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Mitochondrial active and relaxed state respiration after heat shock mRNA response in the heart

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ABSTRACT

Induction of Heat Shock Proteins results in cytoprotection. Beneficial effect results from transcription and translational cellular components' involvement that defends metabolism and thus induce ischemic protection of the tissue. Mitochondrial respiration is also involved in stress- induced conditions. It is not a uniform process. Cytochrome c Oxidase (CytOx) representing complex IV of the Electron Transfer Chain (ETC) has a regulatory role for mitochondrial respiratory activity, which is tested in our study after hsp induction. Moreover, protein translation for mitochondrial components was probed by the detection of MT-CO1 for Subunit 1 of CytOx neosynthesis. Wistar rats were subjected to whole-body hyperthermia at 42.0–42.5 °C for 15 min followed by a normothermic recovery period. Heat shock response was monitored time dependent from LV biopsies of all control and heat treated animals with PCR-analysis for hsp 32, 60, 70.1, 70.2, 90 and MT-CO1 expression at 15, 30, 45, 60, 120 and 360 min recovery (n = 5 in each group), respectively. Enzymatic activity of CytOx were evaluated polarographically. High energy phosphates were detected by chromatographic analysis. The mRNA expression of MT-CO1 peaked at 60 min and was accompanied by hsp 32 (r = 0.457; p = 0.037) and hsp 70.2 (r = 0.615; p = 0.003) upregulation. With hsp induction, mitochondrial respiration was increased initially. Enzymatic activity reconciled from active into relaxed status wherein CytOx activity was completely inhibited by ATP. Myocardial ATP content increased from stress induced point i.e. < 1 μmol g^{-1} protein w/w to finally 1.5 ± 0.53 μmol g^{-1} protein w/w at 120 min recovery interval. Hyperthermic, myocardial hsp- induction goes along with increased CytOx activity representing an increased "active" mitochondrial respiration. In parallel, de -novo holoenzyme assembly of CytOx begins as shown by MT-CO1 upregulation at 60 min recovery time crossing with a final return to the physiological "relaxed" state and ATP -inhibited respiration.

1. Introduction

Respiration is not a uniform process. In the common sense the term is used to define an oxygen uptake via the lungs. But this represents a rather "external respiration" of the organisms. Respiration at the molecular level means reduction of dioxygen and reaction with hydrogen for water production. This means an elementary process to provide ATP as "energy currency" to all eukaryotes as well as to the most prokaryotes. This essential chemical reaction is catalyzed by Cytochrome c Oxidase (CytOx; E.C. 1.9.3.1.) This mitochondrial enzyme represents the complex IV of the electron transmission chain (ETC) and creates in combination with complex I and III a mitochondrial membrane potential by selective proton pumping driven through concomitant electron transmission from complex I to IV. CytOx is composed of 13 subunits and is in a special focus of our interest being the rate limiting enzyme of the respiratory chain (Dalmonte et al., 2009; Kunz et al., 2000; Li et al., 2006; Villani et al., 1997, 1998, 2000; Hüttemann et al., 2012; Arnold, 2012). The CytOx has the center stage for high energy phosphates production. However, different enzymatic kinetics results in a different electron take over on Dioxygen, proton pumping and mitochondrial membrane potential (Δψm) (Ramzan et al., 2010). Hereinafter we define the term "mitochondrial respiration" as Dioxygen binding to the oxygen binding binuclear center heme a_3 – Cu^B in subunit I close to subunit II containing CuA and the cytochrome c binding site (Tsukihara et al., 1996) for electron transfer from cytochrome c and subsequent water production. The enzymatic activity of CytOx is

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dependent on the ADP/ATP- ratio within the mitochondrial intermembrane space, the phosphorylation status of the enzyme, binding of ligands (e. g. Calcium), electron transfer rate from cytochrome c and last but not the least on its subunit composition. At present, although a number of details remained to be clarified, we know two different states of CytOx enzymatic activities of "internal respiration" (Kadenbach et al., 2013). First, the "active state" representing a condition with high enzymatic activity for an elevated mitochondrial membrane potential (Δψm) and maximum ATP synthesis, whereas the second "relaxed state" shows moderate enzymatic activity, low Δψm and a reduced but more effective ATP production. The equal H + /e- stoichiometry (~1) in the "active state" changes to an elevated H + /e- ratio (~2) in the latter case according to the experimental work with reconstituted CytOx in liposomes with 98% ATP content (Frank and Kadenbach, 1996). At present, discrimination between the statuses is performed by the detection of the ATP- dependent inhibitory effect on CytOx.

Moreover, CytOx from hypoxia exposed and stressed cells exhibited altered subunit content and activity (Srinivasan and Avadhani, 2012). Herein, data suggests that CytOx dysfunction is invariably associated with increased mitochondrial reactive oxygen species production and cellular toxicity.

