Mitochondrial performance in heat acclimation—a lesson from ischemia/reperfusion and calcium overload insults in the heart

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Assayag M, Saada A, Gerstenblith G, Canaana H, Shlomai R, Horowitz M. Mitochondrial performance in heat acclimation—a lesson from ischemia/reperfusion and calcium overload insults in the heart. Am J Physiol Regul Integr Comp Physiol 303: R870–R881, 2012. First published August 15, 2012; doi:10.1152/ajpregu.00155.2012.—Long-term heat acclimation (LTHA; 30 days, 34°C) causes phenotypic adaptations that render protection against ischemic/reperfusion insult (I/R, 30 min global ischemia and 40 min reperfusion) via heat acclimation-mediated cross-tolerance (HACT) mechanisms. Short-term acclimation (STHA, 2 days, 34°C), in contrast, is characterized by cellular perturbations, leading to increased susceptibility to insults. Here, we tested the hypothesis that enhanced mitochondrial respiratory function is part of the acclimatory repertoire and that the 30-day regimen is required for protection via HACT. We subjected isolated hearts and mitochondria from controls (C), STHA, or LTHA rats to I/R, hypoxia/reoxygenation, or Ca2+ overload insults. Mitochondrial function was assessed by measuring O2 consumption, membrane potential (ΔΨm), mitochondrial Ca2+ ([Ca2+]m), and complex I activity, respectively, whereas the corresponding LTHA parameters remained unchanged. STHA mitochondria maintained ΔΨm but did not preserve ATP production. LTHA [Ca2+]m was significantly higher than that of C and STHA and decreased in the cardiac mitochondria of heat-acclimated rats, whereas the full protection provided by HACT requires 30 days of heat acclimation (long-term heat acclimation, or LTHA). STHA worsens the deleterious effects of some stressors (9), suggesting that other adaptive features, in addition to the above-mentioned mitochondrial outer-membrane changes, are essential for HACT. Given our previous studies showing that the LTHA heart possesses 1) improved metabolic efficiency and energy potential (29); 2) an attenuated drop in ATP

decreased plasma thyroxin activity in acclimating animals (10). Along these lines, Nichols and Locke (49) established that the mitochondria of heat-adapted guinea-pigs maintain a significantly higher “energy potential” and are less sensitive to uncoupling events. Adaptive responses of oxidative metabolism are largely controlled by transcription factors and their coactivators, which affect both mitochondrial biogenesis and the level of expression of genes encoding the electron-transport chain and oxidative phosphorylation (58). The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), which modulates metabolism in response to multiple stimuli, including temperature (via a thermoregulatory sympathetic pathway and by altering mitochondrial biogenesis) is a master regulator in this process (1, 36, 53). The mammalian model, which according to evidence provided by Seebacher et al. (59), also applies to lower vertebrates, is in agreement with Cassuto’s findings (10) demonstrating that heat acclimation decreases cytochrome-c oxidase (COX), citrate synthase (CS), and downstream targets in the PGC-1α pathway.

Revisiting mitochondrial heat acclimation, Assayag et al. (3) and Umschiew et al. (65) proved that the mitochondria, via heat acclimation-mediated cross-tolerance (HACT) mechanisms, in which heat acclimation confers protection against stressors of a different nature, attenuated apoptosis during ischemia/reperfusion (I/R) insult in the heart and following traumatic brain injury. Assayag et al. (3) also demonstrated that basal levels of cytochrome c, an essential electron carrier that transfers electrons from complex III to the COX complex, decreased in the cardiac mitochondria of heat-acclimated rats, whereas heat stress induced a marked elevation of this protein. The former fits with Seebacher’s COX activity in warm acclimated reptiles (59). The mitochondrial BclXL protein was elevated under both basal-normothermic and heat-stress conditions, implying adaptive properties in the mitochondria-importing complexes and synthesis systems (2) and marked the mitochondria as essential players in thermotolerance, as well as in HACT. Assayag et al. (3) demonstrated that the adaptive features of the outer mitochondrial membrane (such as an elevated BclXL/Bad ratio) are already found after 2 days of heat acclimation, namely short-term heat acclimation (STHA), whereas the full protection provided by HACT requires 30 days of heat acclimation (long-term heat acclimation, or LTHA). STHA worsens the deleterious effects of some stressors (9), suggesting that other adaptive features, in addition to the above-mentioned mitochondrial outer-membrane changes, are essential for HACT. Given our previous studies showing that the LTHA heart possesses 1) improved metabolic efficiency and energy potential (29); 2) an attenuated drop in ATP

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and pH upon global ischemia [31P-NMR studies (35)], and is protected upon reperfusion (35, 41); and 3) preserves mitochondrial complex V (ATP synthase) abundance upon global ischemia in contrast to a significant drop in normothermic ischemic hearts (Mizraji N and Horowitz M, a proteomic approach study, unpublished data), we hypothesize that LTHA improves mitochondrial metabolic performance and mitochondrial biogenesis, and thereby promotes cytoprotection upon stressful conditions.

