensitivity to KCl depolarization without adversely affecting maximum isometric force (Fig. 2, inset) after organ culture. An alteration in ion channel function could result in compromised Ca2+ handling leading to reduced relaxation to hypoxia. It is conceivable that a loss in K+ or Ca2+ vital for the reduction in [Ca2+]i during hypoxia could manifest as an inability to relax to hypoxia.

K+ and Ca2+ channels directly regulate [Ca2+]i in response to depolarization-mediated activation in VSM. We measured force production in response to general K+ channel activation (pinacidil), Kv channel blockade (4-AP), and L-type Ca2+ channel blockade (nifedipine) to determine whether changes in these ion channel activities underlie changes in Ca2+ handling and depolarization-contraction coupling. After organ culture of coronary arteries, activation of K+ channels by pinacidil is markedly reduced (Fig. 3A). Both the magnitude and rate of relaxation of coronary rings after 4 μM pinacidil are decreased. In a previous report

Fig. 1. A: typical tracing of control and organ cultured rat aortas in an organ bath experiment. Cultured aorta exhibit significant reduction in hypoxic vasorelaxation. B: summary of the effects of organ culture on hypoxia-induced relaxation in porcine coronary artery, porcine carotid artery, and rat and mouse aorta. All four vascular smooth muscle types show a marked reduction in relaxation to hypoxia after 24 h of organ culture at 37°C (solid bars). Results represent the means ± SE for 12 experiments (coronary artery and rat aorta), 4 experiments (carotid artery), and 2 experiments (mouse aorta). *P < 0.05 vs. control.
(26), we demonstrated a major role of Kv channels in modulating porcine coronary contractility. Approximately 40% of the pinacidil vasodilation is inhibited by 1 mM 4-AP (Fig. 3C). In addition, there is evidence implicating a role for Kv channels in O2-sensing mechanisms (1, 26, 28). Organ culture results in a decreased force response to Kv channel blockade (Fig. 3B). These data are summarized in Fig. 4. Both relaxation after K+ activation and the increase in force after Kv channel blockade are inhibited by organ culture. The degree of inhibition of relaxation after K+ activation is similar to that observed for relaxation to hypoxia. Relaxation after L-type Ca2+ channel blockade was not significantly affected by organ culture. These results indicate a reduction in K+ channel function, specifically Kv channels after organ culture that correlates with a loss in hypoxic relaxation.

Expression of O2-sensitive Kv channels and Ca2+ channel subtypes after organ culture. The activity of certain Kv channel subtypes and Ca2+ channels has been shown to be O2 sensitive (1, 9, 10). We investigated the expression of a subset of these channels that have been previously reported (19, 31) to be involved in hypoxic vasoconstriction in pulmonary arteries. Whereas our functional database is most extensive for hog coronary artery, many Kv channel molecular tools are not available. RT-PCR was performed using specific sense and antisense primers designed to amplify rat cDNA fragments of the following ion channels: Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv2.1, L-type Ca2+, T-type Ca2+, and P/Q-type Ca2+. Because organ culture also inhibited hypoxic relaxation in rat aorta, this vessel was used in these experiments and for Western blot analysis. The results are shown in Fig. 5. Only Kv1.2, Kv1.3, Kv1.5, Kv2.1, and the three Ca2+ channels subtypes were present in this tissue. RT-PCR was performed on rat brain RNA as a control to confirm that the primers were amplifying the cDNA fragment of the correct size (data not shown). Whereas there was no change in Kv1.2 and Kv1.3 expression, there was a marked downregulation of Kv1.5 and Kv2.1. There was
also no change in Ca\(^{2+}\) channel expression after organ culture. To confirm decreased expression of Kv1.5 and Kv2.1 at the protein level, Western blots were made using antibodies specific to each channel. Figure 6 shows that there is a loss in both Kv1.5 and Kv2.1 protein after organ culture. These results suggest that organ culture inhibits the expression of Kv1.5 and Kv2.1 without affecting that of other Kv or Ca\(^{2+}\) channels present.