Heat stress contributes to the improvement of cardiac mitochondrial respiratory complex activity (Sammut et al., 2001). The expression of heat shock proteins protects myocardium against ischemia or infarction (Currie et al., 1988). After myocardial hsp $72^{+}/73^{+}$ expression, we already found improvement of myocardial contractility, coronary blood flow

and oxygen consumption and reduced myocardial lactate release (Vogt et al., 2000). Especially, in hypoxia/reoxygenation states mitochondria are protected by hsp70 overexpression (Williamson et al., 2008). Preservation of postischemic mitochondrial ultrastructure was found because of the protected potential to synthesize high-energy phosphates (Currie et al., 1988). In a ^{31}phosphorus- NMR- study, we detected in accordance to previous studies, a higher myocardial HEP-content and lower pH reduction during cardiac ischemic arrest (Vogt et al., 2007) after hsp induction so that adaptive changes in respiratory complex structure and activity have to be assumed.

In the present study we investigate the effect of myocardial hsp induction by hyperthermia on mitochondrial respiration and the mRNA expression for subunit 1 (MT-CO1) because we suggest a change between active to relaxed state respiration and induction of protein translation modifying the number of the CytOx holoenzyme and its enzymatic activity. The expression of CytOx subunits is genetically mitochondrial (3 subunits: I, II and III) and nuclear encoded (10 subunits: IV, Va and b, VI a,b and c, VII a,b and c and VIII) according to the *Kadenbach*- classification. We focus on Subunit 1 (MT-CO1) because assembly of all subunits is arranged by the initial alignment of subunit I to subunit IV. Myocardial ischemia and reperfusion (Fang et al., 2007; Prabu et al., 2006) go along with enzymatic activity alterations (Vijayasarathy et al., 1998, 2003) based on exacerbation of CytOx subunit I loss (Yu et al., 2008). A coordinated down regulation of mitochondrial genome-coded CytOx subunit I was found during hypoxic stress. Severe decline in mitochondrial transcription rates, associated decrease of mitochondrial transcription factor A and reduced mitochondrial function were accompanied with reduction of the cellular heme and ATP pools as well (Vijayasarathy et al., 1998). These data indicate alterations in mitochondrial protein synthesis in response to myocardial stress. Therefore, in our study we focussed on mRNA up-regulation of hsp species and MT-CO1 as well together with relaxed and active state respiration and ATP content in the heat- stressed myocardium.

2. Materials and methods

Experiments were permitted by the local committee of animal research (GZ:V 54-19c20/15c MR 20/11) and animals were handled according to the declaration of Helsinki. 35 Wistar- rats were supplied from "Charles River Deutschland" (male, weight between 250 and 300 g, age 6 months, standard feeding). The animals were kept in single cages at 12 h day–night- regiment. Feeding of the rats were stopped 12 h before the experiment but drinking water ad libitum was available.

2.1. Narcose protocol

Animals were carefully taken from the cages and Ketamine/Xylazine (5 mg and 2.5 mg per kg BW) was injected intraperitoneally. Detections of heart rate and respiratory frequency were carried out by ECG and impedance monitoring. Body temperature was measured with a rectal catheter.

2.2. Heat shock treatment

Rats were introduced in a water bath and warmed up to 42.0–42.5 °C. The treatment was continued for further 15 min even when body temperature had reached the required degrees. Thereafter, the animals were released from the bath, dried carefully and kept covered in tissues in their cages for recovery. The recovery times were 0, 15, 30, 60, 120 and 360 min, respectively. For every recovery interval, the same procedure was performed with 5 animals.

2.3. Harvesting the hearts

After recovery, narcosis was induced again and thoracotomy via sternotomy was carried out rapidly. The pericardium was dissected and hearts were removed by cutting the great vessels. Left ventricle biopsies were taken and pieces were stored at –80 °C.

2.3.1. PCR- analysis- RNA extraction

Tissue specimens were minced in liquid nitrogen. For further analysis, a Qiagen RNeasy Kit (Quiagen, Hilden, Germany) was used. The RNA extractions were performed according to the standard protocols by mini prep columns with β- Mercaptoethanol and Ethanol extraction. We normally used myocardial biopsies with the weight of 5 mg. Samples were vortexed for 1 min and minced carefully to shear genomic DNA before loading onto the RNeasy mini columns and eluted in a volume of 50 µl of RNAse-free water.

2.3.2. PCR- analysis- RNA transcription

The RNA was transcribed into cDNA with oligo dT primers (in vitrogene life technologies, U.S.A.) 2 µg RNA were transcribed in the presence of 2 µl Oligo dT Primer (sequence pd(T)12–18) in a final volume of 11 µl with distilled water. All reagents were purchased from PE Applied Biosystems unless otherwise specified. Denaturation was carried out at 70 °C for 10 min in a Gene Amp PCR system 9600 (PE Applied Biosystems), followed by cooling to 4 °C. Master-Mix containing 10 mM dNTP, RT- Buffer and 0.1 M DTT was supplied with reverse transcriptase 200 U/µl and incubated at 37 °C for 70 min and a second step of incubation at 94 °C for 10 min was performed.