A major player in regulating mitochondrial function is mitochondrial Ca2+ ([Ca2+]m). A correlation has been found between increased bioenergetics and increased [Ca2+]m (48). Excess [Ca2+]m, however, opens the permeability transition pore (40, 54), a key factor in apoptosis during ischemia-reperfusion injury (12–17). These reports, together with our findings that LTHA enhances Ca2+ stress-endurance and decreases Ca2+ sensitivity in cardiomyocytes (52, 67), lend further support to the hypothesis that the adaptive features of the mitochondria evolve with heat acclimation and are important for HACT by enhancing respiratory performance during stress.

The aim of this study was twofold, to investigate whether respiratory performance of AC (heat acclimation) mitochondria is enhanced and whether alterations in mitochondrial biogenesis contribute to the LTHA adaptive processes, in general, and to HACT, in particular. For this purpose, we subjected hearts and mitochondria to I/R, hypoxia/reoxygenation, or Ca2+ overload insults, while measuring physiological and biochemical functional parameters and markers of mitochondrial biogenesis. The study was conducted on rats during an acclimation regimen to determine how long the animal must be under acclimatizing conditions for each adaptive process to develop. Our results, combining both parameters, demonstrate that cardiac mitochondria from LTHA, but not STHA, rats, subjected to impaired oxygen demand/oxygen supply balance, exhibited enhanced respiratory functions that are due to improved complex performance and maintenance of mitochondrial membrane integrity. In turn, intact ATP production facilitated the HACT cascade. We also suggest that enhanced mitochondrial biogenesis, primarily during STHA, preconditions the mitochondria (44) for the induction of a mitochondrial energetic gene-regulatory program, leading to enhanced hypoxic/oxygenation tolerance and a possible shift toward fatty-acid utilization during the insults.

MATERIALS AND METHODS

Animals

Male, 3-wk-old Rattus norvegicus (Sabra strain, albino var.), initially weighing 80–90 g, fed Ambar laboratory chow with water ad libitum, were randomly assigned to 1) heat acclimated for 30 days (LTHA), 2) heat acclimated for 2 days (STHA), and 3) control-normothermic (C) groups. Each group was further divided according to experimental conditions into normoxic, ischemic/reperfusion, or anoxic/reoxygenation. All experimental protocols were approved by the Ethics Committee for Animal Experimentation of The Hebrew University and complied with the guidelines of the National Research Council’s Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996).

Experimental Conditions

The C group was maintained at an ambient temperature of 24 ± 1°C; LTHA was achieved by continuous exposure to 34 ± 1°C and 30–40% relative humidity in a light-cycled room (12:12 h) for 30 days; short-term heat acclimation (STHA) was achieved by exposure to ambient conditions as above for 2 days, following maintenance under control conditions for the initial 28 days, as previously described (28).

Physiological Experiments

Ischemic/reperfusion isolated heart preparation. Animals were anesthetized using a mixture of ketamine-xylazine (8.5 mg/100 g ip body wt ketamine in 0.5% xylazine), the hearts were rapidly removed, mounted on a Langendorff perfusion system, and retrogradely perfused with Krebs-Henseleit buffer containing (in mM): 120 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 1.25 CaCl2, 25 NaHCO3, and 11 glucose, at pH 7.4, and aerated with a mixture of 95% O2-5% CO2 at 37°C (35) at a perfusion pressure of 100 cm H2O. After 10 min of equilibration, perfusion was stopped (global ischemia, GI) for 30 min, and the hearts were then reperfused for 40 min.

Hearts were either used immediately for mitochondrial isolation and characterization of the function of the respiratory complexes, ATP production, and mitochondrial membrane potential or frozen (−80°C) until further analyses, including mitochondrial respiratory enzymes and molecular markers of mitochondrial biogenesis.

