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