2.3.3. PCR- analysis- primer selection

Our primers were synthesized by TIB Molbiol (Berlin, Germany) and were always chosen according to the following parameters (Table 1): length between 18 and 25 bases, optimal 20–22 bases; length of amplification product between 200 and 500 bp. Primers were selected manually according to the following criteria: C + G content > 60%; repetitive sequences absent; repetitive bases and stretches of > 3 identical bases were avoided; sequence should be perfectly homologous to the RNA of interest with a 3' end base was preferably G or C. Specificity of all sequences were compared with the Genbank available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). We used PCR primers in the way that the 5'and the 3' primers span different exons so that the amplification product would be

Table 1
Primers used in PCR- analysis after myocardial heat shock protein induction. Specificity of all sequences was compared with the Genbank available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

mRNA	Primer used
hsp-32	**Forward 5`- CCA CgC ATA TAC CCg CTA CC**
hsp-32	**Reverse 5´- TCA CCC TgT gCT TgA CCT C**
hsp-60	**Forward 5´- Tgg Tgg TgC ggT gTT Tgg Ag**
hsp-60	**Reverse 5´- AAg gCT ggg ATg CAC CgA Ag**
hsp-70-1	**Forward 5´- AgA ggC TTT TCT ggC TCT CA**
hsp-70-1	**Reverse 5´- gAA CAT gCA ACC TTA ATT Tg**
hsp-70-2	**Forward 5´- Agg CTC TTT CTg gCg CTC CA**
hsp-70-2	**Reverse 5´- CCA CAg AgT AAA Tgg AAg gC**
hsp-90	**Forward 5´- CCg Tgg TgT ggT TgA TTC Cg**
hsp-90	**Reverse 5´- Tgg CCA CCT gCT CTT Tgc TC**
r-GAPDH	**Forward 5´- TCA ACg gAT TTg gCC gTA TTg**
r-GAPDH	**Reverse 5´- TgA Tgg TAT TCg AgA gAA ggg**
RCytopos	**Forward 5´- CgT TgA CTC TTT TCA ACT AAC C**
RCytopos	**Reverse 5´- gCT CCA ATT ATT AgT CCT ACA A**
RCytoneg	**Forward 5´- TTA gTA gAg ATT CTC TAC A**
RCytoneg	**Reverse 5´- TCA gCT CgA ATT AgA ATA CTC A**

of different length from that obtained from any contaminant genomic DNA. Table 1 contains used primers while Tm was calculated according to the formula reported by Sambrook et al. (1989).

2.3.4. PCR- analysis- determination of cycling parameters

The calculated annealing temperatures were tested in any case. In some cases, there were minimal deviations that we have corrected. The standard program for GAPDH (housekeeping gene), cytochrome c oxidase (positive and negative control) and hsp 70-2 (HSP A1B) is comprised of a cycle of 5 min at 95 °C, 1 min at 94 °C, followed by 1 min at 57 °C, 1 min at 72 °C and finally 5 min at 72 °C. For the hsp 70–1 (HSP A1A) we used a cycle of 5 min at 95 °C, 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C and finally 5 min at 72 °C. For the hsp 32, hsp 60 and hsp 90, we used the following PCR program: 5 min at 95 °C, 1 min at 94 °C, 1 min at 62 °C, 1 min at 72 °C and 5 min at 72 °C. The amplification products were stored at 4 °C. For the optimization, we studied the number of cycles in several experiments. Finally the optimal number of cycles was determined as 28 cycles for each primer. The amplification products were clearly visible although the amplification was still in the exponential range.

2.4. Cytochrome c oxidase (CytOx) activity measurements using myocardial tissue homogenates

Frozen myocardial biopsies (−80 °C) were immediately minced and taken in 1 ml of 50 mM potassium phosphate buffer (pH 7.4) 1% Tween 20, 1 mM EDTA. The kinetics of CytOx activity were measured polarographically at 25 °C using an Oxygraph System (Hansatech, Norfolk, UK) in a final volume of 0.5 ml. Homogenates were dissolved in 50 mM potassium phosphate (KPi buffer, pH 7.4), 2 mM EGTA, 5 mM MgSO4, 10 mM NaF, 0.5% BSA and 2% Tween 20 after protein estimation with the Bicinchoninic Acid (BCA) Protein Assay (Stoscheck, 1990). Oxygen consumption was measured at increasing concentrations of cytochrome *c* (0.2–100 µm) in the presence of 18 mM ascorbate and either 5 mM ADP or 5 mM ATP and an ATP -regenerating system (RS) consisting of 10 mM phosphoenolpyruvate and 200 units/ml pyruvate kinase. The activity of CytOx was analyzed with a micro Clark-type oxygen electrode (Oxygraph systems, Hansatech, U.K.). The measurements were performed with 3 separate biopsies at 25 °C and were correlated to the protein concentration of the samples determined by the BCA (Bicinchoninic Acid) method using a commercially available protein assay kit (Bio-Rad) (Stoscheck, 1990). Data were plotted as Δ nmol oxygen/ min x mg protein.

