Hypoxia and Stretch Regulate Intercellular Communication in Vascular Smooth Muscle Cells Through Reactive Oxygen Species Formation

Douglas B. Cowan, Mara Jones, Lina M. Garcia, Sabrena Noria, Pedro J. del Nido, Francis X. McGowan Jr

Objective—We hypothesized that the alterations in vasomotor tone and adaptive remodeling responses that occur in the circulation because of hypoxia were dependent on changes in cell to cell communication through regulation of gap junction protein expression and function. Consequently, we studied the amount, distribution, and permeability of the principal vascular smooth muscle cell (VSMC) gap junction protein, connexin43, in rat aortic cultures exposed to oxygen partial pressures of 150 or 15 mm Hg.

Methods and Results—Immunohistochemical staining, immunoblot assays, and Northern blot analyses demonstrated that connexin43 expression was reversibly increased in hypoxic cultures. As a result, hypoxic cells exhibited greater intercellular communication as determined by fluorescence recovery after photobleaching experiments. Using a fluorogenic substrate, hypoxic VSMCs showed increased reactive oxygen species generation, which could be prevented by the glutathione peroxidase mimic ebselen and the mitochondrial complex I inhibitor rotenone but not with the redox-sensitive thiol pyrrolidine dithiocarbamate. The rise in connexin43 expression attributable to hypoxia could be attenuated by ebselen and rotenone treatment. Interestingly, the previously reported induction of connexin43 expression by tensile stretch was also contingent on oxidative activity.

Conclusions—Hypoxia and stretch increased gap junctional intercellular communication in VSMCs attributable to enhanced connexin43 expression initiated by reactive oxygen species formation. (Arterioscler Thromb Vasc Biol. 2003; 23:1754-1760.)

Key Words: gap junction ■ reactive oxygen species ■ hypoxia ■ stretch ■ smooth muscle cell

The coordination and propagation of motor responses in blood vessels are brought about by neurohormonal modulation, ion channel conductance, and intercellular communication. Although acute vasomotion leads to circulatory homeostasis through the maintenance of local vessel tone, sustained motor responses initiate structural remodeling of the tissue. Because vasomotor and arterial remodeling responses require extensive signaling between the endothelium and medial smooth muscle layers, as well as between adjacent smooth muscle cells, we have been studying cell to cell communication in the vasculature.

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The structure of the arterial circulation can be adjusted in both the circumferential and radial direction, resulting in changes to vessel diameter or wall thickness. For instance, vascular tissue responds to variations in the mechanical forces imposed on it by blood pressure (ie, tensile stretch) and blood flow (ie, shear stress) with rapid adjustments to vasomotor tone and with changes in tissue geometry when alterations in these forces persist. These responses are specific for the type of hemodynamic force that is imposed on the vessel wall. Whereas an increase in tensile stretch causes vasoconstriction in the short term and arterial thickening in the long term, an increase in blood flow induces acute vasodilation and chronic enlargement of arterial diameter when the time-averaged shear force is maintained.

Vascular remodeling can also result from exposure to low levels of environmental oxygen. Chronic hypoxia causes pulmonary vasoconstriction and muscularization of precapillary resistance arterioles in the lung. Paradoxically, hypoxia results in vasodilation and structural enlargement of many systemic blood vessels. In addition, remodeling after acute changes in vasomotor tone occurs in response to vessel injury, as seen in postangioplasty restenosis, graft vasculopathy, and hyperlipidemia-induced atherosclerosis.

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From the Departments of Anesthesiology, Perioperative and Pain Medicine (D.B.C., M.J., F.X.M.) and Cardiac Surgery (L.M.G., P.J.D.), Children’s Hospital Boston and Harvard Medical School, Boston, Mass, and Department of Laboratory Medicine and Pathobiology (S.N.), University of Toronto, Ontario, Canada.
Correspondence to Douglas B. Cowan, PhD, Department of Anesthesiology, Perioperative and Pain Medicine, Enders Room 1355, Children’s Hospital Boston, 300 Longwood Ave, Boston, MA 02115. E-mail douglas.cowan@childrens.harvard.edu © 2003 American Heart Association, Inc.

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Whereas the means by which mechanical forces, hypoxia, and vascular injury are transduced into cellular signals is not fully understood; it seemingly involves the cytoskeleton, cellular junctions, ion channels, transmembrane receptors, second messengers, signaling proteins, and reactive oxygen species (ROS) generation. In any case, the transition from vasomotor to structural remodeling responses most likely involves expression of many genes that affect cell proliferation and death as well as connective tissue elaboration and organization.

