cardiac fibers also have diffusion restrictions. This is surprising because rainbow trout cardiomyocytes are thinner and have fewer intracellular membrane structures than adult rat cardiomyocytes. However, results from fibers may be affected by incomplete separation of the cells. The aim of this study was to verify the existence of diffusion restrictions in trout cardiomyocytes by comparing ADP-kinetics of mitochondrial respiration in permeabilized fibers, permeabilized isolated cardiomyocytes and isolated mitochondria from rainbow trout heart. We developed a new solution specific for trout cardiomyocytes, where they retained their shape and showed stable steady state respiration rates. The apparent ADP-affinity of permeabilized cardiomyocytes was different from that of fibers. It was higher, independent of temperature and not increased by creatine. However, it was still about ten times lower than in isolated mitochondria. This suggests that intracellular diffusion of ADP is indeed restricted in trout cardiomyocytes. The difference between fibers and cardiomyocytes suggest that results from trout cardiac fibers were affected by incomplete separation of the cells. The lack of a creatine effect indicates that trout heart lacks mitochondrial creatine kinase tightly coupled to respiration. These results from rainbow trout cardiomyocytes are similar to those from neonatal mammalian cardiomyocytes. Thus, it seems that metabolic regulation is related to cardiac performance. It is likely that rainbow trout can be used as a model animal for further studies of the localization and role of diffusion restrictions in low-performance hearts. Next step will be to identify the contribution of mitochondrial outer membrane and cytosolic factors in intracellular diffusion restriction.

3823-Pos

Novel Method for Investigation of Interactions between Mitochondrial Creatine Kinase and Adenine Nucleotide Translocase

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The aim of this study was to elaborate fluorescent labeling of mitochondrial creatine kinase (MtCK) and adenine nucleotide translocase (ANT) to investigate the mechanism of their functional coupling with Förster resonance energy transfer (FRET) technique. New alternative fluorescent labeling technique - Fluorescein Arsenical Hairpin (Flash/tetracystein) binder technology was exploited to fluorescently label MtCK. Implementation of fluorescent proteins such as GFP for MtCK fluorescent tagging was excluded because of the functional importance of MtCK C- and N-terminal part and insertion of large fluorescent protein inside the MtCK protein imposes potential risk to interfere the structure, localization and function of the fused protein. Tetracysteine motifs were introduced into five different positions in MtCK by mutagenesis. Sequentially the recombinant MtCK constructs were expressed in different eukaryotic cells lines and activity of the constructs were determined. The cells were stained with Flash labeling reagent and the expression of tetracysteine tagged MtCK mutants were visualized *ab inito* with epifluorescent and confocal microscopy. Improved variant of cyan fluroescent protein Cerulean as an appropriate FRET partner for Flash was chosen to fluorescently label ANT. Both N- and C-terminally fused ANT-Cerulean constructs were generated. ANT fusion proteins were expressed in different eukaryotic cell lines and their expression was visualized with epifluorescent and confocal microscopy. Functional constructs of MtCK and ANT-Cerulean were selected for studies of their interaction in cardiomyocytes by applying FRET technique.

3824-Pos

VDAC Phosphorylation Regulates Interaction with Tubulin

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Mitochondria and mitochondrial bioenergetics are believed to be involved in glycogen synthase kinase (GSK3β)-related cardioprotection. Recently it was suggested that cardioprotection could be achieved through the preservation of mitochondrial binding of hexokinase II (HXKII), or/and through GSK3β phosphorylation of voltage dependent anion channel (VDAC) (Pastorino et al., *Cancer Res.,* 2005; Das et al., *Circ. Res*., 2008). VDAC, the most abundant channel in the mitochondria outer membrane (MOM), is known to be responsible for most of the metabolite and ATP/ADP fluxes across MOM. Recently we have found that dimeric αβ-tubulin regulates mitochondrial respiration by directly blocking VDAC and hence, permeability of MOM for ATP/ADP (Rostovtseva et al., *PNAS*, 2008). Here, using mammalian VDAC reconstituted into planar lipid membrane, we show that tubulin-VDAC interaction appears to be very sensitive to the state of VDAC phosphorylation. When VDAC is phosphorylated *in vitro* by either GSK3β or protein kinase A (PKA), the on-rate of tubulin binding increases up to 100 times compared with untreated VDAC. Importantly, the basic properties of VDAC, such as single-channel conductance, selectivity, and voltage gating, remain almost unaltered after phosphorylation. Nonspecific alkaline phosphatase and tyrosine kinase inhibitor PP2A dephosphorylate VDAC, which results in decreased tubulin binding. Gel analysis and subsequent phospho-staining confirm that VDAC contains motifs recognized by both GSK3β and PKA. Phosphorylation causes a pronounced asymmetry of tubulin binding to VDAC. These findings allow us to point to the tentative GSK3β and PKA serine/threonine phosphorylation sites positioned on the cytosolic loops of VDAC. The results show that VDAC phosphorylation enhances tubulin-induced VDAC closure and thus could reduce MOM permeability and mitochondria respiration. We suggest that GSK3β cardioprotective effect is more complex that was initially thought because along with HXKII it involves tubulin as a potent regulator of VDAC and hence, cellular respiration.