2.5. HPLC analysis for the determination of myocardial high energy phosphates (HEP)

The determination of adenine nucleotides was performed according to the method of Volonte et al. (2004). The chromatographic conditions were: RP 18 column (Macherey- Nagel, Düren, Germany); mobile phase composed by KH(2)PO(4) (215 mM), tetrabutylammonium hydrogen sulfate (2.3 mM), acetonitrile (4%) and KOH (1 M 0.4%); flow rate 1 ml min^{-1}; temperature 25 degrees C; injection volume 20 µl; detection at 220 nm and height of the peak as the integration parameter. The method was validated by means of linearity and sensitivity evaluations using calibration curves done with five concentration levels of each compound. Biopsies from the rat hearts were homogenized (n = 5) with a mechanical homogenizer for 3 min at 0 °C and then 5 ml of 0.4 N $HClO_4$ was added. After precipitation with 0.8 ml of 2 M KOH, the extract was shaked for 2 min and later centrifuged at 0 °C for 10 min. The supernatant was kept on ice, filtrated and injected into the HPLC system (Shimadzu, Duisburg, Germany). Data were evaluated and adjusted to protein content.

2.6. Statistical data analysis

The PCR signals were averaged from at least 5 replicates. To compare differences in expression, the Mann- Whitney *U*-test was used (SPSS™). For CytOx levels and enzymatic activity compared between ADP respiration and ATP dependent respiration and HPLC analysis, an unpaired, two-tailed *t*-test and a Mann-Whitney Rank Sum Test was used. A Fisher-test was used to demonstrate homogeneity of variances while in case of inhomogeneity, a Welch-test was performed. A p-value of less than or equal to 0.05 was considered significant. The significance threshold was set at $p < 0.05$. All tests and subsequent correlation analysis were performed using SPSS for Windows (SPSS™, München, Germany).

3. Results

3.1. hsp and COX1 induction

The mRNA expression of MT-CO1 peaked at 60 min after heat exposure and were accompanied from hsp 32 ($r = 0.457$; $p < 0.037$) and hsp 70.2 ($r = 0.615$; $p < 0.003$) (Fig. 1). Fluorescence enhanced

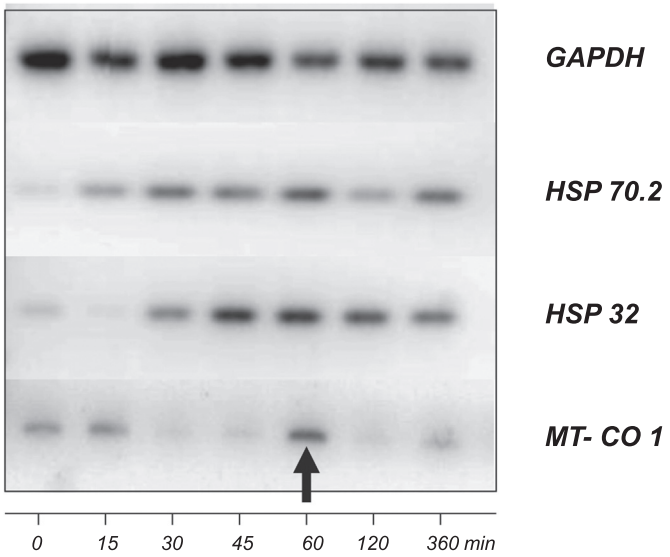

Fig. 1. Original lanes of GAPDH-, hsp70.2, hsp32- and MT-CO1 mRNA expression in the myocardium of heat shocked rats. After 60 min the MT-CO1 mRNA expression peaked.