Because arterial remodeling attributable to chronic changes in oxygen tension likely involves extensive cell to cell communication, we reasoned that gap junctions would play a significant role in mediating early remodeling responses analogous to those demonstrated for mechanical forces. Gap junctions are transmembrane channels that directly link the cytoplasm of neighboring cells, thereby allowing for low-resistance electrical coupling between cells in excitable tissue. The channels are formed when a hemichannel, or connexon, in one cell docks to another in an adjacent cell. Six connexin proteins associate to form the hemichannel, and at least 20 connexin isoforms have been predicted to exist in vertebrates. Connexons composed of different isoforms exhibit distinct electrophysiological properties with respect to conductance, voltage dependence, and permeability. Although vascular tissues are known to express multiple connexins, connexin43 (Cx43, gap junction protein α1) is the predominant isoform expressed in the smooth muscle layers of major arteries.

Because smooth muscle cells are the primary cell type found in the vessel media and are nominally the final effectors of vasomotor tone and structural remodeling responses, we tested whether vascular smooth muscle cells (VSMCs) exhibit oxygen tension-sensitive expression of Cx43. We found that hypoxic smooth muscle cell cultures had increased Cx43 mRNA and protein levels that resulted in greater gap junctional intercellular communication. Furthermore, we demonstrated that inhibition of enhanced ROS production during hypoxia prevented the increase in Cx43 expression and cell to cell communication. Intriguingly, the previously reported rise in Cx43 protein induced by mechanical stretch was also found to be mediated through the formation of ROS.

**Methods**

**Cell Culture**

Animal procedures received institutional approval and were conducted according to the NIH guidelines. Wistar rat thoracic aorta VSMCs were isolated and cultured as described earlier. Confluent passage 4 cultures were exposed to a normobaric partial pressure of oxygen (PO2) of 150 or 15 mm Hg for various times in either a standard culture incubator or a hypoxia chamber set to 2.2% oxygen (Coy Laboratory Products). Oxygen levels were reduced in the humidified chamber using 95% N2/5% CO2 replacement and verified by dissolved gas measurements on culture media using a Stat Profile phOx Plus analyzer (Nova Biomedical). Other VSMCs were subjected to mechanical strain (20% static stretch) as previously specified. Where applicable, cells were subjected to a 10-minute pretreatment with 10 μmol/L pyrroline dithiocarbamate (PDTC) (Sigma), 50 μmol/L ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) (Calbiochem), or 10 μmol/L rotenone (Sigma) before treatment with either hypoxia or stretch in the presence of these compounds.

**Immunohistochemistry**

Fixed VSMCs were stained with 10 μg/mL anti-Cx43 monoclonal antibody (Chemicon) and detected with a 1:200 dilution of antirabbit Alexa 568-conjugated secondary antibody mixed with 4 U/mL Alexa 488-phalloidin (Molecular Probes). Slides were visualized using a BioRad MRC1024 confocal microscope as described previously.

**Immunoblot Analyses**

Immunoblotting was performed as described before. Cx43 was detected with 2 monoclonal antibodies to distinguish between total protein and unphosphorylated protein. The Chemicon antibody (MAB3067) was used at a concentration of 1 μg/mL, whereas the Zymed antibody (13-8300) was used at 0.5 μg/mL. Primary antibodies were detected with horseradish peroxidase–labeled antimouse secondary antibodies (1:2500) and the ECL kit (Amersham). Densitometric analysis was performed using the public domain Image software developed by the NIH and Scion Corporation. Integrated area measurements were analyzed as described earlier.

**Northern Blot Analysis**

Total RNA isolation, electrophoresis, and transfer to nitrocellulose were performed as previously described. The rat Cx43 cDNA clone and GAPDH cDNA clone (Ambion) were radioactively labeled and hybridized to RNA as detailed earlier.

**Fluorescence Recovery After Photobleaching**

VSMCs were grown on bovine plasma fibronectin (Sigma)-coated 25-mm No. 1 glass coverslips and loaded for 10 minutes with 2 μmol/L calcein AM (Molecular Probes) in phenol red-free medium containing 199 (Gibco). After rinsing the cells 3 times, coverslips were mounted in a Sykes-Moore chamber (Beltco) and images were digitally acquired with a MicroMAX 1300Y CCD camera (Princeton Instruments) at a 100 magnification using a Zeiss Axiovert 35 fluorescence microscope fitted with standard excitation and emission filters and a 75-W xenon arc lamp. Cells in the center of the acquired fields were photobleached for 30 seconds using a ×63 Zeiss Achroplan objective lens (numerical aperture [NA] 0.75; working distance [WD] 0 to 1.5 mm). Images were subsequently obtained at 1-minute intervals with a ×10 Achroplan (NA 0.25) objective lens. Average fluorescence intensity region measurements were made using MetaMorph 6.0 software (Universal Imaging).

