like ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA), are cytoprotective and inhibit cell death. The mechanisms associated with these distinct effects are not entirely clear. However, the effect of hydrophilic bile acids seems to be related with the blockage of a series of processes that converge on mitochondrial damage. Bax is a pro-apoptotic protein that belongs to the superfamily of the Bcl-2 proteins and is involved in mitochondrial pore formation. Submicellar concentrations of cytoprotective bile acids have been shown to modulate Bax concentration in mitochondria, suggesting that these molecules may interact directly with the protein. In this study, our objective was to evaluate the affinity of bile acids to recombinant Bax protein, making use of fluorescence spectroscopy (FRET and fluorescence anisotropy), as well as Fluorescence Correlation Spectroscopy (FCS). Our results show that the cytoprotective bile acids UDCA and TUDCA associate with recombinant Bax protein with high affinity, while the cytotoxic bile acid DCA only seems to be able to adsorb to the protein with much lower affinity. Notably, the binding site for UDCA seems to be located in a hydrophobic pocket of the protein. This interaction could be responsible for the disruption of Bax translocation to the mitochondrial outer membrane in the presence of UDCA and/or TUDCA.

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3071-Pos Board B501

MAC Inhibitors Neutralize the Pro-Apoptotic Effects of Tbid

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Since our initial characterization of the iMACs, different di-bromocarbazole derivatives with anti-apoptotic function have been developed and tested in several mouse models of brain injury and neurodegeneration [13-21]. Owing to the increased therapeutic potential of anti-apoptotic di-bromocarbazole derivatives, we sought to expand our knowledge of the mechanism of action of these small molecule inhibitors. We investigated the kinetics of MAC inhibition in mitochondria from wild type, Bak, and Bax knockout cell lines using patch clamp electrophysiology, fluorescence microscopy, ELISA, and quantitative western blot analyses. Our results show that iMACs work through at least two mechanisms: 1) by blocking relocation of the cytoplasmic Bax protein to mitochondria and 2) by disassembling Bax oligomers in the outer membrane. A comparison of the inhibitory effects over channel conductance and cytochrome c release suggests that the iMACs interacted with both Bax and Bak with similar kinetics. Interestingly, wild type mitochondria were more susceptible to inhibition than the Bak or Bax knockouts. A quantitative western blot analysis showed that wild type mitochondria had lower steady state levels of Bak, which suggests an uneven stoichiometry of the MAC components.

3072-Pos Board B502

Tyrosine Phosphorylation of Mitochondrial Ca^{2+} Uniporter Regulates Mitochondrial Ca^{2+} Uptake

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Mitochondrial Ca^{2+} has a critical role for balancing cell survival and death. Ca^{2+} influx into mitochondrial matrix is mediated primarily by the mitochondrial calcium uniporter (MCU). However, the signaling pathways that regulate MCU channel functions via post-translational modifications of MCU are completely unknown. Here we show that adrenergic signaling induces MCU tyrosine phosphorylation and accelerates mitochondrial Ca^{2+} uptake in cardiac cells. Adrenergic signaling induces activation of proline-rich tyrosine kinase 2 (Pyk2) and translocation into the mitochondrial matrix; enhancing the interaction between Pyk2 and MCU, which subsequently accelerates mitochondrial Ca^{2+} uptake via Pyk2-dependent MCU tyrosine phosphorylation. MCU contains 15 tyrosine residues (5 in the N-terminus, 0 in the pore-forming region, 4 in transmembrane domains and 6 in the C-terminus), which are conserved across all eukaryotic species. Among them, only 3 of these tyrosine residues (Y157 at N-terminus, Y288, and Y316 at C-terminus in mouse MCU) remained as potential phosphorylation candidate sites for protein tyrosine kinases using phosphorylation prediction programs. We mutated these tyrosine residues to phenylalanine and generated non-phosphorylation mimetic MCU mutants (MCU-YFs). We confirmed that only two tyrosine sites were phosphorylated in response to adrenergic stimulation *in situ* using cell lines stably expressing MCU-YFs. In addition, overexpression of these MCU-YFs failed to increase mitochondrial Ca^{2+} uptake in response to cytosolic Ca^{2+} elevation by thapsigargin, whereas wild-type MCU transfection dramatically accelerates mitochondrial Ca^{2+} uptake compared to non-transfected cells. In summary, MCU contains Pyk2-specific phosphorylation site(s) and Pyk2-dependent tyrosine phosphorylation of MCU can modulate its channel functions and regulate mitochondrial Ca^{2+} uptake.

