Nox5, Cholesterol-rich Microdomains And Ang II Signalling In Human Vascular Smooth Muscle Cells

I. Background: NADPH oxidase 5 (Nox5), a generator of Reactive Oxygen Species (ROS), has been identified in human vessels (1). Although, Nox5 have been found to be essential in redox signalling involved in growth and inflammatory responses by Ang II in human endothelial cells (2), the trafficking, regulation and function of vascular Nox5 is unclear. We questioned the function of Nox5 and other Nox isoforms in signaling associated with vascular smooth muscle cell contraction, growth and cytoskeletal organisation and assessed whether lipid rafts/caveolae play a role in Nox5 regulation.

II. Methods: Human vascular smooth muscle cells (VSMCs) isolated from small arteries were studied. Nox isoforms were sequentially downregulated by siRNA. siRNA to p22phox reduced activity of Nox1,2 and 4, since these isoforms are p22phox-dependent. Nox5 was targeted by Nox5 siRNA. Signaling pathways associated with contraction (MLC, MYPT1), cell growth (PCNA, p53) and cytoskeletal organisation (Ezrin-Radixin-Moiesin) were assessed by immunostudies. Cells were stimulated with Ang II in the absence and presence of disrupters and enhancers of cholesterol-rich microdomains. Effects of Ang II, MCD and Nystatin were determined relative to vehicle, with the control normalized to 100%. Results are presented as mean±SEM and compared by one-way ANOVA followed by Student *t* test when appropriate. Values of $P<0.05$ were considered to be significant. Nox5 interaction with lipid rafts/caveolae was examined by immunohistochemistry in intact vessels and by sucrose gradient cell fractionation in cultured cells.

III. Results: Nox5 inhibition by siRNA in hvSMCs was associated with reduced Ang II-induced phosphorylation of MLC20 and MYPT1 (by 0.74-fold and 0.5-fold respectively in Ang II 5 mins stimulation, N=3, $p<0.05$). p22phox siRNA did not significantly alter activation of MLC20 or MYPT1. siRNA to p22phox and Nox5 decreased Ang II-induced increase in PCNA expression (by 0.18-fold and 0.3-fold in Ang II 2h and 8h stimulation respectively, N=3, $p<0.05$) and decreased phospho-p53 levels (by 0.4-fold in Ang II 5 mins stimulation, N=3, $p<0.05$). In isolated human small arteries Nox5 co-localized with caveolin-1 as assessed by immunohistochemistry. In fractionated cells, Nox5 and Nox1 localised in fractions 3 and 4, which are rich in cholesterol microdomains. To further explore the role of lipid-rafts/caveoale in Nox function, we disrupted lipid-rafts/caveolae using methyl- -cyclodrexin (MCD) and nystatin. MCD and nystatin increased Ang II-induced Nox-derived superoxide formation and increased Ang II-induced phosphorylation of MLC20 (by 3.45-fold and 1.12-fold respectively, N=3, $p<0.05$) and Ezrin-Radixin-Moiesin (by 1.2-fold in MCD, Ang II 15 mins stimulation and 1.2-fold in nystatin, Ang II 5 mins stimulation, N=3, $p<0.05$).

IV. Conclusions: These results indicate that in human VSMCs Ang II stimulates contractile pathways through Nox5-dependent pathways whereas signaling associated with VSMC growth and cytoskeletal organisation involve multiple Nox isoforms. Nox5 co-localises in lipid rafts/caveolae, which may play a role in Nox5 trafficking. Our data identify novel redox signaling processes through cholesterol-rich microdomains and highlight a potentially important function of Nox5 in vascular contraction.

(1) Pandey D *et al*. (2012). Am J Physiol Heart Circ Physiol. **302**: H1919-1928.

(2) Montezano AC *et al*. (2010). Circ Re. **106**: 1363-1373.