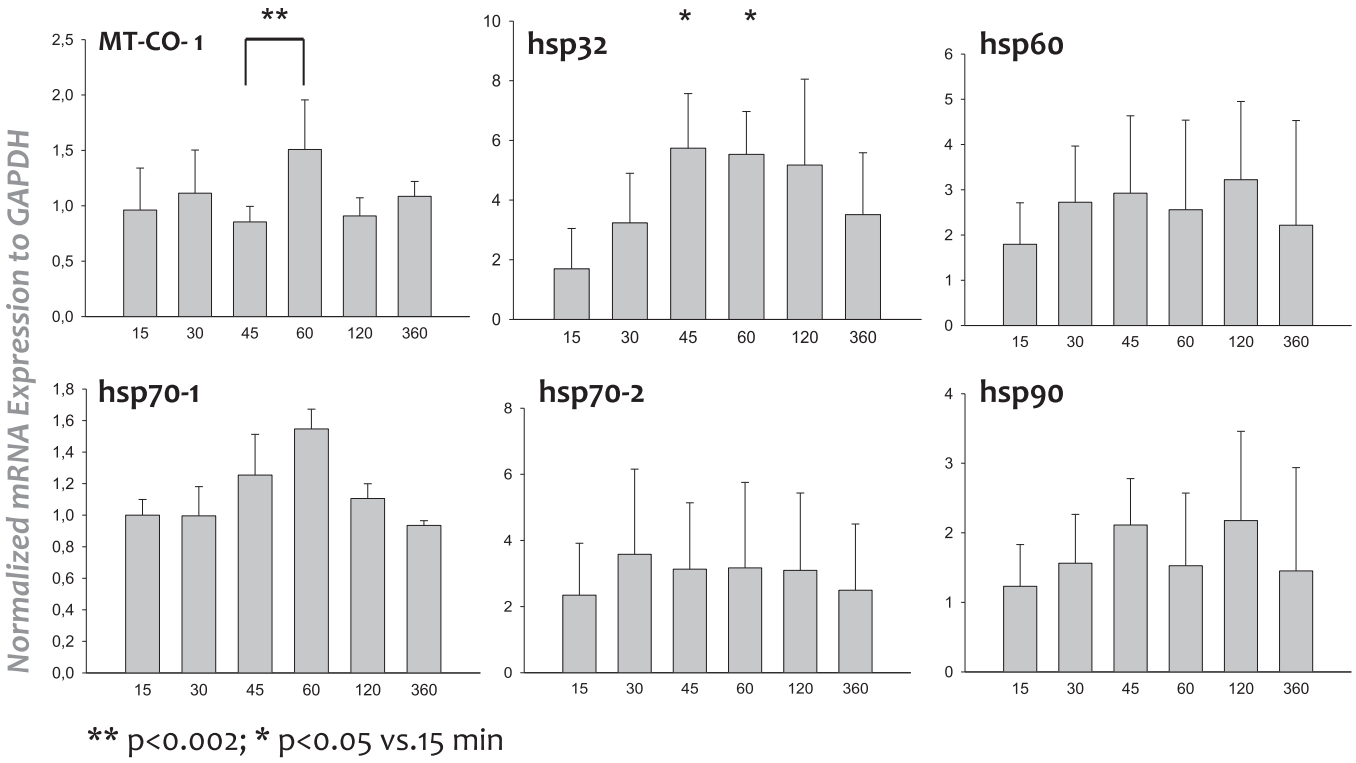

Fig. 2. Results of the PCR- analysis after myocardial heat shock: Evaluation of the mRNA expression was performed with fluorescence spectroscopy and was correlated to the normalized mRNA expression of GAPDH. Significant increase was found for MT-CO1 and hsp 32, both. Correlation data in PCR- analysis between gene expressions were detected. According to the significance, we found correlations between the mRNA expressions (n = 5, each) of hsp 70.2 and hsp 60 (r = 0.632; p < 0.003), hsp 32 and hsp 70.1 (r = 0.786; p < 0.036) and hsp 90 and hsp 60 (r = 0.759; p < 0.0001), respectively. Basic line data were included and subtracted from the normalized mRNA expression to GAPDH, so all data (n) from 15 min to 360 min are presented as ($x_n - x_{0\,min}$). Correlations were performed with *spaeman-rho*-test (SPSS™). The PCR signals were averaged from at least 5 replicates. To compare differences in expression, the Mann- Whitney *U*-test was used (SPSS™).

analysis of the normalized mRNA expression to GAPDH is shown in Fig. 2. Correlations between the mRNA expressions (n = 5, each) of hsp 70.2 and hsp 60 (r = 0.632; p < 0.003), hsp 32 and hsp 70.1 (r = 0.786; p < 0.036) and hsp 90 and hsp 60 (r = 0.759; p < 0.0001) were found.

3.2. Polarographic cell respiration measurements

The heat stress induced CytOx- activity representing tissue respiration was studied with a polarographic measuring cell. Procedures were performed according to the detailed known procedure (Vogt et al., 2013; Helling et al., 2012; Kadenbach et al., 2011; Ramzan et al., 2010). Polarographic measurements were developed by Ferguson-Miller et al. based on its kinetics when mitochondria respire (Ferguson-Miller et al., 1978). In contrast to the traditional axis whereas oxygen consumption per protein or aa_3 concentration per time is plotted against the cytochrome c concentrations (see Supplemental material), Figs. 3 and 4 represent enzymatic activities of CytOx (in Δ nmol O_2 /min. mg protein) with constant concentrations of 2 μM and 10 μM cytochrome per time, respectively. Immediately, after heat shock an increased respiration in the presence of ATP + RS is found. Under physiological conditions (see fig. S1, serving as control group) mitochondrial respiration is approximately zero at 2 μM cytochrome c. In the recover time, enzyme activities decreased and reached almost zero (white triangle line in Fig. 3). Contrastingly, when 5fold higher concentrations were used in the same setting, the return to basic values for respiration was also seen but not down to zero (grey zone, Fig. 4). So after a delay of respiratory excitation and hsp/MT-CO1 induction a return to physiological enzymatic activity was found representing an ATP- dependent inhibition of CytOx - activity *ex vivo* that confirms former reports (Arnold et al., 1997; Kadenbach et al., 1998).