3825-Pos

Free Tubulin and cAMP-Dependent Phosphorylation Modulate Mitochondrial Membrane Potential in Hepg2 Cells: Possible Role of VDAC

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BACKGROUND: Conductance of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane has been proposed to limit mitochondrial metabolism in cancer cells and contribute to the Warburg effect. Since tubulin binding and phosphorylation promote VDAC closure, we hypothesized that free tubulin and cAMP-dependent phosphorylation by protein kinase A (PKA) modulate $\Delta\Psi$ in cancer cells by regulating VDAC-dependent flux of substrates into mitochondria. Our **AIM** was to modulate VDAC closure and opening in intact cells by increasing and decreasing endogenous free tubulin and by promoting and blocking PKA activation. **METHODS**: HepG2 human hepatoma cells were incubated in Hank's solution with 5% CO_2/air, and $\Delta\Psi$ was assessed by confocal microscopy of TMRM. Free and polymerized tubulin was determined using a commercial kit. **RESULTS**: Myxothiazol (10 μM), a respiratory inhibitor, caused only a slight decrease of (TMRM fluorescence), but subsequent addition of oligomycin (10 μg/ml), a F_1-F_0-ATPase inhibitor, collapsed $\Delta\Psi$ nearly completely, showing that inhibition of both respiration and ATPase are required to collapse $\Delta\Psi$. Stabilization of microtubules by paclitaxel (10 μM) increased $\Delta\Psi$ by 60%, whereas disruption by colchicine (10 μM) or nocodazol (10 μM) decreased $\Delta\Psi$ by 60-70%. Paclitaxel pretreatment prevented the depolarizing effect of colchicine and nocodazol. Dibutyryl cAMP (1 mM) decreased $\Delta\Psi$ by 45% whereas H89 (1 μM), a specific inhibitor of PKA, increased $\Delta\Psi$ by 94% and blocked the effect of dibutyryl cAMP. **CONCLUSION**: Free tubulin and cAMP/PKA-dependent phosphorylation modulate mitochondrial $\Delta\Psi$ in HepG2 cells, most likely by regulating VDAC conductance. Up and down regulation of $\Delta\Psi$ by tubulin polymerization/depolarization and PKA dependent phosphorylation/dephosphorylation is consistent with the hypothesis that VDAC is rate-limiting for mitochondrial metabolism in cancer cells and responsible, at least in part, for the Warburg effect.

3826-Pos

Hypothermic Cardioprotection Attenuates Mitochondrial Permeability Transition Pore Opening and Calcium Loading in Isolated Cardiac Mitochondria

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Ischemia-reperfusion injury (IRI) is associated with mitochondrial permeability transition pore (mPTP) opening and impaired mitochondrial respiration. Hypothermia attenuates IRI. We examined mitochondrial function in mitochondria obtained from isolated hearts subjected to warm or cold ischemia. Guinea pig isolated hearts were perfused at constant pressure with Krebs-Ringer's solution at 37°C and subjected to 30 min global ischemia at 37°C or 17°C. After 5 min of reperfusion mitochondria were isolated. Mitochondrial $[Ca^{2+}]_m$, membrane potential ($\Delta\Psi_m$), and NADH were measured by spectrophotometry at appropriate wavelengths with indo-1, BCECF, rhodamine 123 fluorescent dyes, and autofluorescence, respectively. After energizing with pyruvic acid, 0-100 μM $CaCl_2$ (0.03-60 μM free $[Ca^{2+}]_e$) was added followed by 250 μM ADP. Ca^{2+} -induced mPTP opening was assessed by collapse of $\Delta\Psi_m$. 10 μM $[Ca^{2+}]_e$ resulted in mPTP opening after 37°C IRI, but only at 35 μM $[Ca^{2+}]_e$ after 17°C IRI. ADP decreased $\Delta\Psi_m$ and NADH and increased $[Ca^{2+}]_m$ in all mitochondria, but the fall in $\Delta\Psi_m$ was greater and the responses to ADP with Ca^{2+} overloading were worse after 37°C IRI vs. 17°C IRI. The incidence of no state 4 respiration was 25% with no added

