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Tnfα Alters Mitochondrial Function And Ca^{2+} Homeostasis In Ventricular Cardiomyocytes: A Key Role For Caspase-8 Activation

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Tumor necrosis factor α (TNFα), a pro-inflammatory cytokine, is associated with major cardiomyopathy. In the heart, TNFα binding to the TNF receptor 1 (TNFR1) has been implicated in TNFα mediating negative inotropic effects as well as apoptosis. TNFα-TNFR1 activates caspase-8 which leads to caspase-3 activation either directly or following mitochondrial disruption. Here we investigated whether caspase-8-induced mitochondrial dysfunction could lead to TNFα-induced alterations of Ca^{2+} homeostasis. All experiments were performed on freshly isolated rat ventricular cardiomyocytes using multi-photons or confocal microscope. One hour of TNFα application (10 ng/ml) activates caspase-8 as well as caspase-3 measured with carboxyfluroscein-derived specific probes. TNFα depolarized mitochondrial membrane potential (measured with TMRM), and increased mitochondrial superoxyde production (measured with MitoSox). In the mean time, mitochondrial Ca^{2+} decreased, preceding an elevation in resting cytosolic Ca^{2+} fluorescence (Rhod-2 and Fluo-4 measurements respectively) and an increase in spontaneous ryanodine receptors activities (sparks frequency). Alternatively, on field stimulated cells (0.5 Hz), TNFα decreased Ca^{2+} transients' amplitude and SR load. TNFα-mediated alteration in SR Ca2+ function was normalized by antioxidant (NAC; 20 mM). In addition, a broad-spectrum caspase inhibitor (Q-VD-oph; 10 μM) or specific caspase-8 inhibitors (TRP801 and z-IETD-fmk; 10 μM), blocked TNFα effects both on mitochondria and Ca^{2+} handling. On an ischemia-reperfusion model, intra-peritoneal injection of TRP801, 15 min minutes prior reperfusion, prevented long term morpho-functional remodeling. In conclusion, caspase-8 activity appears to mediate TNFα-induced mitochondrial dysfunction which in turn alters global Ca^{2+} handling independently of caspase-3 activation. Caspase-8 inhibition presents a potential therapeutic target.

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Blocking Mitochondrial Ca^{2+} Uptake Increases Matrix Reactive Oxygen Species During Excitation-contraction Coupling In Cardiac Myocytes

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Mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) is taken up by the Ca^{2+}-uniporter (mCU) and stimulates NADH- and ATP-production. Furthermore, the NADH redox state is in equilibrium with the NADPH- and glutathione-pools, and glutathione is required for glutathione peroxidase to eliminate H_2O_2. Thus, we hypothesized that inhibiting mitochondrial Ca^{2+}-uptake could increase H_2O_2 formation. Experiments were performed in guinea-pig cardiac myocytes (n=10-13/group). To monitor $[Ca^{2+}]_m$, myocytes were loaded with rhod-2AM, and then patch-clamped and dialyzed with a pipette solution containing indo-1 to detect cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$). Alternatively, myocytes were loaded with the H_2O_2-sensitive dye CM-DCF, which locates primarily to mitochondria, and then dialyzed with DCF-free pipette solution to remove cytosolic DCF. In these cells, NADH autofluorescence was monitored together with DCF. In voltage-clamp mode, cells were depolarized from -80 to +10mV at 3Hz and exposed to isoproterenol (10/100 nM) for 12 min. Under control conditions, beat-to-beat oscillations of $[Ca^{2+}]_m$ were observed during cytosolic Ca^{2+} transients. Isoproterenol increased the amplitude of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ transients and led to diastolic accumulation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$. When $[Ca^{2+}]_c$ transients increased in response to isoproterenol, NADH transiently oxidized, but recovered when diastolic $[Ca^{2+}]_m$ increased. During this transient NADH oxidation, net formation of H_2O_2 increased but returned to baseline levels when diastolic $[Ca^{2+}]_m$ increased and NADH recovered. When inhibiting mitochondrial Ca^{2+}-uptake with the mCU-blocker Ru360 (1μM in pipette solution), diastolic accumulation of $[Ca^{2+}]_m$ was abolished and the recovery of oxidized NADH blunted. Consequently, net formation of H_2O_2 increased compared to control conditions (F/F_0 after 12 min of isoproterenol: 1.7 ± 0.2 vs 1.2 ± 0.1; $p<0.05$). We conclude that mitochondrial Ca^{2+} uptake is required for (a) matching energy supply and demand and (b) keeping the mitochondrial matrix in a reduced redox state to prevent formation of H_2O_2.