3.3. HPLC- analysis of high energy phosphates

All estimations of myocardial phosphorylated adenosine contents were correlated to the protein concentrations. Immediately after heat shock exposure, the high energy phosphate (HEP) contents were as follows: ADP/ATP: 0.72 ± 0.19 vs. 2.63 ± 0.93 μmol g^{-1} protein w/w. In further equal time intervals, a reduction especially of ATP was seen below > 1.0 μmol g^{-1} protein w/w (p < 0.001). After 60 min, myocardial ATP content increased again up to 1.5 ± 0.53 μmol g^{-1} protein w/w (Fig. 5).

4. Discussion

Cells have developed various intricated protein quality control systems that recognize dysfunctional proteins. In the heart, an “unfolded protein response” is activated during acute stress, including ischemia/reperfusion, as well as following long term stress that lead to cardiac hypertrophy and heart failure (Glembotski, 2008). Concomitance of heat shock protein expression and increased activity of mitochondrial complex was already shown (Sammut et al., 2001). Our study addressed the mitochondrial respiration and the induction of COX1 -mRNA- expression after heat shock. We assume direct promotion of gene induction for mitochondrial protein complexes and further involvement of translocation machineries of the mitochondrial inner and outer membrane (Tim and Tom complexes) as known from the ischemic preconditioning. Herein, cardio protection comes from their role in

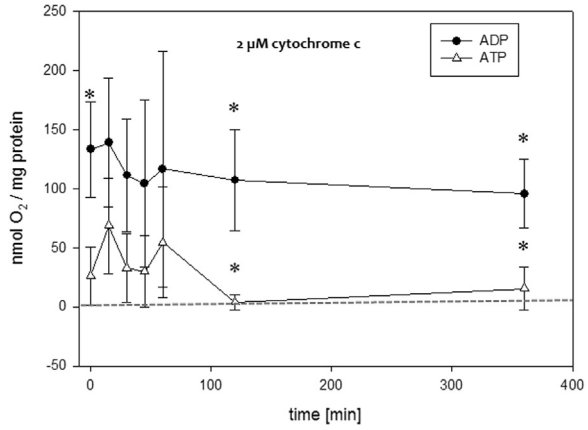

Fig. 3. Polarographic measurements of cytochrome c oxidase (CytOx) activity in the presence of ADP and separately ATP and regenerating system (RS) using myocardial tissue homogenates (n = 3 each point). This figure summarizes all respiratory measurements at one single concentration of cytochrome c as an electron donor. See Supplemental Fig. S1 for physiological conditions. The activity of CytOx was analyzed at the concentration of 2 µM cow heart cytochrome c. Details of the polarographic method are already described (Ferguson-Miller et al., 1978; Ramzan et al., 2010). After heat shock treatment, myocardial respiration (nmol O2 /mg protein) increases immediately expressing the "active state" (no ATP inhibition) but returns to the values corresponding to the "relaxed status" (ATP inhibition) after 2 h recovery time. Dotted line illustrates the respiration inhibition between 60 min and 120 min recovery time where the change of the respiratory states are visible. Significance between ADP respiration curve data and ATP respiration curve data was measured according to *Mann- Whitney U-test (SPSS™). Level of* * $p < 0.05$ was considered as significant.

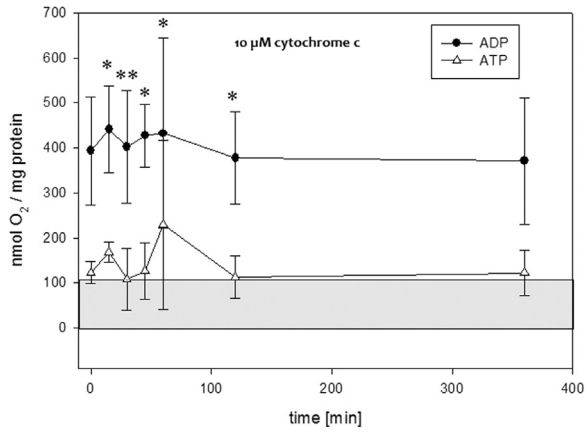

Fig. 4. Polarographic measurements of cytochrome c oxidase (CytOx) activity in the presence of ADP and ATP using myocardial tissue homogenates (n = 3 each point) for monitoring of myocardial respiration. Data were evaluated with the same experimental setting as in Fig. 3. In contrast, activity of CytOx was analyzed at a concentration of 10 µM cow heart cytochrome c (used as electron donor) corresponding to the upper limit of physiological concentrations in mitochondria. Significance according to *Mann- Whitney U-test (SPSS™)* with * $p < 0.05$.