**Oxidative Activity Measurements**

The 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(ace- toxymethyl ester) (H2DCFDA-AM) (Molecular Probes) isomer was loaded into VSMCs at 5 μmol/L in the presence or absence of PDTC, ebselen, and rotenone. Cells were carefully harvested with a rubber policeman and analyzed directly with a Packard Fusion 3.02 fluorescent plate reader or fixed with 4% paraformaldehyde in PBS pH 7.4 and then stained with a 1:200 dilution of an anti-smooth muscle actin-Cy3 conjugate (Sigma) and 300 nmol/L 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) before mounting and viewing with a Zeiss fluorescent microscope.

**Results**

**Cx43 Expression in Hypoxic VSMCs**

The effect of hypoxia on cultured VSMC Cx43 protein distribution and quantity was determined by immunohistochemical staining (Figure 1) and immunoblot analysis (please see the online supplement, available at http://atvb.ahajournals.org). Confluent monolayers of cells (A through C) exhibited a large increase in Cx43 staining (D through F) when exposed to...
hypoxia (B, E, and H). Staining of filamentous actin is depicted in green (A through C, G through I), and Cx43 staining is shown in red (D through F, G through I). Staining of the same VSMCs as depicted in A through C, with an anti-Cx43 antibody detected with an Alexa 568-conjugated secondary antibody (red). G through I, Merged images of A and D, B and E, and C and F, respectively. Scale bars are shown in the lower left corner of each panel, and results are representative of 4 experiments.

Figure 1. Projected series of confocal photomicrographs depicting immunofluorescently stained VSMCs cultured at a P O2 of 150 mm Hg continuously (A, D, and G), 15 mm Hg for 24 hours (B, E, H), or 15 mm Hg for 24 hours followed by 150 mm Hg for an additional 24 hours (C, F, and I). A through C, Staining of the actin-cytoskeleton with Alexa 488-phalloidin (green). D through F, Staining of the same VSMCs as depicted in A through C, with an anti-Cx43 antibody detected with an Alexa 568-conjugated secondary antibody (red). G through I, Merged images of A and D, B and E, and C and F, respectively. Scale bars are shown in the lower left corner of each panel, and results are representative of 4 experiments.

Immunoblots of Cx43 protein levels in response to hypoxia from 0 to 24 hours are shown in Figure I of the online supplement (available at http://atvb.ahajournals.org). Equal protein concentrations were loaded in each lane, and protein quality was assessed by staining identical gels with Coomassie Brilliant Blue R-250 (not shown). The top panel shows total Cx43 protein, whereas the bottom panel shows only the unphosphorylated protein. Hypoxia induced the protein (P<0.001) by 4 hours, and the amount of Cx43 remained elevated for at least 24 hours (compare lane 1 to lanes 2 through 5). As well, there was no readily apparent difference in the ratio of phosphorylated protein to unphosphorylated protein (lanes 1 through 5). These observations were confirmed by densitometry and statistical analyses.15

Cx43 mRNA levels in response to P O2s of 150 mm Hg (lane 1), 15 mm Hg for 24 or 48 hours (lanes 2 and 3), or 15 mm Hg for 24 hours followed by reoxygenation at 150 mm Hg for 4, 8, and 24 hours (lane 4, 5, and 6) are depicted in Figure 2A. Cx43 mRNA was considerably increased in the low-oxygen environment (compare lane 1 with lanes 2 and 3) and, on return to a P O2 of 150 mm Hg for 4, 8, and 24 hours (lane 4, 5, and 6) hours. Samples were electrophoresed on a 1.0% formaldehyde-agarose gel, transferred to nitrocellulose membranes, hybridized to rat Cx43 and GAPDH cDNA probes, and washed using standard procedures.6 Membranes were exposed to x-ray film overnight with intensifying screens. The depicted blots are representative of 4 independent experiments.