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Effects Of Oxysterols On The Sr Ca^{2+} Cycling In Ventricular Myocytes

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Oxysterols are biologically active molecules generated during the oxidation of low density lipoprotein (LDL). Several oxysterols are found in macrophage-derived 'foam cells' from human atherosclerotic tissue. Lipophilic oxysterols penetrate cell membranes and, therefore, can diffuse into the surrounding epithelial, smooth muscle, and cardiac cells from macrophages located in the atherosclerotic plaques or from inflammatory zones. Some cholesterol oxides have been shown to injure vascular endothelial and smooth muscle cells. 7β- and 25-hydroxycholesterol (HC) are the most toxic and the most abundant agents in the group.

We employed confocal microscopy and fluorometry to study the effects of 0.1-10 μM 7β-HC and 25-HC on the mechanisms underlying contraction in rat ventricular myocytes. Our experiments showed that both oxysterols:

(1) inhibit cell responses to electrical stimulations (2 Hz) in a dose-dependent manner;

(2) increase resting cytoplasmic $[Ca^{2+}]$ two-fold (1 Hz stimulation);

(3) slow Ca^{2+} removal from the cytosol in stimulated cells (1 Hz);

(4) reduce the caffeine-induced sarcoplasmic reticulum (SR) Ca^{2+} release by 30-45%;

(5) reduce the appearance of spontaneous Ca^{2+} waves in Ca^{2+}-overloaded intact ventricular myocytes by ~40 % and abolished them in Ca^{2+}-overloaded permeabilized ventricular myocytes;

(6) do not change the frequency of Ca^{2+} sparks in permeabilized ventricular myocytes during 5 minutes after the exposure under normal conditions (100 nM Ca^{2+}) but reduce it by ~40 % in Ca^{2+}-overloaded myocytes (120 nM Ca^{2+});

(7) increased the time constant of the SR Ca^{2+} uptake up to 3 fold in cardiac SR microsomes.

We conclude that oxysterols inhibit SR Ca^{2+} uptake (probably by decreasing the turnover rate of the SR Ca^{2+} ATPase). Our data suggest that the pathological actions of macrophage oxysterols may depend on dysfunctional Ca^{2+} signaling at the cellular and subcellular levels.

2644-Pos Board B614

Modulation Of Cardiac Contractility By Antagonism Of Pleckstrin-homology Domain And Akt-1 Silencing

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The pleckstrin-homology (PH) domain is involved in PI3-Kinase-mediated membrane recruitment, and subsequent activation, of signaling pathways, including Akt. PI3-Kinase pathway may modulate beta-adrenergic inotropic effect and Akt disregulation has a central role in diabetic cardiomyopathy. Recent data suggest that Akt may directly modulate sarcoplasmic reticulum (SR) function. Aims: to investigate modulation of cardiac excitation-contraction (EC) coupling by 1) two chemically unrelated compounds with PH-domain affinity (compounds A and B); 2) selective Akt-1 isoform silencing by small RNA-interference (sRNAi). Methods: rat ventricular myocytes were studied at 36.5 °C. Twitch amplitude was measured during field stimulation (2 Hz). Intracellular Ca2+ transients (FLUO 4-AM) was recorded in V-clamped myocytes; SR Ca2+ uptake function was estimated from Ca2+−transient features under inhibition of the Na+/Ca2+ exchanger (by Na+−free conditions). Akt-1 silencing was performed by myocyte transfection with Akt1-specific ds-iRNA oligos, labelled with the fluorescent probe Cy3. Akt phosphorylation levels and activity were tested by western-blot and ELISA. The effect of all interventions was tested in basal conditions and under weak adrenergic activation (isoproterenol 10 nM). Results: Akt-1 phosphorylation and activity were decreased by compounds A and B. Both compounds increased ($p<0.05$) twitch amplitude in basal condition; these effects was enhanced during weak beta-AR stimulation, also the compounds effects are significantly reduced during beta-AR blockade. Akt-1 silencing increased twitch amplitude, enhanced its response to beta-AR stimulation and completely occluded the effect of compounds. The compounds increased SR Ca reuptake rate and EC-coupling gain. Conclusions: 1) chemical antagonism of PH-domain increased contractility by stimulating SR Ca2+ uptake; 2) this effect is likely to result from inhibition of the Akt-1 pathway and involve interaction of the latter with beta-AR-mediated signaling; 3) PH-domain is a novel putative target for inotropical support through enhancement of SR function.