prevention of ischemia induced decrease of Tom 20 (Wagner et al., 2009). Heat shock results in an increased, "active" respiratory activity of complex IV (CytOx) of the mitochondrial electron transmission chain (ETC). After 60 min, higher myocardial MT-CO1- mRNA expression is found together with a return to a "relaxed" myocardial respiration, but also with an increased ATP content. The concomitance of protein genesis and relaxed respiration seems to be an important factor for the maintenance of myocardial contractility and defence against subsequent ischemic episode. We know about different CytOx activities in relation to different subunit compositions, tissue specificity and oxygen

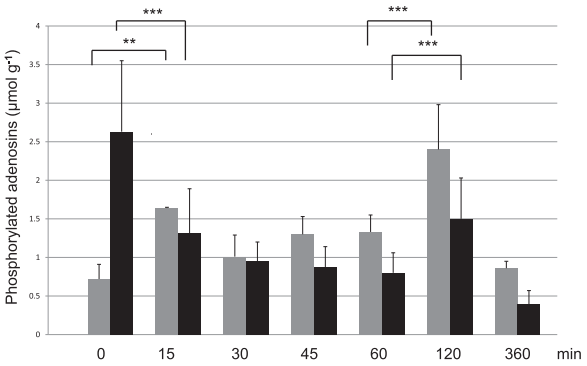

Fig. 5. Measurement of the myocardial ADP and ATP content (n = 5) in the time course after heat shock induction by HPLC. Myocardial ADP and ATP content (n = 5) was detected by HPLC- method (grey bars = ADP, black bars = ATP). The determination of adenine nucleotides was performed according to the method of Volonte et al. (2004). Note, recurrent ATP data 120 min after heat shock.

availability (e.g. hypoxia) (Vijayasarathy et al., 1998, 2003; Yu et al., 2008; Patel and Katyare, 2005; Benard et al., 2006). Therefore, it seems to be justified when increased respiratory enzyme activities (Sammut and Harrison, 2003) after heat shock protein induction are assumed to be related to alterations in subunit composition. Biogenesis of the eukaryotic enzyme requires the coordinated action of two genes and more than 20 additional nuclear- encoded factors, all acting at different levels of the biogenetic process (Fontanesi et al., 2006). Studies with yeast mutants indicate that most catalytic core unassembled subunits are post-translationally degraded in terms of specific processing of apoproteins. From previous works it is known that the assembly of the 13 subunits of CytOx starts with the association of subunit I and IV (Nijtmans et al., 1998). Subunit 1 is mitochondrially encoded and is a part of catalytic center around which the full complex is assembled (Fontanesi et al., 2006, 2008) and is located directly inside the inner mitochondrial membrane, so it can be hardly modified from both sides. It is essential for the enzymatic activity although the holoenzyme alternates its activity dependent on its phosphorylation status (Lee et al., 2005).

The apparent enzyme kinetics of the ETC by cytosolic ADP represents a second order reaction and not like a first order reaction as assumed, previously (Jeneson et al., 1996). To maintain ATP content in the cell, mitochondria must sense cellular ATP utilization and transduce this demand to the F0-F1-ATPase. The inhibition of CytOx by high intramitochondrial ATP is accompanied by a change of hyperbolic into sigmoidal kinetics and a "relaxed state" workload associated with low level ROS production (Kadenbach et al., 2013). In a review article we have given more details of the "second mechanism of respiratory control" (Kadenbach et al., 2010; Vogt et al., 2016). According to this mechanism the conditions of relaxed and active state respiration have an elementary impact on myocardial cytoprotection because of different amounts of ROS release.

We measured the enzymatic activity of CytOx in a polarographic set up and studied the time course dependent enzymatic activity change at two different Cytochrome c concentrations (2 and 10 µM) and reduction rates of the enzyme. In both cases the relaxed state of the enzymatic activity were found 120 min after heat shock induction. In this state we suggest a lower, but high effective ATP synthesis. After 60 min of hsp induction ATP content was increased again although it was still below the initial value. The coincidence of elevated ATP levels and a rather normalized cellular respiration indicates a beneficial effect of hsp induction. Although mitochondrial hsp70 and Tim44 facilitate the translocation of precursor proteins from the inner membrane into the matrix, it has to be pronounced that transport across the inner membrane is also dependent on the mitochondrial membrane potential and

ATP hydrolysis. Interestingly, after hsp induction the myocardial ATP contents fell down to < 1.0 μmol g^{-1} protein w/w ($p < 0.001$) comparable to the suggested hsp associated ATP consumption for protein genesis (Boengler et al., 2006; Wagner et al., 2009). Ischemic ATP depletion was attenuated and in reperfusion, the ATP content was quickly restored. Heat shock treatment provides transient protection against myocardial injury and improves postischemic functional recovery (Vogt et al., 2000 and 2007; Vogt et al., 2011). The enzymatic activities of mitochondrial respiratory complexes were already found to be increased after heat shock expression (Sammut et al., 2001; Sammut and Harrison, 2003). Brief ischemic episodes activate a protective gene machinery including a strong upregulation of mRNA transcripts for heat shock proteins (Simkhovich et al., 2003; Das and Maulik, 2006). Heat shock proteins are known as molecular chaperones and regulators of the cellular hemostasis.