Figure 2. Northern blot analyses of Cx43 (A) and GAPDH (B) mRNA levels in total RNA (C) extracted from VSMCs cultured at a P O2 of 150 mm Hg for 48 hours (lane 1), a P O2 of 15 mm Hg for 24 hours (lane 2), a P O2 of 15 mm Hg for 48 hours (lane 3), and a P O2 of 15 mm Hg for 24 hours and then 150 mm Hg for 4, 8, and 24 hours (lanelane 4, 5, and 6) hours. Samples were electrophoresed on a 1.0% formaldehyde-agarose gel, transferred to nitrocellulose membranes, hybridized to rat Cx43 and GAPDH cDNA probes, and washed using standard procedures. Membranes were exposed to x-ray film overnight with intensifying screens. The depicted blots are representative of 4 independent experiments.

Intercellular Communication in Hypoxic VSMCs
Because we noticed much of the Cx43 protein in hypoxic VSMCs was located intracellularly rather than on the cell surface, we sought to determine whether the increases in Cx43 expression correlated with enhanced intercellular communication. To assess gap junction function, we used a modified fluorescence recovery after photobleaching (FRAP) technique16,19 on confluent monolayers of cells. VSMCs were
Figure 3. Representative fluorescent images of VSMCs used in FRAP analyses (top). Cells were cultured at a PO2 of 150 mm Hg (A through C) or 15 mm Hg for 24 hours (D through F), and the same field of cells is depicted in each row of photomicrographs. The calcein-loaded cells before photobleaching are shown in A and D. The photobleached area is shown in B and E, whereas the same area is shown in C and F after 2 minutes of recovery from photobleaching. Cells within the photobleached area regain fluorescence attributable to the transfer of calcein from unbleached neighboring cells through gap junctions. A graph of the average fluorescence intensity of the circular target area before photobleaching (total fluorescence), immediately after photobleaching (photobleached), and 2 minutes after photobleaching (recovered) in VSMCs cultured at PO2s of 150 mm Hg (control) or 15 mm Hg for 24 hours (hypoxia) (bottom). Standardized measurements were made using MetaMorph 6.0 software, and arbitrary values are expressed as mean ± SE (n=8). *Statistically significant difference (P<0.001) in fluorescence recovery in control (▪) vs hypoxic cultures (□) as determined by the Tukey-Kramer test.

ROS Formation in Hypoxic VSMCs
The effect of hypoxia on VSMC oxidative activity was investigated using the intracellular probe H2DCFDA-AM. Figure II of the online supplement shows photomicrographs depicting fluorescent emission resulting from interaction of oxidized H2DCFDA with reactive oxidant species (green) after exposure to a PO2 of 150 mm Hg (A) or a PO2 of 15 mm Hg for 1 (B), 2 (C), and 4 hours (D). The cells were cross-stained for nuclei with DAPI (blue) and smooth muscle actin using an anti-α-smooth muscle actin-Cy3 antibody (red). Figure 4 graphically depicts DCF fluorescence from VSMCs (n=3) cultured at a PO2 of 150 mm Hg (control) or 15 mm Hg for 1, 2, and 4 hours. Results are also presented for 1-hour hypoxia treatment in the presence of 50 μmol/L ebselen, 10 μmol/L rotenone, or 10 μmol/L PDTC (bars, left to right). The results are from 6 independent experiments and expressed as mean±SE. *Statistically significant increase (P<0.001) in DCF fluorescence vs control (bar on the left). **Statistically significant decrease (P<0.001) in DCF fluorescence vs 1 hour of hypoxia. ***Significant increase (P<0.001) in DCF fluorescence vs 1 hour of hypoxia.

ROS Inhibition and Cx43 Expression
To ascertain if oxidative activity in VSMCs was associated with gap junction protein α1 expression, we examined Cx43 levels in hypoxic cells treated with specific inhibitors of bleached isolated cells (ie, not connected by gap junctions) and confluent VSMCs loaded with 5 μmol/L CellTracker CM-DiI. Cells lacking neighbors were not able to recover fluorescence, and cells labeled with the membrane tracer DiI demonstrated an inability to transfer fluorescence from one cell to the next (not shown). In addition, photobleached cells were confirmed as viable, because they could repeatedly be loaded with calcein AM and subjected to photobleaching and recovery.
VSMCs cultured for 6 hours at a P O2 of 15 mm Hg (A) or sub-

grown on fibronectin-coated silicone membranes (B). Lane 2, VSMCs cultured for 6 hours at a P O2 of 15 mm Hg (A) or subjected to 20% static stretch for 6 hours (B). Lanes 3 through 5, VSMCs grown at a P O2 of 15 mm Hg for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively, or stretched for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively. Lanes 6 through 8, VSMCs grown at a P O2 of 150 mm Hg in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively. Lane 1, Untreated VSMCs cultured at a PO2 of 150 mm Hg (A) or grown on fibronectin-coated silicone membranes (B). Lane 2, VSMCs grown on fibronectin-coated silicone membranes (B). Lane 1, Untreated VSMCs cultured at a PO2 of 150 mm Hg (A) or grown on fibronectin-coated silicone membranes (B). Lane 2, VSMCs grown at a P O2 of 15 mm Hg for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively, or stretched for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively. Lanes 6 through 8, VSMCs grown at a P O2 of 150 mm Hg in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively, or cultured on fibronectin-coated silicone membranes in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively. Results are representative of 4 experiments.

