without TNFα (100 U/ml for 30 minutes) and examined for ROS production (lucigenin emiluminescence) and MAPK activation (Western blot). Compared with cells transfected with an empty vector control) or cells transfected with wild-type p47phox (overexpression), S303/304A mutation had no significant effect on the basal level (without TNFα) of ROS production. When cells were stimulated with TNFα, there was a twofold increase in ROS production by control and p47phox overexpressed cells. TNFα-induced ROS production was significantly reduced (~60%) in cells transfected with the S303/304A mutation. However, there was no significant difference in TNFα-induced phosphorylation of ERK1/2, p38MAPK and JNK between controls and the cells transfected with the S303/304A mutation. In conclusion, p47phox phosphorylation at S303/S304 plays an important role in TNFα-induced ROS production, but is not involved in TNFα-induced MAPK activation.

008 NEURONAL NITRIC OXIDE SYNTHASE-MEDIATED REGULATION OF MYOCARDIAL REDOX STATE AND β3 ADRENERGIC RECEPTOR RESPONSES

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Recent evidence suggests that increased superoxide production may decrease the bioavailability of endothelial nitric oxide synthase (eNOS)-derived nitric oxide in the myocardium of neuronal nitric oxide synthase (nNOS)$^{-/-}$ mice. To test the functional relevance of this observation, we compared the eNOS-dependent inotropic and $[Ca^{2+}]_i$ transient effect of beta3-adrenergic receptor (β3-AR) stimulation in left ventricular myocytes from eNOS$^{-/-}$ and nNOS$^{-/-}$ mice and their wild-type littermates. β3-AR stimulation (with BRL 37344, 10 μmol/l in the presence of the β1 and β2-AR blocker nadolol, 10 μmol/l) resulted in a significant reduction in cell shortening and $[Ca^{2+}]_i$ transient amplitude in wild-type myocytes. In contrast, BRL plusnadolol had no effect in myocytes from eNOS$^{-/-}$ and nNOS$^{-/-}$ myocytes (in the absence of changes in β3-AR messenger RNA levels or in eNOS protein expression in the latter) or in nNOS$^{+/+}$ myocytes pretreated with the nNOS-specific inhibitor, SMTC (0.1 μmol/l). Inhibition of xanthine oxidoreductase (XOR) or NADPH oxidases caused a similar reduction in basal superoxide production in nNOS$^{-/-}$ myocytes; however, whereas apocynin had no effect, XOR inhibition restored the negative inotropic response to β3-AR stimulation in nNOS$^{-/-}$ myocytes but not in eNOS$^{-/-}$ myocytes. eNOS activity was uncoupled in the myocardium of nNOS$^{-/-}$ mice in the absence of changes in BH4 and biopterin levels or arginase activity. However, L-arginine transport was impaired in nNOS$^{-/-}$ myocytes and excess L-arginine (10 mmol/l) restored the response to β3-AR stimulation. In summary, increased superoxide production by XOR (but not by NADPH oxidase) selectively abolished the negative inotropic effect of β3-AR stimulation in nNOS$^{-/-}$ myocytes by decreasing L-arginine transport and the bioavailability of eNOS-derived nitric oxide. These findings demonstrate that the source and subcellular localisation ofsuperoxide production can account for the diverse and specialised actions of reactive oxygen species in the heart.