**DISCUSSION**

Recently, we (29) have shown that organ culture specifically reduces relaxation to hypoxia in the porcine coronary artery. In this study, we demonstrate that this is also observed for the porcine carotid artery and rat and mouse aorta. We have shown that inhibition of relaxation to hypoxia after organ culture involves changes in [Ca\(^{2+}\)]\(_i\) handling (29). Others have shown that organ culture alters overall Ca\(^{2+}\) handling in VSM (15). Figure 2 shows that the KCl-force relation is shifted rightward in cultured arteries, suggesting altered Ca\(^{2+}\) handling. Stimulation by depolarization-mediated agonist involves reduced K\(^+\) influx through K\(^+\) channels and activation of voltage-gated Ca\(^{2+}\) channels. Our data indicate that there is a change in the excitation-contraction coupling pathway for KCl after organ culture (Fig. 2) that is likely due to reduced Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels. We have previously shown that there is a reduction in [Ca\(^{2+}\)]\(_i\) during hypoxic relaxation under low levels of activation (24). In a recent study, we demonstrated that organ culture for 24 h at 37°C abolishes not only relaxation to hypoxia but also the concomitant decrease in [Ca\(^{2+}\)]\(_i\) (29). An inability to reduce [Ca\(^{2+}\)]\(_i\) may reflect altered function or expression of ion channels, presumably K\(^+\) channels and Ca\(^{2+}\), that are responsible for regulating Ca\(^{2+}\) homeostasis specifically during hypoxia.

K\(^+\) channels play a major role in modulating force and ultimately Ca\(^{2+}\) homeostasis in vascular smooth muscle. After organ culture there is a reduction in the vasodilator response to pinacidil, a general K\(^+\) channel activator (Figs. 3A and 4). This suggests that K\(^+\) channels are not functional or that there is a decrease in the number of K\(^+\) channels. The specific type of K\(^+\) channel activity that is altered cannot be inferred from

**Fig. 4.** Summary of the decrease in force (left y-axis) to hypoxia, 4 μM pinacidil, and 1 μM nifedipine in the porcine coronary artery. Summary of isometric force generation (right y-axis) to 1 mM 4-AP. *Inset*, 4-AP-generated force as a percentage of maximum isometric force to 40 mM KCl. Responses to all treatments except nifedipine are decreased. Results represent the means ± SE of 4 experiments. *P < 0.05 vs. control.

**Fig. 5.** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Kv channels (A) and Ca\(^{2+}\) channel subtypes (B) in control and organ cultured rat aortas. Total RNA samples were reverse transcribed and K\(^+\) channel cDNA fragments were amplified by PCR with the specific primers outlined in METHODS. There is a decreased expression of Kv1.5 (600 bp) and Kv2.1 (585 bp) but not Kv1.2 (407 bp) and Kv1.3 (235 bp) after organ culture. There was also no change in L-type (400 bp), T-type (164 bp), and P/Q-type (393 bp) Ca\(^{2+}\) channel expression. The ribosomal protein S16 was analyzed in the same samples for loading control and showed no change after organ culture.

**Fig. 6.** Western blot analysis of K\(^+\) channels and L-type Ca\(^{2+}\) channels using specific polyclonal anti-rat antibodies to each protein. Protein levels of both Kv1.5 (60 kDa) and Kv2.1 (100 kDa) channels are markedly reduced after organ culture. Kv1.3 (90 kDa), Kv1.2 (140 kDa), and L-type Ca\(^{2+}\) (205 kDa) channel protein were unchanged. The smooth muscle-specific protein calponin (34 kDa) was analyzed for loading control.
these experiments because pinacidil has been shown to activate several different K⁺ channel subtypes (3). However, 40% of the pinacidil response is 4-AP sensitive suggesting a major Kv channel contribution (Fig. 3C). Shimizu et al. (26) showed that 4-AP-sensitive K⁺ channels were the most significant K⁺ channels in terms of modulating force and [Ca²⁺]ᵢ in porcine coronary artery. There is evidence that hypoxia relaxation of arteries can involve hyperpolarization mediated through ATP-sensitive K⁺ channels and Ca²⁺-sensitive K⁺ channels (12, 16, 19). However, using a variety of K⁺ channel inhibitors, only 4-AP treatment resulted in a significant decrease in the hypoxic relaxation (24). However, because 4-AP pretreatment also increased the prehypoxic force, interpretation of the attenuated hypoxic relaxation in terms of K⁺ channel blockade is not straightforward.