We have demonstrated a reversible increase in Cx43 expression in response to mechanical loading, and rotenone in either treatment group (lanes 4 and 5). PDTC treatment, on the other hand, had little effect (P>0.05) on Cx43 protein levels in hypoxic or stretched cells (compare lanes 2 and 3 in Figures 5A and 5B). In untreated cells, PDTC seemed to increase unphosphorylated Cx43 protein levels (lane 6); however, this observation was not statistically significant. Alternatively, ebselen and rotenone were both found to depress (P<0.05) baseline Cx43 expression with little effect on state of protein phosphorylation (lanes 7 and 8). FRAP experiments confirmed that ebselen and rotenone (but not PDTC) reduced (P<0.001) cell to cell dye transfer in hypoxic VSMCs (not shown).

ROS Formation in Stretched VSMCs

Because ebselen and rotenone attenuated the induction of Cx43 expression in response to mechanical loading,6 we measured oxidative activity in stretched VSMC cultures using H2DCFDA-AM (Figure 6). There was a significant increase in ROS generation (P<0.001) in stretched cells. The rise in DCF fluorescence attributable to mechanical strain could be reduced to levels lower than seen in controls with ebselen and rotenone treatment. In contrast, PDTC enhanced the production of ROS as a result of VSMC stretch (P<0.01) (compare stretch to stretch plus PDTC). There was no difference in oxidative activity between cells cultured on standard tissue culture dishes versus those cultured on un-stretched fibronectin-coated silicone membranes.

Figure 5. Immunoblot analyses of total (A and B, top) and unphosphorylated (A and B, bottom) Cx43 protein levels in lysates from VSMCs treated with 2.2% oxygen (A) or mechanically strained (B) in the presence or absence of inhibitors of ROS. Lane 1, Untreated VSMCs cultured at a PO2 of 150 mm Hg (A) or grown on fibronectin-coated silicone membranes (B). Lane 2, VSMCs grown at a P O2 of 15 mm Hg for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively, or stretched for 6 hours in the presence of 10 μmol/L PDTC, 50 μmol/L ebselen, and 10 μmol/L rotenone, respectively. Results are representative of 4 experiments.

Discussion

We have demonstrated a reversible increase in Cx43 expression and gap junctional intercellular communication in rat thoracic aorta VSMCs cultured in a low oxygen tension environment. Other investigators have shown Cx43 expression can increase (ie, in the carotid body and petrosal...
because of its charge.25,26 Most of the calcein fluorescence pass through gap junction channels and well-retained in cells. The intensely fluorescent product, calcein, was small enough to rescent until it is hydrolyzed by esterases within VSMCs. The version of the dye calcein, which is colorless and nonfluorescent, is associated with improved intercellular coupling. Despite this observation, functional assays (ie, FRAP analysis) could either be recycled to the surface or degraded.22

Similarities in the magnitude and timing of Cx43 induction during hypoxia and that reported earlier for tensile stretch indicate that these 2 stimuli may share a common mechanism of activation. Comparable to the hypoxic cultures, the increased Cx43 protein levels observed on static mechanical strain of VSMCs was attenuated by treating stretched cells with either rotenone or ebselen. In addition, stretched VSMCs were found to have higher oxidative activity compared with static cultures. Combined, these results indicated that regulation of intercellular communication by hypoxia and mechanical strain was mediated through the amplification of ROS formation in VSMCs.

Examination of individual optical sections from confocal images revealed that much of the Cx43 protein translated in response to hypoxia seemed to be distributed intracellularly rather than localized to the cell surface. The perinuclear pool of Cx43 may have represented proteins localized to the endoplasmic reticulum, Golgi apparatus, and transport vesicles destined for the cell surface or possibly contained within compartments originating from the plasma membrane that could either be recycled to the surface or degraded.22–24 Despite this observation, functional assays (ie, FRAP analyses) revealed that the increase in Cx43 expression was associated with improved intercellular coupling.