009 ENDOTHELIAL-SPECIFIC OVEREXPRESSION OF NOX2 ENHANCES ANGIOTENSIN II-INDUCED CARDIAC DYSFUNCTION AND FIBROSIS

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Nox2-containing NADPH oxidases are reported to be involved in the development of cardiac fibrosis in response to chronic angiotensin II infusion, but the cellular source(s) of Nox2 involved in fibrosis remains unclear. We investigated the role of endothelial Nox2 in angiotensin II-induced left ventricular hypertrophy (LVH). Male transgenic mice with endothelial-specific overexpression of Nox2 were compared with matched wild-type (wt) littermates after angiotensin II (1.1 mg/kg per day) or saline infusion for 14 days. Basal blood pressure and left ventricular NADPH oxidase activity were similar in wt and transgenic mice. After angiotensin II infusion, both wt and transgenic groups developed similar hypertension (170.2±11.6 vs 170.4±12.3 mm Hg; n=10) and hypertrophy (left ventricular/body weight ratio 4.8±0.2 vs 4.7±0.2 mg/g; and echocardiographic septal thickness increased by 34% wt and 37% transgenic mice; n>10). NADPH oxidase activity was higher in angiotensin II-infused transgenic compared with wt left ventricles ($p<0.05$). Interestingly, transgenic hearts showed significantly more interstitial cardiac fibrosis (2.3±0.5 vs 1.3±0.1% by Sirius red staining; $p<0.05$; n=6). In concurrence with a greater increase in inflammatory cell infiltration in transgenic left ventricles compared with wt (55% increase in CD45 staining; $p<0.05$; n=6). Left ventricular systolic function assessed in vivo by left ventricular pressure–volume analysis was similar in both angiotensin II groups (ESPVR, 8.9±0.7 in wt vs 8.9±1.3 mm Hg/μl in transgenic mice; p=ns; n=10), whereas diastolic stiffness increased significantly only in the angiotensin II transgenic group (EDPVR increased from 0.2±0.0 to 0.5±0.1 mm Hg/μl in transgenic mice; $p<0.05$; and from 0.2±0.0 to 0.3±0.0 mm Hg/μl in wt; n=10). These results indicate that endothelial-specific overexpression of Nox2 significantly enhances the development of angiotensin II-induced cardiac fibrosis and left ventricular diastolic dysfunction, independent of changes in hypertrophy.

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010 CARDIOMYOCYTE-SPECIFIC OVEREXPRESSION OF NOX4 ATTENUATES ADVERSE CARDIAC REMODELLING AFTER MYOCARDIAL INFARCTION

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Background Reactive oxygen species (ROS) production is implicated in the development of cardiac remodelling after myocardial infarction (MI). NADPH oxidases are major sources of cardiovascular ROS, with cardiomyocytes expressing both Nox2 and Nox4 isoforms. We previously showed that Nox2 contributes significantly to the processes underlying adverse cardiac remodelling and contractile dysfunction post-MI, but the effect of Nox4 remains unclear. The aim of this study was to investigate the role of Nox4 in cardiac remodelling after MI.

Methods and Results We generated transgenic mice with cardiomyocyte-specific overexpression of Nox4, expressing the mouse full-length Nox4 complimentary DNA under control of the α-MHC promoter. Transgenic mice were backcrossed onto a C57BL6/J background and wild-type littermates (wt) used as controls. Nox4 transgenic mice were grossly similar to wt in terms of body weight, growth, activity, fertility and basal cardiac function. Transgenic mice displayed heart-specific expression of the Nox4 transgene, had significantly higher Nox4 protein expression, over 60% higher heart NADPH oxidase activity and 42% increased hydrogen peroxide production (all $p<0.05$). Compared with wt, p22phox protein expression was increased by 2.4-fold, which may be related to an increased protein stability. There was no change in the protein expression level of Nox2 or eNOS. MI was achieved by permanent left coronary ligation for 4 weeks. Compared with wt/MI, transgenic/MI groups displayed significantly high survival rate (72% vs 95%) and less hypertrophy at 4 weeks post-MI in terms of heart/body weight ratio (6.95±0.16 vs 6.44±0.14, $p<0.01$), although the

infarct size was similar (38.3±2.0% vs 39.9±2.8%). Transgenic mice also exhibited less apoptosis and interstitial cardiac fibrosis (0.52±0.03% vs 0.65±0.05%, $p<0.05$), and lower increases in the expression of fibronectin and procollagen III mRNA. Both echocardiography and cardiac catheterisation demonstrated less left ventricular cavity dilatation and a preservation of cardiac function in transgenic than wt mice.

Conclusions In contrast to Nox2, which contributes to cardiac dilation, contractile dysfunction and fibrosis, cardiomyocyte Nox4 was found to exert protective effects against adverse remodelling post-MI.

011 A NEW MOLECULAR MECHANISM FOR FAMILIAL DILATED CARDIOMYOPATHY BASED ON STUDIES WITH INTACT MUTANT TRANSGENIC MOUSE AND HUMAN EXPLANTED HEART MUSCLE