2645-Pos Board B615

Peroxynitrite Increases Protein Phosphatase Activity and Promotes the Interaction of Phospholamban with Protein Phosphatase 2a in the Myocardium

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Nitric oxide and superoxide react to form the potent oxidant peroxynitrite. The production of peroxynitrite increases during the pathogenesis of heart failure

and is detrimental to myocardial function. We previously demonstrated that high levels of peroxynitrite decrease myocardial contraction by reducing phospholamban (PLB) phosphorylation through a protein phosphatase-dependent mechanism. However, we did not examine the direct effect of peroxynitrite on protein phosphatase activity in the myocardium or the specific protein phosphatase which is activated. Here we test: 1.) the effect of SIN-1 (peroxynitrite donor) on protein phosphatase activity in whole heart homogenates using a colorimetric assay, and 2.) the effect of SIN-1 on the interaction of PLB with protein phosphatase 1 (PP1) and protein phosphatase 2a (PP2a) using co-immunoprecipitation. SIN-1 induced a 63% increase in total protein phosphatase activity (1.6 ± 0.2 vs. 2.6 ± 0.3 nmol/min/mg, $p<0.05$ vs. Control), which was abolished with specific PP1/PP2a inhibition using okadaic acid (1.4 ± 0.2 nmol/min/mg, $p<0.05$ vs. SIN-1). Since okadaic acid prevented the effects of SIN-1, we next examined the effect of SIN-1 on the interaction of PLB with PP1 and PP2a. SIN-1 increased the interaction of PLB with PP2a by 350% ($0.6 \pm$ 0.3 vs. 2.7 ± 0.7 A.U., $p<0.05$ vs. Control), but had no effect on the interaction with PP1. The peroxynitrite scavenger, urate, prevented both the SIN-1-induced increase in protein phosphatase activity and the interaction of PLB with PP2a, thus implicating peroxynitrite as the causal species. The results of this study provide further insight into the mechanism through which high levels of peroxynitrite serve to decrease PLB phosphorylation and myocardial contraction. Therefore, increased peroxynitrite production may play a key role in heart failure where protein phosphatase activity is increased and PLB phosphorylation is decreased, ultimately leading to contractile dysfunction.

2646-Pos Board B616

Epac Effect on the Cardiac RyR: Involvement of PLC, PKC and IP3R

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Epac is a protein directly activated by cAMP whose actions are independent of PKA. We recently show that Epac induces activation of CaMKII and phosphorylation of the Ca^{2+} release channel, the RyR, in rat cardiac myocytes. The effects included an increase in the Ca^{2+} sparks frequency and a slight decrease in the $[Ca^{2+}]_i$ transient amplitude. Here we investigated the signaling cascade from Epac activation to its effects on Ca^{2+} release. Ventricular myocytes were enzymatically isolated from rat heart ventricles. Cells were loaded with the fluorescence Ca^{2+} indicator Fluo-3 AM and viewed by confocal miscroscopy. $[Ca^{2+}]_i$ transients were evoked by field stimulation at 1 Hz. Ca^{2+} sparks were recorded in quiescent cells and SR Ca^{2+} load was estimated by rapid caffeine exposure. Epac activation was analyzed in presence of 8-CPT and of various antagonists. The possible involvement of Rap was checked on cells infected with adenoviruses coding for Rap-GAP and GFP. The results show that Rap is not involved in Epac effect on cardiomyocyte Ca^{2+} release. Inhibition of PLC by U73122 completely prevented Epac actions on Ca^{2+} sparks and $[Ca^{2+}]_i$ transients, indicating that PLC is involved in Epac actions. Blocking PKC by chelerytrine completely prevented Epac effect on $[Ca^{2+}]_i$ transient but not on Ca^{2+} sparks, suggesting that there are two separates pathways. Because PLC activation produces IP3, we checked whether activation of IP3 receptors (IP3R) is involved in Epac actions. Blockade of IP3R by 2-APB attenuated the effects of Epac on Ca^{2+} release events. Thus we conclude that activation of Epac by cAMP leads to Ca^{2+} release events modulation via a cascade involving PLC, PKC and IP3R. The resulted increase in the local Ca^{2+} release might be involved in the prohypertrophic actions of Epac on cardiac myocytes.