$CaCl_2$ after 37°C IRI and 0% after 17°C IRI. This study shows that hypothermia prevents IRI damage through pathways restricting mitochondrial Ca^{2+} loading and preserves mitochondrial redox state and respiration. Moreover, mitochondria protected during ischemia with hypothermia were more resistant to Ca^{2+}-induced mPTP opening and oxidative phosphorylation was better preserved. Hypothermia might prevent conformational changes in the F_1F_0-AT-Psynthase and the ADP/ATP carrier, leading to better mitochondrial function and a resistance to mPTP opening as the ADP/ATP carrier is associated with mPTP opening.

3827-Pos

Buffer Magnesium Limits Mitochondrial Calcium Uptake but not Matrix Calcium Buffering in Response to ADP

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Mg^{2+} is known to limit Ca^{2+} uptake by mitochondria through the Ca^{2+} uniporter. Changes in matrix Ca^{2+} concentration are an important signaling pathway in mitochondrial function as well as in apoptosis. In a previous study we showed an increase in matrix free Ca^{2+} in response to added ADP in $MgCl_2$ free buffer. Because of the presumed role of Mg^{2+} in mitochondrial regulation of Ca^{2+} we explored the effects of buffer Mg^{2+} on matrix Ca^{2+} uptake and buffering in isolated mitochondria. Guinea pig heart mitochondria were isolated by differential centrifugation, loaded with the fluorescent dye Indo 1 AM and then suspended in respiration media, containing 1 mM of EGTA, with or without added 1 mM $MgCl_2$. To the mitochondrial suspension was added 0.5 mM pyruvic acid, either 0.25, 0.5 or 0.75 mM $CaCl_2$, and 250 μM ADP. Adding 0.25, 0.5 and 0.75 mM Ca^{2+} caused a dose-dependent increase in matrix Ca^{2+} of 14, 35 and 45%, respectively, in the group without Mg^{2+} in the buffer, and 6, 18 and 42%, respectively, in the group with Mg^{2+} in the buffer. The differences in uptake between Mg^{2+} and no Mg^{2+} groups were significant in the 0.25 and 0.5 mM groups, but not in the 0.75 mM group. The additional increase in matrix free Ca^{2+} in response to ADP without Mg^{2+} was 9, 11 and 9% for the 0.25, 0.5 and 0.75 mM Ca^{2+} groups, respectively. These additional increases in matrix free Ca^{2+} with ADP were not significantly altered by Mg^{2+}. We conclude that external Mg^{2+} alters the uptake of Ca^{2+} into the mitochondrial matrix, but does not alter the increase in matrix ionized Ca^{2+} after addition of ADP.

3828-Pos

Complex I and F_0F_1-ATP Synthase Mediate Membrane Depolarization and Matrix Acidification by Isoflurane in Mitochondria

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Introduction: Short application of volatile anesthetic isoflurane at reperfusion after ischemia exerts strong protection of heart and cardiac mitochondria against injury. Mild depolarization and acidification of mitochondrial matrix are involved in the protective mechanism, but the molecular basis for these changes is not known. In this study we investigated the electron transport chain, F_0F_1-ATP synthase and mitochondrial ion channels as potential targets of isoflurane in mitochondria.

Methods: We have measured mitochondrial respiration, membrane potential, matrix pH, matrix swelling, and H_2O_2 release in isolated mitochondria in the presence and absence of isoflurane (0.5 mM). Pyruvate/malate, succinate/rotenone, or ascorbate/TMPD, were used as substrates for complex I, II and IV, respectively. Guanosine-diphosphate (GDP), oligomycin, paxilline and 5-hydroxydecanoic acid (5-HD) were used to probe involvement of uncoupling proteins, F_0F_1-ATP synthase, mitochondrial ATP- and Ca^{2+}-sensitive K^+ channel. Nigericin, a K^+/H^+ exchanger, was used to manipulate the matrix pH.

Results: With pyruvate/malate as substrates, isoflurane inhibited mitochondrial respiration by $23 \pm 4\%$, depolarized membrane potential by $2.7 \pm 0.7\%$ and decreased matrix pH by $11 \pm 3\%$. With complex II and complex IV-linked substrates, respiration was not changed, but isoflurane still decreased matrix pH and depolarized $\Delta\Psi_m$. Depolarization and matrix acidification were only attenuated by oligomycin, but not GDP, paxilline, or 5-HD. Isoflurane did not induce matrix swelling, but decreased H_2O_2 release in the presence of succinate in an oligomycin and matrix pH sensitive manner.