3073-Pos Board B503

Cardioprotective Roles of Neuronal Ca^{2+} Sensor-1 during Stress

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Dysregulation of Ca^{2+} homeostasis in cardiomyocytes often results in heart failure. Identifying molecular targets that regulate cardiomyocyte survival is of therapeutic importance. Neuronal Ca^{2+}-sensor-1 (NCS-1) is an EF-hand Ca^{2+}-binding protein, which is important for excitable cell functions. We previously found that NCS-1-deficient *($Ncs1^{-/-}$)* mice had excess neonatal mortality (*Circ. Res. 2011*). The aim of the present study is to examine whether NCS-1 plays beneficial roles in cardiac survival during stress and the possible mechanisms underlying these effects. Neonatal mouse ventricular myocytes or whole hearts from wild-type (WT) and $Ncs1^{-/-}$ mice were subjected to stressors, and the resistance to stress was evaluated. $Ncs1^{-/-}$ mouse hearts were more susceptible to stress induced by oxidative impairment and ischemia-reperfusion injury. Stress-induced activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, a major survival pathway, was substantially reduced in the $Ncs1^{-/-}$group, and overexpression of NCS-1 or the constitutive active form of Akt increased the survivalrate of $Ncs1^{-/-}$myocytes. Cellular ATP levels, as well as mitochondrial respiration rates (both basal and maximal O_2 consumption) were significantly depressed in $Ncs1^{-/-}$ myocytes; especially with oxidative stress. Furthermore, intracellular Ca^{2+} handling was more easily dysregulated in stressed $Ncs1^{-/-}$ myocytes than WT myocytes. Since NCS-1 levels were increased by stress, the data suggest that NCS-1 is a survival-promoting factor, which is upregulated by stress stimuli. Interestingly, however,supra-physiological NCS-1 expression was toxic to cells. Taken together, our data suggest that moderate NCS-1 expression during stress promotes cardiomyocyte survival by maintaining proper Ca^{2+} handling, which is required for activation of Akt survival pathways and mitochondrial function.

3074-Pos Board B504

Initiation of Electron Transport Activity and a Decrease of Oxidative Stress Occur Simultaneously during Embryonic Heart Development

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Mitochondria in early embryonic hearts are not thought to produce ATP, yet they do produce reactive oxygen species (ROS) that regulate myocyte differentiation. To assess changes in ATP and ROS generation in the developing heart, we measured mitochondrial oxygen consumption, the activity of the complexes (Cx) 1 and 2 of the electron transport chain (ETC), ETC supercomplex assembly, and ROS in embryonic mouse hearts. At embryonic day (E) 9.5, mitochondrial ETC activity and oxidative phosphorylation (OXPHOS) are not coupled, even though the ETC complexes are present. We show that Cx-1 is able to accept electrons from the Krebs cycle, but enzyme assays that specifically measure electron flow to ubiquinone or Cx-3 show no activity at this early embryonic stage. At E11.5, mitochondria appear functionally more mature; ETC activity and OXPHOS are coupled and respond to ETC inhibitors. In addition, the assembly of highly efficient respiratory supercomplexes containing Cx −1, −3, and −4, ubiquinone, and cytochrome *c* begins at E11.5, the exact time when Cx-1 becomes functional activated. At E13.5, ETC activity and OXPHOS of embryonic heart mitochondria are indistinguishable from adult mitochondria. In contrast, generation of reactive oxygen species (ROS), as measured with Amplex Red, is high at E9.5 and drops significantly by E11.5, coinciding with activation of the ETC. In summary, our data suggest that between E9.5 and E11.5 dramatic changes occur in the mitochondria of the embryonic heart, which result in a decrease of ROS generation and an increase in OXPHOS due to the activation of Cx-1 and the formation of supercomplexes.