Data in Fig. 3B (and summarized in Fig. 4) show a significantly reduced reactivity to 4-AP blockade after organ culture. The blunted force increase to 4-AP may indicate Kv channels not affected by organ culture that contribute to the small remaining hypoxic relaxation observed (Fig. 1, lane 1). The reduction in the hypoxic relaxation after organ culture also paralleled the decreased relaxation to pinacidil (Fig. 4). The loss of K⁺ channel function was also paralleled by the inability of hypoxia to reduce [Ca²⁺]ᵢ during hypoxia could be explained by altered Ca²⁺ channel function as well. However, our results indicate that voltage-dependent Ca²⁺ channel function was not altered by organ culture (Fig. 4).

Our conclusions based on functional evidence are strongly supported by our biochemical results. Organ culture is known to alter the expression of several ion channel subtypes. It has been reported (21) that chronic hypoxia decreases the expression of Kv1.5 and Kv2.1 in pulmonary myocytes (5). We investigated the effect of organ culture on K⁺ channels known to be O₂ sensitive. Our results indicate that expression of Kv1.5 and Kv2.1 is decreased in the rat aorta after organ culture. Functional results with K⁺ channel modulators in both the hog coronary and rat artery support these findings (Fig. 4). Expression of other K⁺ channel subtypes did not change (Fig. 5). This is consistent with the ability to contract to depolarization. The lack of change in expression of Ca²⁺ channel subtypes not only supports the maintenance of L-type Ca²⁺ channel function, but also suggests that inhibition of relaxation to hypoxia after organ culture primarily involves altered K⁺ channel expression. The functional and molecular data presented here suggest that at least Kv1.5 and Kv2.1 are important for hypoxic vasorelaxation after organ culture.

The mechanism(s) by which organ culture results in altered ion-channel expression is unknown. VSM cell culture is associated with differentiation and progression from a contractile to a proliferative phenotype (23). Organ culture is also known to alter both smooth muscle-specific and ubiquitously expressed genes (27). These changes could manifest into altered ion channel expression and/or function by several mechanisms including inhibition of gene transcription, decrease in mRNA or protein expression, or modulation of transcription factors that regulate ion channel expression. A change in transcription factors and promoter elements necessary for regulation of Kv channel expression (8), for example, could occur during organ culture and cause the downregulation of Kv1.5 and Kv2.1 that we observe. The exact mechanism may actually be a combination of several and warrants continued investigation.

Our results provide compelling evidence for the involvement of K⁺ channels in the hypoxic relaxation. However, it is possible that mechanisms not involving K⁺ channels may be involved. We have championed the hypothesis that a Ca²⁺-independent mechanism of hypoxic relaxation also exists (24). Moreover, hypoxic vasodilation of some vessels is in fact, not associated with hyperpolarization (11). In this investigation, we demonstrate that organ culture inhibits hypoxia-induced relaxation in four different vascular smooth muscle tissues. We previously showed that altered Ca²⁺ handling contributes to this attenuated relaxation response. Our current data show that K⁺ channels, specifically Kv1.5 and Kv2.1, but not Ca²⁺ channels are decreased in parallel to the hypoxic relaxation by organ culture. Further investigation is needed to unambiguously link these K⁺ channels to the hypoxic relaxation.

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