For the FRAP experiments, we used a cell-permanent version of the dye calcein, which is colorless and nonfluorescent until it is hydrolyzed by esterases within VSMCs. The intensely fluorescent product, calcein, was small enough to pass through gap junction channels and well-retained in cells because of its charge.25,26 Most of the calcein fluorescence was detected in the region surrounding the VSMC nuclei, presumably because the cytoplasmic volume was the greatest in that region. Dye transfer was apparent between fluorescent donor (unbleached) cells immediately adjacent to nonfluorescent recipient (bleached) cells (first-order transfer) as well as between more distant nonfluorescent cells (second-order transfer) located in the middle of the bleached field and not in direct contact with unbleached cells. We attributed the higher rate of dye transfer in hypoxic cultures to the corresponding increase in Cx43 expression because it was by far the most highly expressed gap junction protein present in these cells.1,6,12 and induced by exposure to low oxygen tension. Because VSMCs have previously been shown to generate reactive oxygen intermediates in response to hypoxia,27–30 we investigated whether oxidant activity may regulate Cx43 expression in a manner similar to that described for various inflammatory proteins such as vascular cellular adhesion molecule-1, monocyte chemotactic protein-1, and E-selectin.9 Initially, the effect of hypoxia on VSMC oxidative activity was investigated using the nonfluorescent leuco dye H2DCFDA-AM, which can freely enter cells and be oxidized to yield fluorescent dichlorofluoroscein (DCF). The oxidation to the parent dye can occur through interaction with several ROS, including H2O2, hydroxyl radicals, and nitric oxide, but not through interaction with superoxide radicals. The lack of DCF reactivity with superoxide, however, does not preclude the possibility that this radical is generated by hypoxia in VSMCs, because O2·− can be reduced by either the cytosolic copper/zinc or mitochondrial manganese forms of superoxide dismutase to yield H2O2 and molecular oxygen. Consequently, dichlorofluoroscein may be considered a sensitive but not specific indicator of overall cellular oxidant activity.27–30

We have shown that VSMCs formed significantly more reactive oxygen intermediates after 1 and 2 hours at a Po2 of 15 mm Hg compared with cells cultured at a Po2 of 150 mm Hg or for longer periods of time (ie, >4 hours) in the hypoxic environment. The rapid, hypoxia-mediated rise in DCF fluorescence could be almost completely prevented by pretreatment with ebselen and rotenone but not with PDTC. As a matter of fact, PDTC significantly increased DCF emission but had no effect on Cx43 expression. These apparently contradictory effects were likely the result of prooxidant activity at low concentrations (10 μmol/L) rather than the more commonly referenced antioxidant effects seen at higher concentrations (ie, ≥100 μmol/L).31,32 In contrast, stretched aortic myocyte cultures caused production of ROS as measured by DCF fluorescence analogous to that observed for hypoxic VSMCs. Although it has been reported that mechanical stretch of VSMCs enhances ROS production through NAD[P]H oxidase,27–29,33,34 the fact that peroxide production could be prevented with rotenone indicates that mitochondrial ROS generation contributed significantly toward regulating gap junctional intercellular communication.

In summary, our findings support the notion that coordinated conduction of vasomotor responses attributable to a change in oxygen concentration or hemodynamic forces is dependent on gap junctional intercellular communication. As a result, electrical impulses could be propagated in both the transverse and longitudinal direction of the vascular wall, thereby orchestrating acute vasomotor and chronic remodeling responses throughout the blood vessel.

Acknowledgments

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Figure 1. Immunoblot analysis of connexin 43 protein levels in total lysates (20 µg per lane) from VSMCs cultured at a PO₂ of 15 mmHg for 0, 4, 8, 16, and 24 hours (lanes 1 to 5). The upper panel shows phosphorylated and unphosphorylated forms of the protein, while the lower panel shows only unphosphorylated connexin43. Results are representative of five independent experiments.
**Figure II.** Representative fluorescent photomicrographs of VSMCs cultured at PO$_2$s of 150 mmHg (panel A) or 15 mmHg for 1 (panel B), 2 (panel C), and 4 (panel D) hours. Prior to exposure to the different partial pressures of oxygen, the cells were loaded with H$_2$DCFDA-AM to determine if reactive oxygen species were formed (green). Cells were fixed and stained with an anti-smooth muscle actin-Cy3 conjugate (red) and DAPI (blue) before visualization. Scale bars are shown in the lower left corner of each panel.