

Title: HYPOXIC PULMONARY VASOCONSTRICTION IN CELL CULTURE

Authors: TR Murray, M.D., Ph.D., EJ Macarak, Ph.D., BE Marshall, M.D., F.R.C.P.

Affiliation: Center for Research in Anesthesia, University of Pennsylvania School of Medicine and Department of Histology, University of Pennsylvania School of Dental Medicine, Philadelphia, Pennsylvania 19104

**Introduction** The use of vascular smooth muscle (VSM) cells in tissue culture for the study of the molecular and biochemical events underlying the hypoxic pressor response of the pulmonary vasculature offers several potential advantages. Environmental conditions are easily controlled and hemodynamic influences as well as those of endothelial cells are eliminated by the use of isolated VSM cells in culture. However, a critical prerequisite to using the isolated smooth muscle cell preparation for such studies is the demonstration that they will react appropriately in culture when exposed to hypoxia. Previous work showed morphologic evidence that fetal PA VSM cells would reversibly increase tension in culture when exposed to hypoxia and other agonists<sup>1</sup>. Cells grown on a thin layer of polymerized silicone fluid will demonstrate tension generated by the cells as elastic distortions and wrinkles of the cross-linked silicone<sup>2</sup>. Hypoxia causes PA VSM cells to induce prominent distortions in the growth surface which are reversible with a return to normoxia<sup>1</sup>. The present work was undertaken to show that the distortions of the elastic growth surface seen with exposure to hypoxia indeed represent contraction of VSM cells in response to hypoxia. Phosphorylation of the 20,000 dalton myosin light chain (LC<sub>20</sub>) plays a regulatory role in controlling tension generated by VSM and can serve as a biochemical marker for activation of the contractile apparatus. Incorporation of <sup>32</sup>P into the myosin LC<sub>20</sub> is used here as a means of assessing the extent of phosphorylation in response to a hypoxic stimuli.

**Methods:** Cultures of PA VSM cells were established, passaged at weekly intervals and used from the third to the sixth passage. Experimental cultures were incubated for 4 hours in serum-free media (M 199) containing 50  $\mu$ Ci of <sup>32</sup>P per 25 cm<sup>2</sup> flask. Thereafter, cells were exposed to normoxia (PO<sub>2</sub>  $\approx$  140 mmHg) or hypoxia (PO<sub>2</sub>  $\leq$  30 mmHg) for 5 minutes. Cultures were treated with 1M perchloric acid and rapidly frozen to prevent further changes in phosphorylation. Two-dimensional gel electrophoresis was performed on aliquots of the homogenized samples containing equivalent amounts of protein. Isoelectric focusing was carried out in the first dimension and polyacrylamide gel electrophoresis in the second. Gels were stained, dried, and autoradiographs were made. Portions of the gels containing the myosin light chains were excised, solubilized, and placed in scintillation fluid for determination of the amount of incorporated <sup>32</sup>P. Phosphorylation of myosin LC<sub>20</sub> in normoxic versus hypoxic cells was assessed by comparing the amount of radiolabeled phosphorus incorporated into the myosin LC<sub>20</sub> bands. Results are expressed as the mean  $\pm$  S.D. of (3) determinations.

**Results:** Multiple 2-D gels showed there to be some of the phosphorylated form of myosin LC<sub>20</sub> present in the myosin bands of unstimulated cultures. This generally represented less than 30% of the total LC<sub>20</sub>. Addition of <sup>32</sup>P into the culture media of cells exposed to normoxia during the 4 hours preincubation period with <sup>32</sup>P followed by a 5 minute experimental period showed a baseline level of incorporation of the label into myosin LC<sub>20</sub> (see Table). Cultures similarly preincubated for 4 hours in normoxia but then exposed to hypoxia during the 5 minute experimental period showed a marked increase in <sup>32</sup>P incorporation over controls.

**Table** - Incorporation of <sup>32</sup>P into Myosin LC<sub>20</sub> (mean CPM  $\pm$  S.D.) \*p = .0167 in Student's t-test

Condition	<sup>32</sup> P Incorporated (CPM)
Normoxia	242 $\pm$ 85
Hypoxia	535 $\pm$ 97 *

**Discussion:** Contraction of VSM involves phosphorylation of myosin LC<sub>20</sub> as an early step in the interaction of actin and myosin which ultimately leads to cell shortening. This work demonstrates for the first time that PA VSM cells in culture will respond to hypoxia by activating this process and increasing the amount of myosin LC<sub>20</sub> which is phosphorylated. Phosphorylation of the myosin LC<sub>20</sub> in the normoxic cultures is probably related to the resting tension of the cultured cells, as a low level of tension was typically seen in unstimulated cultures grown on the distensible silicone elastic surface<sup>1</sup>. The present study employing a biochemical marker for VSM contraction compliments the prior studies showing morphologic changes in response to hypoxia. Taken together, these results strongly suggest that cultured PA VSM cells are capable of generating the hypoxic vasoconstricting response known to occur *in vivo* and in isolated lung and vessel preparations. Thus, this culture system may prove to be a powerful tool for elucidating the cellular events leading to hypoxic pulmonary vasoconstriction as well as the mechanism through which inhalational anesthetic agents inhibit the response.

Supported in part by GM5T3207612 and GM29628

#### References:

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