3075-Pos Board B505

The Stoichiometry between MICU1 and MCU Determines the Different Mitochondrial Ca^{2+} Uptake Phenotypes in Heart and Liver

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Mitochondrial Ca^{2+} uptake is central to oxidative metabolism and cell death signaling. The first clues to its molecular mechanism have emerged from the recent identification of the mitochondrial Ca^{2+} uniporter's pore forming protein (MCU) as well as its regulators. Among the regulators, MICU1 shows striking

co-expression and co-evolution with MCU. MICU1 has been demonstrated to be a Ca^{2+}-sensing protein, which both sets a threshold for low Ca^{2+} concentration while it assures cooperative activation during high Ca^{2+} rises. Mitochondrial Ca^{2+} uptake shows tissue specific differences and interestingly, mRNA level for MICUs and MCUs also displays tissue specificity. We set out to investigate if the stoichiometry between MICU1 and MCU could account for the previously described differences between heart and liver in mitochondrial Ca^{2+} uptake. Immunoblotting showed higher expression for all MICU1, MICU2 and MCU in mouse liver versus heart mitochondria, and a 4.5 fold higher MICU1 to MCU ratio in liver. In fluorometric measurements of mitochondrial Ca^{2+} uptake, heart mitochondria displayed a decreased threshold and lesser cooperativity compared to liver mitochondria. Additionally, NAD(P)H elevation was detectable after exposure to moderate $[Ca^{2+}]$ elevations only in heart mitochondria. Overexpression of MICU1 in the heart using AAV9-MICU1 tail-vein injection significantly increased the MICU1 protein level without any changes of MICU2 or MCU. This increased the MICU1 to MCU ratio in the heart and led to increased thresholding and cooperativity, reproducing the liver-like mitochondrial Ca^{2+} uptake phenotype. Vice versa MICU1 downregulation in the liver has been shown to lower the threshold and cooperativity of mitochondrial Ca^{2+} uptake in hepatocytes. Thus, heart and liver mitochondria show different levels of Ca^{2+} threshold and cooperative activation of Ca^{2+} uptake, which seem to result from differential quantitative relationship between MICU1 and MCU.

3076-Pos Board B506
ER Calcium Release is Tuned by Mitochondrial Redox Nanodomains

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Spatially and temporally controlled increases of H_2O_2 emerge as an intracellular signal. We hypothesized that ROS and Ca^{2+} interact locally, in the restricted volume of the ER-mitochondrial interface. These physically tethered structures host enrichments of ion transport proteins such as the IP_3 receptor, which support elevated nanodomains of Ca^{2+} during signalling events and are sensitive to H_2O_2. We used the genetically encoded H_2O_2 sensor HyPer incorporated into an inducible linker system to probe the redox environment at the ER-mitochondrial interface in HepG2 cells. We found a moderately elevated H_2O_2 nanodomain which developes into a H_2O_2 transient following IP_3 receptor-mediated ER Ca^{2+} release and mitochondrial Ca^{2+} uptake. Pharmacological inhibition showed that the transient was dependent upon ER Ca^{2+}, mitochondrial membrane potential and functional electron transport chain. HyPer measurements of the mitochondrial intermembrane space revealed significantly elevated H_2O_2 within this volume. Using electron microscopy we found that HepG2 mitochondria possess a cohort of dilated cristae, which disappeared following IP_3-linked Ca^{2+} release. Paxilline that inhibits mitochondrial BKCa channels blocked the cristae reshaping and also abolished the H_2O_2 transient at the interface. Furthermore, paxilline caused suppression of the IP_3-linked calcium signal, whereas interface targeted killer red, a photoactivated H_2O_2 source, induced sensitization to the IP_3-linked agonist. We conclude that the intermembrane/cristae volume of mitochondria represents an oxidized pool fed by the electron transport chain. Ca^{2+}-uptake stimulates expansion of the mitochondrial matrix via K^+ and concomitant water uptake, squeezing the oxidized volume of the cristae to the interface. Here, a transient H_2O_2 nanodomain sensitizes IP_3 receptors to further stimulation. We demonstrate a physiological setting where Ca^{2+} release may autoregulate using mitochondrial H_2O_2 released from the cristae.

3077-Pos Board B507
Reactive Oxygen Species (ROS) Suppress Mitochondrial Motility

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Mitochondrial distribution and transport play pivotal roles for many cellular functions, including cell differentiation, cell division to ensure proper inheritance, apoptosis, ATP supply at the local sites of demand, Ca^{2+} buffering for intracellular Ca^{2+} homeostasis.

