AuPS Meeting - Melbourne 2008

Symposium: Role of ROS in Cardiovascular Function and Disease

Wednesday 3 December 2008 − Wright Theatre

Chair: Livia Hool and David Allen

NADPH oxidase: role in muscular dystrophy

N.P. Whitehead and D.G. Allen, Discipline of Physiology, Bosch Institute, University of Sydney, NSW 2006, Australia.

Duchenne muscular dystrophy (DMD) is a degenerative muscle disease caused by the absence of dystrophin, a large (427 kDa) protein connecting the cytoskeleton to the sarcolemma. The dystrophin gene is located on the X chromosome and therefore DMD occurs almost exclusively in males, with an incidence of 1 in 3500 births. In young DMD patients, muscle damage is followed by regeneration but as the disease progresses, regeneration is compromised and muscle fibres are replaced with connective tissue and fatty deposits. This causes profound muscle weakness resulting in loss of mobility by the age of 10-12, and eventually death by about 20, due to respiratory and/or cardiac failure.

Reactive oxygen species (ROS) have been implicated in a wide range of human diseases. Over two decades ago, ROS were considered to be involved in the pathogenesis of DMD, which led to a number of clinical trials using antioxidants. Overall, these trials were disappointing and so the ROS hypothesis lost favour for a number of years. However, recent studies on the *mdx* mouse, an animal model of DMD, have shown that antioxidants can ameliorate dystrophic damage in both skeletal (Hnia *et al*., 2007; Whitehead *et al*., 2008) and cardiac (Williams & Allen, 2007) muscle. These findings suggest that a re-evaluation of the role of ROS in DMD is warranted.

An important follow up question is: what is the source of the increased ROS in dystrophic muscle? Disatnik *et al*., (1998) provided evidence of oxidative stress in *mdx* muscle before the onset of muscle necrosis. This suggests that the primary source of excessive ROS is produced by the dystrophic muscle fibres rather than secondary factors associated with muscle damage, such as inflammatory cells. NADPH oxidase is a ROS-producing, membrane-bound protein complex first discovered in phagocytes. Preliminary evidence from our laboratory suggests that skeletal muscle NADPH oxidase is a primary source of oxidative damage in *mdx* muscle. We have found that the expression of NADPH oxidase proteins, $gp91^{phox}$ (NOX2), $p67^{phox}$ and rac1, is increased approximately 2-fold in pre-necrotic *mdx* muscles compared to wild type. We have also found that NADPH oxidase inhibitors significantly reduce stretch-induced damage in *mdx* muscle fibres. In addition, Jung *et al*., (2008) have recently shown that ROS produced by NADPH oxidase triggers the rise in intracellular Ca^{2+} concentration following hypotonic swelling of *mdx* cardiomyocyes. Finally, Spurney *et al*., (2008) have shown differential gene regulation of NOX isoforms between skeletal and cardiac muscle in *mdx* mice, with NOX2 higher in skeletal muscle and NOX4 greater in cardiac muscle. Since NOX4 is associated with tissue fibrosis, this might explain why *mdx* cardiac muscle undergoes fibrosis while the skeletal muscles are relatively spared. Therefore, this recent experimental evidence suggests that targeting NADPH oxidase could be an effective therapeutic approach for DMD.

Disatnik MH, Dhawan J, Yu Y, Beal MF, Whirl MM, Franco AA & Rando TA (1998). *Journal of Neurological Sciences*, **161**: 77–84.

Hnia K, Hugon G, Rivier F, Masmoudi A, Mercier J & Mornet D (2007). *American Journal of Pathology*, **170**: 633–643.

Jung C, Martins AS, Niggli E & Shirokova N (2008). *Cardiovascular Research*, **77**: 766-773.

Spurney CF, Knoblach S, Pistilli EE, Nagaraju K, Martin GR & Hoffman EP (2008). *Neuromuscular Disorders*, **18**: 371-381.

Whitehead NP, Pham C, Gervasio OL & Allen DG (2008). *Journal of Physiology*, **586**: 2003-2014.

Williams IA & Allen DG (2007). *American Journal of Physiology. Heart and Circulatory Physiolology*, **293**: H1969–H1977.