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We created a transgenic mouse that expressed the dilated cardiomyopathy (DCM) mutation *ACTC* E361G at 50% of total cardiac actin. We isolated F-actin from transgenic and non-transgenic (NTG) mice and reconstituted thin filaments using native human cardiac troponin and tropomyosin (from donor heart). In in-vitro motility assays we could observe no differences between E361G and non-transgenic mouse thin filaments; however, when troponin was fully dephosphorylated with acid phosphatase we observed that E361G Ca^{2+} sensitivity was lower than non-transgenic, as previously observed with the recombinant proteins (EC_{50} E361G/NTG 2.2±0.1). When we compared natively phosphorylated and dephosphorylated thin filaments we observed that Ca^{2+} sensitivity did not change in E361G mouse thin filaments (EC_{50} P/unP=1.0±0.1) but the Ca^{2+} sensitivity increased 3.0±0.3-fold on dephosphorylation of non-transgenic mice as expected. Thus the only functional change induced by the E361G mutation in cardiac actin was a blunted response to troponin I phosphorylation. We also studied troponin extracted from the explanted heart of a DCM patient carrying the cardiac TnC G159D mutation. In-vitro motility assay investigation of reconstituted thin filaments showed that the cTnC G159D mutation also showed little change in Ca^{2+} sensitivity when TnI was dephosphorylated (EC_{50} P/unP=1.2±0.2). In contrast, with donor heart control troponin, Ca^{2+} sensitivity was increased (EC_{50} P/unP=4.7±1.9). We conclude that Ca^{2+} sensitivity per se is not the prime determinant of familial DCM. The causative property shared by mutations in contractile proteins that cause DCM is a blunted response to changes in troponin I phosphorylation that could impair the normal response to adrenergic stimulation.

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012 IS NITRIC OXIDE SYNTHASE PRESENT IN MITOCHONDRIA?

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In addition to the three known forms of nitric oxide synthase (NOS) in the heart, it has recently been proposed that NOS is also present in mitochondria. However, studies are controversial due to the possibility of contamination by non-mitochondrial NOS, and because none of the known forms of NOS contain a mitochondrial targeting sequence. We investigated whether NOS was present in isolated mitochondria using antibodies against all three forms of NOS (endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)). Crude fractions of heart and liver mitochondria were obtained by differential centrifugation, and 35% Percoll was used to obtain highly purified mitochondria, as tested using antibodies against subcellular marker proteins: cyclophilin D, mitochondrial marker; monocarboxylate transporter-1, plasma membrane marker; ryanodine receptor, sarcoplasmic reticulum marker (heart mitochondria only) and catalase, peroxisomal marker (liver only). Western blotting using antibodies against eNOS and iNOS revealed that these isoforms were not present in either heart or liver purified mitochondria (whereas whole heart or liver lysate tested positive). We used five different antibodies against nNOS, and again failed to detect anything in purified heart mitochondria. However, in purified liver mitochondria one of the nNOS antibodies revealed the presence of a band at the correct molecular weight. We are currently determining whether this is indeed nNOS. In addition, we will assay for NOS activity in the purified mitochondria. Nitric oxide can inhibit mitochondrial respiration, so the existence of mitochondrial NOS may provide an important modulatory mechanism for respiration under either physiological or pathological conditions.

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013 REGULATION OF GENE TRANSCRIPTION BY HYDROGEN PEROXIDE IN CARDIOMYOCYTES

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Introduction Reactive oxygen species (ROS) levels rise during ischaemia and reperfusion and may contribute to myocardial injury, remodelling and progression to heart failure. ROS (hydrogen peroxide; H_2O_2) activates both apoptotic and pro-survival signalling pathways, although at higher concentrations cardiomyocyte death invariably results. Here, transcriptional responses of cardiomyocytes to varying concentrations and duration of H_2O_2 were investigated.

Methods Neonatal rat ventricular myocytes were exposed to 0.05–1.0 mM H_2O_2 for up to 6 h. Messenger RNA expression of selected genes was analysed by quantitative PCR. Cycloheximide was used to define immediate early genes (IEG) and second-phase genes.

Results Transcription factors of activating protein 1 (AP-1) and early growth response (EGR) families were upregulated rapidly and transiently by 0.1–1 mM H_2O_2, concentrations, which induce cardiomyocyte apoptosis and are IEG. In contrast, upregulation of transcripts for antioxidant proteins (second-phase genes) was slower and more sustained, occurring at a lower concentration of H_2O_2 (0.05–0.5 mM).

Conclusions AP-1, EGR and antioxidant transcripts were regulated by H_2O_2 in a time and concentration-dependent manner. Induction of antioxidant expression at lower concentrations of H_2O_2 could represent enhancement of ROS-scavenging capacity to prevent apoptosis. At higher H_2O_2 concentrations, the AP-1 and EGR transcription factor IEG may mediate apoptosis. These studies increase the understanding of transcriptional responses in cardiomyocytes to ROS.

014 THIN FILAMENTS RECONSTITUTED WITH TROPONIN EXTRACTED FROM PATIENTS WITH HYPERTROPHIC OBSTRUCTIVE CARDIOMYOPATHY ARE FUNCTIONALLY ABNORMAL

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Tissue obtained from a septal myectomy represented an opportunity to characterise the molecular phenotype of hypertrophic