We previously showed that mitochondrial motility (mito-motility) is regulated by the cytosolic Ca^{2+} concentration ($[Ca^{2+}]$c), providing the basis for a homeostatic circuit in which the organelles decrease their movements along microtubules to locally buffer high $[Ca^{2+}]$c and contribute to ATP supply. Mitochondria are also a major site for production and scavenging of ROS that serve as both a messenger and regulator of calcium signaling and are particularly relevant for the control of mitochondrial function. Here we tested the hypothesis that ROS target mito-motility to control mitochondrial function. H9c2 myoblast cells were transfected with a mitochondrial matrix targeted YFP and then loaded with fura2, to monitor the mito-motility simultaneously with $[Ca^{2+}]$c. H2O2 (100 μM) caused a decrease in mito-motility (64 ± 8 %) and an elevation in $[Ca^{2+}]$c (from 55 ± 8 to 91 ± 8 nM) at the same time. When the cells were incubated in a Ca^{2+}-free medium and were pretreated with thapsigargin to prevent Ca^{2+} entry and intracellular Ca^{2+} mobilization, respectively, H2O2 continued to inhibit the mito-motility dose-dependently without any changes in $[Ca^{2+}]$c. These results indicate that H2O2 can cause suppression of mito-motility through a Ca^{2+} independent mechanism we are currently analyzing.

3078-Pos Board B508
Miro1 is Dispensable for Calcium-Mediated Inhibition of Mitochondrial Movement

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Miro1 and 2 are (*Rhot1 and 2*) are two highly similar GTPases that are bound to the surface of mitochondria and possess EF-hand calcium binding motifs. Several groups have reported that Miro is involved in mitochondrial motility and inheritance, and particularly its calcium regulation, but the roles of the two isoforms have not been established. Genetic deletion of Miro1 in mouse is lethal at birth (Nguyen et al., 2014) and fibroblasts (MEFs) derived from Miro1 KO embryos show abnormal mitochondrial distribution, but the calcium-dependent inhibition of motility is unaffected and the respiratory and calcium buffering capacities are normal. Neuron-specific knockout of Miro1 leads to progressive deficits of upper motor neuron function, however mitochondria in processes of cortical neurons from Miro1 KO and wild-type embryos showed comparable calcium-sensitive motility inhibition. While no significant increase in Miro2 protein was observed in Miro1 KO MEFs, these data raise two possibilities: Miro1 and 2 are interchangeable with regard to calcium regulation of mitochondrial motility or Miro2 is the key player in this regard. To finally resolve this question, we are in the process of generating Miro2 KO and Miro1/2 KO cell lines.

3079-Pos Board B509
Mitochondrial Fusion Dynamics in the Heart

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Heart physiology depends on oxidative metabolism that likely requires dynamic and permanent reorganization of mitochondria by fusion and fission. To directly evaluate mitochondrial fusion dynamics in cardiomyocytes (CM), mitochondrial matrix-targeted photoactivatable GFP and DsRed were introduced either in vitro or in vivo by adenovirus and were followed by confocal microscopy. Four conditions were analyzed: 24 to 48 h cultured neonatal and in vitro transduced adult CM, and CM from in vivo infected rat hearts. In the latter case, CM were isolated 7-10 days after infection and were imaged promptly or 24-48 h post harvesting. Neonatal CM mitochondria form a highly connected network, whereas both in vitro and in vivo transformed cultured CM displayed only some connectivity. Impressively, in vivo transduced adult CM that were imaged promptly after harvesting, unveiled a significantly higher connectivity among mitochondria than the 24-48h cultured adult CM. Furthermore, fusion events (f.e./75 square micrometers/min) were almost absent in cultured in vitro transduced CM, meanwhile in vivo transduced and cultured CM showed 0.4 ± 0.2 f.e./min, whereas isolated, freshly-imaged CM displayed 1.4 ± 0.1 f.e./min. Imaging in perfused whole heart ex vivo, showed considerable mitochondrial continuity and fusion activity in ventricular CM. To study more directly the role of CM's contractile activity in mitochondrial fusion, CM were incubated with Verapamil (10μM), that blocked spontaneous contraction and partially suppressed the fusion activity of mitochondria. Also, mitochondrial fusion activity appeared to be higher after spontaneous contraction or short term field stimulation in isolated freshly-imaged CM. Thus, mitochondria are dynamic in both neonatal and adult CM, but under culture conditions, adult CM lose mitochondrial fusion activity. This might be at least in part, because cardiomyocyte contractile activity is altered in culture and contractions likely provide some factors to support mitochondrial fusion activity.

3080-Pos Board B510
Mechanistic Characterization of the Thioredoxin System in the Removal of Hydrogen Peroxide

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The thioredoxin system plays a critical role in the defense against oxidative stress by removing harmful hydrogen peroxide (H2O2). Specifically,