NADPH oxidases in vascular biology and disease

C.P. Judkins,[1] H. Diep,[1] B.R.S. Broughton,[1] A.E. Mast,[1] G.J. Dusting,[2] C.G. Sobey[1] and G.R. Drummond ,[1]
[1]Department of Pharmacology, Monash University, Clayton, VIC 3800, Australia and [2]Bernard O'Brien Institute of Microsurgery, Fitzroy, VIC 3065, Australia. (Introduced by David Allen)

Until recently, reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), were thought to be mere by-products of cellular metabolism. However, it is emerging that ROS play key roles in a variety of normal cell signaling processes. Within blood vessels, ROS have been implicated in transcriptional and post-transcriptional regulation of gene expression, as well as activation and inactivation of kinases and phosphatases, respectively. ROS have even been touted as endothelium-derived relaxing factors. However, during vascular pathophysiological states such as hypertension, diabetes and hypercholesterolemia, the production of ROS in the blood vessel wall is markedly elevated such that cellular antioxidant defense mechanisms are overwhelmed and a state of 'oxidative stress' arises. Oxidative stress is thought to be an early trigger for many of the cellular processes involved in initiation of atherosclerotic lesions in the blood vessel wall including inactivation of the vasoprotective molecule, nitric oxide (NO), oxidation of lipoproteins, increased expression of adhesion molecules, and activation of pro-inflammatory signaling pathways. Hence, identification of the sources of excessive ROS production in blood vessels may lead to novel therapies to prevent atherosclerosis. There are a number of potential sources of ROS within vascular cells including endothelial nitric oxide synthase (eNOS), xanthine dehydrogenase/oxidase and components of the mitochondrial respiratory transport chain. However, for all of these enzymes, ROS production probably only occurs as a by-product of their normal catalytic function (e.g. mitochondrial respiration), or from a dysfunctional variant of the enzyme (e.g. in the cases of eNOS and xanthine dehydrogenase). Indeed, the only enzymes whose primary function appears to be the production of ROS are the NADPH oxidases, of which at least two isoforms (Nox2 and 4) are relevant to vascular (patho)physiology.

We have previously shown that, during normal physiology, blood vessels from mice express a single isoform of NADPH oxidase - Nox4 - which produces ROS, primarily in the form of H_2O_2, in a controlled manner for use in cell signaling processes (Ellmark *et al.*, 2005). However, in hypercholesterolemic apolipoprotein E-deficient (ApoE-/-) mice, the NADPH oxidase isoform expression profile in the vascular wall is substantially altered (measured by Western blotting) such that Nox4 levels in the aorta are diminished by ~80%, and Nox2 expression is elevated by up to 300% ($p < 0.05$; n=4). Immunofluorescence staining of aortic sections revealed that endothelial cells, adventitial fibroblasts and macrophages residing in the neointima were the major cellular sources of elevated Nox2 expression in ApoE-/- mice. Nox2/NADPH oxidases are likely to generate superoxide as opposed to H_2O_2. Indeed, L012-chemiluminescence studies revealed that blood vessels from ApoE-/- mice produce markedly more superoxide than those from wild-type controls ($p < 0.05$; n≥7). A likely implication of excessive superoxide production, particularly in endothelial cells, is the chemical inactivation of NO. This would not only be expected to reduce the bioavailability of this important vasodilatory and anti-inflammatory molecule, but would also give rise to the powerful oxidizing species, peroxynitrite ($ONOO^-$), thereby triggering/accelerating atherosclerosis by inducing a pro-oxidant and pro-inflammatory state in the blood vessel wall. We have recently shown that genetic deletion of Nox2 in ApoE-/- mice (*via* the generation of a novel strain of Nox2-/-/ApoE-/- double knockout mice) restores aortic superoxide production back to wild-type control levels ($p < 0.05$ *vs* ApoE-/-; n=4). Moreover, deletion of Nox2 markedly delayed the progression of atherosclerosis such that the percentage of the descending aorta covered by lesions in Nox2-/-/ApoE-/- double knockouts versus ApoE-/- mice was approximately 36% ($p < 0.05$; n≥3) and 48% at 12 and 19 weeks of age, respectively. Interestingly, by 25 weeks of age, differences in lesion size between the two strains were no longer apparent suggesting that the importance of Nox2/NADPH oxidase and oxidative stress to lesion development may diminish in the advanced stages of the disease.

In conclusion, we have provided evidence that elevated Nox2/NADPH oxidase activity is not merely a symptom of atherosclerosis but a major contributing factor to lesion development, at least in the early and intermediate stages of the disease. These findings highlight Nox2/NADPH oxidase as a possible therapeutic target for vascular disease.