In our study the increased expression of MT-CO1 correlates with hsp 32 ($r = 0.457$; $p < 0.037$) and hsp 70.2 ($r = 0.615$; $p < 0.003$) mRNA expression, respectively. The latter stabilizes in conjunction with other heat shock proteins existing proteins against aggregation and mediates the folding of newly translated proteins in the cytosol and in organelles. N-terminal ATPase domain of the hsp 70 proteins binds ATP and hydrolyzes it to ADP. This fact may relate to the decrease of myocardial ATP after heat shock. The hsp70 family is identified as an ATPase involved in the protein import (Truscott et al., 2001).

Hsp 32 or heme- oxygenase (HO-1) is an essential component of the catabolism of the heme molecule. Induction of stress inducible HO-1 occurs in response to heat shock and oxidative stress. Low oxygen is a potent inducer of HO-1. Heme is involved in the transcriptional regulation of nuclear COX structural genes by oxygen concentration and by carbon source availability (Fontanesi et al., 2006, 2008). Inhibition of cell respiration by endogenous CO through its interaction with CytOx contributes to cell activation under hypoxic conditions (D'Amico et al., 2006). An enhancement of mitochondrial transport carriers and CytOx activity was found in kidney after up-regulation of HO-1. Interestingly, increased phosphorylation of AKT and levels of BcL-XL proteins were also found so that the cytoprotective mechanisms of HO-1 against oxidative stress is based on multiple steps including increase of anti-apoptotic proteins (Di Noia et al., 2006). Carbon dioxide alters oxygen-dependent functions by binding heme proteins in competitive inverse relation to oxygen partial pressure (Cronje et al., 2004). It means an important fact because protein expression of CytOx subunit I which is encoded by the mitochondrial genome is dependent on heme availability (Converso et al., 2006). These results may confirm recent observations where a loss of subunit I was found in case of severe myocardial ischemia (Yu et al., 2008). Stress induced ROS production could result in a selective oxidation of subunit I on Tryptophan 334 (Lemma-Gray et al., 2007). So, conformational alterations could affect the enzymatic activity and prevent malfunction to avoid ROS production. Hsp 32 induction correlated with the nuclear hsp 70.1 mRNA increases and suggests a participation in CytOx subunit I induction. Perhaps this is the key for understanding the results of Vijayasarathy et al. (1999), whereas the tissue heme level regulates not only the mammalian COX gene expression, but also the catalytic activity of the enzyme probably by affecting its stability. hsp 60 binds in combination with hsp 10 to the newly synthesized polypeptides and facilitates their folding to native state via one or more rounds of ATP hydrolysis. In this concern hsp 70.2 mRNA induction coincides with hsp 60 (Endo, 1991; Pfanner et al., 1991). A link between hsp 60- and hsp 90 mRNA seems feasible because the effect of hsp 90 onto the magnitude while duration of proliferative activation and survival- promoting signaling responses is known (Meares et al., 2004). It is further found that HSP90 is responsible for Akt accumulation in mitochondria. Akt as a protein kinase B could interact for phosphorylations of CytOx subunits (Hüttemann et al., 2012) The level of hsp90 alpha –mRNA is increased by growth factors via tyrosin kinase receptors (Jérôme et al., 1991) and the association of hsp90 with endothelial nitric oxide synthase (eNOS) is well recognized for increasing NO production, essential for oxygen transportation to the subunit IV (CytOx) binding site (Kocha et al., 2015).

5. Conclusion

Hyperthermic stress to the heart results in an induction of hsp –mRNA from different hsp species. After translation they are suggested to interact conforming to the synthesis of mitochondrial proteins. For instance, MT-CO1 mRNA was found upregulated for increased expression of subunit 1 of the Cytochrome c -Oxidase (complex IV) corresponding to the rate limiting step for ATP production. Any kind of stress results in higher workload and increases the oxygen demand for efficient ATP content production required for myocardial function but also protein synthesis (hsp70 and 90). Correspondingly, a stress related switch into “active state” respiration is found in case of hyperthermia, but relapsing to “relaxed state” respiration after 2 h recovery. These findings should be taken into account for preconditioning protocols in clinical settings (e.g. heart surgery).

Disclosure section

No disclosures have to be reported and no conflict of interest exists.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtherbio.2019.01.007.

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