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A Quantitative Assessment Of Selective Pharmacological Inhibition Of Serca In Isolated Rabbit Working Hearts

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Decreased SERCA2a activity has been associated with contractile dysfunction in animal models of heart failure. An isolated working rabbit heart preparation and direct SERCA activity measurements were used to assess the level of SERCA inhibition necessary to terminate cardiac output under a standardised set of haemodynamic conditions. Hearts were perfused with a physiological extracellular solution whilst preload and afterload were set at $10cmH_2O$ and $85cmH_2O$ repectively. Ventricular function was assessed through the use of a pressure-volume catheter. Following initial stabilisation of cardiac function, 2.8μM thapsigargin (TG) was added to the circulating solution. Functional parameters were assessed continuously before and during application of TG. Cardiac function steadily declined in the presence of TG until the working heart configuration could not be sustained. The time to termination of aortic flow ranged from 15 to 60min. In the last minute prior to failure haemodynamic characteristics were markedly impaired (steady state *vs*. TG, n=4, $p<0.05$). Peak systolic pressure (107.78 ± 3.66 *vs*. 82.40 ± 2.15mmHg) and the maximum rate of rise of pressure (dp/dt_{max}) (1951 ± 177 *vs*. 995 ± 22mmHg.s^{-1}) were significantly reduced whilst dp/dt_{min} (-2422 ± 178 *vs*. -1470 ± 122mmHg.s^{-1}), relaxation time constant (0.028 ± 0.004 *vs*. 0.066 ± 0.009ms) and end diastolic pressure (8.33 ± 1.85 *vs*. 11.65 ± 0.62mmHg) were significantly increased. In all hearts coronary flow was maintained (80.5 ± 1.26 *vs*. 79.50 ± 1.7ml.min^{-1}). On cessation of aortic flow the left ventricle was snap frozen and homogenised in a protease-phosphatase buffer solution before biochemical analysis. Oxalate-dependent SERCA-mediated Ca^{2+}-uptake was used to assess SR Ca^{2+} uptake at a range of homogenate protein concentrations. Initial measurements indicate that termination of aortic flow occurs when SERCA activity (V_{max}) is reduced to <15% of control (DMSO vehicle). These data indicate the minimum level of SERCA activity required to sustain cardiac output in the rabbit working heart preparation.

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Contribution of Cycle Length History to Myocardial Contractility in Isolated Rabbit Myocardium under Physiological Conditions

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Modulation of contractile force via changes in heart rate can occur through processes that are either immediate (intrinsic) and/or through processes that involve prolonged exposure to a given situation and act via post-translational modification. Because the contractile strength of the steady state force-frequency relationship (FFR) and post-rest potentiation (PRP) involve both instant intrinsic responses to cycle length as well as slower acting components such as post-translational modification based mechanisms, it remains unclear how cycle length intrinsically affects cardiac contraction and relaxation. To dissect the intrinsic impact of cycle length changes from slower acting signaling components of the FFR, twitch contractions of right ventricular rabbit trabeculae at 5 different cycle lengths were randomized around a physiological stimulation baseline of 2.85 Hz. Patterns of previous cycle lengths that resulted in changes in force and/or relaxation times were identified. We found that the duration of the cycle length prior to the analyzed twitch contraction (primary) positively correlated with force. In sharp contrast, the cycle length one more removed from the analyzed twitch (''secondary'') was found to have a negative correlation with force. The ''tertiary'' cycle length impacted force similar to the secondary cycle length, albeit with a lesser magnitude. Using this novel stimulation protocol we can quantify the intrinsic effect of cycle length on contractile strength, as well as avoiding run-down and lengthiness that are often complications of FFR and PRP assessments. The data show that the history of at least 3 cycle lengths prior to a contraction influences myocardial contractility under near physiological conditions, and the secondary/tertiary cycle lengths affect cardiac twitch dynamics in the opposite direction than primary cycle length with decreasing importance as the cycle length is further removed from the current beat.

2649-Pos Board B619

Nitroxyl (HNO) Modifies Cysteine Residues in Phospholamban to Increase Myocyte Ca^{2+}-Cycling and Contractility

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HNO donors enhance cardiac inotropy by increasing SR Ca^{2+} re-uptake/release. Given its thiophylic nature, HNO likely modifyies critical cysteine residues in E-C coupling proteins. Phospholamban (PLN) is a potential target for HNO, and its genetic removal or mutation of PLN cysteines should abolish/blunt HNO cardiac effects. Cardiomyocytes were isolated from PLN knockout (PLN-/-) and wildtype (WT) mice, field-stimulated and assessed for Ca^{2+} transients and sarcomere shortening (SS). HNO effects on the SR-Ca^{2+}ATPase (SERCA2a) were evaluated by isolating SR vesicles from PLN-/- and WT mice and measuring Ca^{2+} uptake by stopped-flow mixing. Dephosphorylation of SERCA2a (a measure of E_2P hydrolysis) was investigated in ER microsomes from Sf21 insect cells expressing SERCA2a ± PLN (WT or Cys 36-41-46->Ala mutant). PLN-/- myocytes showed enhanced myocyte contraction and a blunted response to isoproterenol. When challenged with the HNO donor