Ellmark SH, Dusting GJ, Ng Tang Fui M, Guzzo-Pernell N & Drummond GR. (2005) *Cardiovascular Research*. **65:** 495-504

Crosstalk between the L-type Ca^{2+} channel and the mitochondria

L.C. Hool, H.M. Viola and P.G. Arthur, Cardiovascular Electrophysiology Laboratory, School of Biomedical Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

The L-type Ca^{2+} channel is responsible for initiating contraction in the heart. Mitochondria are responsible for meeting the cellular energy demands. We examined whether activation of the L-type Ca^{2+} channel alone is sufficient to alter mitochondrial function in guinea-pig ventricular myocytes. We performed experiments in quiescent myocytes with consistent ATP utilisation or where we held ATP concentration constant (in the patch pipette) since this allowed us to more readily explore the effects of channel activation on mitochondrial function. The L-type Ca^{2+} channel was activated directly with the dihydropyridine agonist BayK(−) or voltage-clamp of the plasma membrane. We also activated the channel by depolarization of the plasma membrane with 45 mM KCl. Activation of the channel increased superoxide production (assessed as changes in dihydroethidium fluorescence), mitochondrial NADH production and metabolic activity (assessed as formation of formazan from tetrazolium) in a calcium-dependent manner. Activation of the channel also increased mitochondrial membrane potential assessed as changes in JC-1 fluorescence. The response was reversible upon inactivation of the channel during voltage-clamp of the plasma membrane. Actin filaments can regulate the function of the L-type Ca^{2+} channel. Actin also associates with mitochondria *via* phalloidin binding sites to stabilise mitochondria within the cell. We tested whether changes in mitochondrial membrane potential were mediated through the cytoskeleton by movement of the channel. Depolymerization of actin or exposing cells to a peptide directed against the α-interacting domain of the α1C subunit of the channel (thereby preventing movement of the β subunit) attenuated the increase in mitochondrial membrane potential. We conclude that activation of the L-type Ca^{2+} channel can regulate mitochondrial function and the response also appears to involve movement through the cytoskeleton.

Reactive oxygen species (ROS) and insulin resistant hypertrophic cardiomyopathy

L.M.D. Delbridge, Department of Physiology, University of Melbourne, VIC 3010, Australia.

Reactive oxygen species (ROS) such as superoxide are known to be implicated in the induction of cardiac hypertrophy in various pathologic states. The role of ROS in the etiology and/or exacerbation of insulin resistant hypertrophic cardiomyopathy is not well understood. Our recent studies have utilized Cre-lox mice with cardiac GLUT4 deletion (GLUT4-knockout), superimposed on global GLUT4 suppression (GLUT4-knockdown) to explore the relationship between insulin resistance and ROS-mediated myocardial pathology. In the GLUT4-knockout mice (compared with the knockdown mice) a marked cardiac hypertrophy is observed, characterized by an increase in cardiac weight index, elevated expression of the hypertrophy marker gene B-type natriuretic peptide (BNP). These hearts also exhibit moderate fibrosis associated with elevated expression of the pro-fibrotic gene, pro-collagen III. Of particular interest is the upregulated expression of the gp91(phox) and Nox1 subunits of NADPH oxidase.

In one series of experiments the influence of *in vivo* anti-oxidant treatment on myocardial measures of hypertrophy and oxidative stress was examined (Ritchie *et al.*, 2007). Anti-oxidant treatment using tempol significantly attenuated all of these abnormalities in GLUT4-knockout mice. Surprisingly the antioxidant treatment did not significantly reduce the NADPH-driven superoxide generation in this experimental setting of relatively short term treatment (4 week). These findings suggest that while suppression of oxidative stress in the insulin-resistant myocardium exerts an antihypertrophic effect, NADPH generated ROS may not be the only cellular target of the antioxidant therapy.

Additional studies using DNA microarray analyses to profile mRNA expression differences between GLUT4-knockout GLUT4-knockdown hearts have been informative in relation to global metabolic remodeling which occurs in these insulin-resistant hypertrophic hearts (Huggins *et al.*, 2008). In GLUT4-knockout hearts DNA microarray analysis detected downregulation of a number of genes centrally involved in mitochondrial oxidation and upregulation of other genes indicative of a shift to cytosolic beta-oxidation of long chain fatty acids. In particular gene expression evidence suggests that peroxisomal production of ROS in these hearts may be increased. Thus the benefit of antioxidant therapy in the GLUT4-knockout hearts may offset the deleterious effects of both NADPH- and peroxisomal-generated ROS.

These structural and molecular findings have important implications for understanding the role of ROS in the etiology of cardiac hypertrophy in the setting of insulin resistance, and highlight a potential role for antioxidant therapy in the treatment of diabetic cardiomyopathy.

Ritchie RH, Quinn JM, Cao AH, Drummond GR, Kaye DM, Favaloro JM, Proietto J, Delbridge LMD. (2007) *Journal of Molecular Cellular Cardiology* **42**, 1119-1128.

Huggins CE, Domenighetti AA, Khalil N, Favaloro J, Richie ME, Smyth GK, Proietto J, Pepe S, Delbridge LMD. (2008) *Journal of Molecular Cellular Cardiology* **44**, 270-80.