Conclusion: Our results indicate that isoflurane inhibited the electron transport chain at the site of complex I and also modified F_0F_1-ATP synthase. Both effects lead to an acidification of the mitochondrial matrix which is beneficial at the time of reperfusion. K^+ channels and uncoupling proteins are likely not involved in these direct effects of isoflurane on isolated mitochondria.

3829-Pos

Ca^{2+} Enhances ROS Generation from Inhibited Complex I but not from Inhibited Complex III with Nadh-Linked Substrate

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Mitochondrial electron transport chain complexes can be major sources of ROS. Several mechanisms are responsible for modulating ROS production, possibly including mitochondrial Ca^{2+} uptake. Here we tested effects of added buffer $CaCl_2$ on ROS generation from complex I in the presence of rotenone, and from complex III in the presence of antimycin A. Guinea pig heart mitochondria (n=6) were isolated by differential centrifugation and suspended in respiration media containing amplex red and horseradish peroxidase to measure the rate of H_2O_2 generation. Increasing concentrations of buffered $CaCl_2$ were added to the mitochondrial suspension. Complex I substrate pyruvate (10 mM) or complex II substrate succinate (10 mM) was added followed by either rotenone (10 μM) or antimycin A (5 μM) to block complex I or III, respectively. Compared to no added $CaCl_2$ in the respiratory buffer, the slope of the H_2O_2 signal in the presence of pyruvate + rotenone increased respectively by 1.3 ± 0.1, 2.1 ± 0.2, 3.4 ± 0.4, 4.5 ± 0.3 times with 10, 25, 50, and 100 μM added external $CaCl_2$. In contrast, H_2O_2 generation from complex III in the presence of antimycin A did not change with increasing $CaCl_2$, whereas H_2O_2 generation from complex I in the presence of succinate (due to reversed electron flow) decreased with increasing buffer $CaCl_2$. Moreover, H_2O_2 generation from complex III in the presence of antimycin A and rotenone in mitochondria supported with succinate did not change with increased buffer $CaCl_2$. We conclude that adding $CaCl_2$ to the buffer enhances H_2O_2 generation from complex I only during blocked downstream electron transport. This emphasizes the impact of matrix Ca^{2+} loading on electron leak leading to free radical formation only under conditions of inhibited electron flow at complex I.

3830-Pos

Identification of the Mitochondrial Carrier that Provides *yarrowia Lipolytica* with a Fatty Acid- Induced and Nucleotides- Sensitive Uncoupling Protein- Like Activity

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Uncoupling proteins (UCPs) are mitochondrial carriers distributed throughout the eukaryotic kingdoms. While genes coding for UCPs have been identified in plants and animals, evidences for the presence of UCPs in fungi and protozoa are only functional. Here, it is reported that in the yeast Yarrowia lipolytica there is a fatty acid-promoted and GDP-sensitive uncoupling activity indicating the presence of a UCP. The in silico search on the Y. lipolytica genome led to the selection of two genes with the highest homology to the UCP family, XM_503525 and XM_500457. By phylogenetic analysis, XP_503525 was predicted to be an oxaloacetate carrier while XP_500457 would be a dicarboxylate carrier. Each of these two genes was cloned and heterologously expressed in Saccharomyces cerevisiae and the resulting phenotype was analyzed. The transport activity of the two gene products confirmed the phylogenetic predictions. In addition, only mitochondria isolated from yeasts expressing XP_503525 showed bioenergetic properties characteristic of a UCP: the proton conductance was increased by linoleic acid and inhibited by GDP. It is concluded that the XM_503525 gene from Y. lipolytica encodes for an oxaloacetate carrier although, remarkably, it also displays an uncoupling activity stimulated by fatty acids and inhibited by nucleotides.

3831-Pos

Silybin Derivatives Modulate Thyroid Hormone-Mediated UcP2 Expression in Neonatal Rat Cardiomyocytes

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Thyroid hormones (TH) govern cardiac phenotype including myocardial bioenergetics, a finely tuned process, possibly by affecting expression of a number of proteins. Chronic hyperthyroidism is associated with cardiac hypertrophy, which may lead to serious heart problems perhaps through higher expression of uncoupling protein 2 (UcP2), which is present in the failing heart. We were investigating effects of silybin (SB) and dehydrosilybin (DHSB) on TH-regulated cardiomyocyte bioenergetics, including UcP2 expression levels.