Cardiomyocyte mitochondrial Ca2+ levels under basal conditions. Animals were euthanized, and the hearts were rapidly removed as described above. Cardiomyocytes were isolated according to Cohen et al. (15). The cell preparations contained 50–70% rod-shaped cells. Briefly, the intracellular and mitochondria Ca2+ concentrations ([Ca2+]i, [Ca2+]m, respectively) were measured using the acetoxyethyl ester form of Indo-1 (2 μM and 0.075% pluronic acid for 20 min), according to Miyata et al. (47). The myocyte suspension was examined in a chamber with a quartz base using an inverted epifluorescence microscope (Nikon Diaphot 200) and perfused with Krebs-Henseleit bicarbonate buffer solution at 37°C. Cells were excited at 340 nm, and emission was recorded at 405- and 495-nm wavelengths, using a PTI (Photon Technology International, Birmingham, NJ) fluorimetric system. For [Ca2+]m, cells were perfused with 200 μM MnCl2 for 20 min, resulting in a quenching of the [Ca2+]i; Ca2+ levels were presented as the ratio of fluorescence 409 nm/495 nm. Isolated cardiomyocytes of C and AC groups were also subjected to hypoxia/reoxygenation protocol to investigate whether AC mitochondria serve as a Ca2+ sink. The time lapses to the onset of ATP depletion-dependent rigor (defined as T1) and the duration of continued anoxia after the rigor until reoxygenation (T2 = total anoxia – T1) were measured as described by Miyata et al. (47) and Pronobesh et al. (62). For anoxia, ultrapure argon was used.

In vitro hypoxia model. A modified Elrod et al. (19) preparation was used for the measurements of ATP production and mitochondrial membrane integrity in a sequence of oxygenated-anoxic-reoxygenated states. Freshly isolated mitochondria (see Isolated mitochondria preparation) were placed into an Eppendorf test tube, and following equilibration were bubbled with ultrapure argon gas for 3 min, and then the tube was sealed and left for 30 or 45 min. To reoxygenate, the Eppendorf test tube was opened, and the mitochondria were aerated for 20 or 40 min. ATP production and the mitochondrial membrane potential were measured at the end of each anoxic and reoxygenation session.

Isolated mitochondria preparation. For mitochondrial fraction separation, the left ventricle of the heart was homogenized in mitochondrial isolation buffer containing (in mM): 10 HEPES, 200 mannitol, 70 sucrose, and 1 EGTA. The homogenate was centrifuged for 10,000 × g, 14,000 g, at 4°C, the cytosolic fraction was removed and the pellet (mitochondrial fraction) was resuspended, recentrifuged, resuspended, and immediately processed. For the assessment of Ca2+ effects on the respiratory activity and ATP production, we added 100 μM Ca2+ to the mitochondrial sample just before processing. Protein concentra-
tion was determined using Bradford reagent (Bio-Rad Laboratories, Richmond, CA).

**Oxygen consumption.** Oxygen consumption was assessed on freshly isolated mitochondria using a Clark oxygen electrode, as described previously (4, 57). Briefly, freshly isolated mitochondria were introduced to a respiration buffer containing (in mM): 5 K2HPO4, 10 Tris, 100 KCl, 5 MgCl2, 0.005 EDTA, 75 mannitol, 25 sucrose, 0.6 mg/ml fatty-acid free BSA. State 4 respiration was monitored in the presence of substrate glutamate with malate (GM): complex I linked substrate, or succinate (SR): complex II linked substrate in the presence of rotenone-complex I inhibitor. State 3 respiration was monitored in the presence of ADP. The data are presented as nanomoles O2 per milligram protein per minute. The respiratory control index (RCI, defined as state 3 divided by state 4) reflects the control of oxygen consumption by phosphorylation ("coupling"). The P/O ratios (number of ADP molecules added for each oxygen atom consumed) are an index of the efficiency of oxidative phosphorylation. These indexes were calculated as described by Chance and Williams (12).

**Mitochondrial membrane potential.** The mitochondrial membrane potential (ΔΨm) was measured by using a cationic dye JC-1 (Sigma Aldrich). This dual-emission mitochondrial potentiometric dye exhibits a potential dose-dependent accumulation in mitochondria. This reaction is indicated by a fluorescence emission shift from green to red, when the positively charged dye enters the negatively charged mitochondrial aggregates, and fluoresces red. When mitochondrial ΔΨm collapses, JC-1 is distributed throughout the inner membrane in a monomeric form that fluoresces green while the amount of red fluorescence drops. As a result, mitochondrial depolarization and decreased ΔΨm are indicated by a decrease in red/green fluorescence intensity ratio (60). The ratio of red-to-green JC-1 fluorescence is relatively independent of putative interfering factors, including mitochondrial mass, shape, or density (22, 61) and is, therefore, advantageous compared with nonratiometric dyes, whereas ΔΨm is measured in isolated mitochondria. Freshly isolated mitochondria (5 μg protein) were stained with 0.2 μg/ml JC-1 diluted in JC-1 assay buffer, as indicated by the manufacturer. After 20 min of incubation in the dark, fluorescence was measured. JC-1-loaded mitochondria were excited at 485 nm, and the emission was detected at 585 nm (JC-1 aggregates) and 516 nm (JC-1 monomers) using a spectrofluorimeter (FluoStar, BMG LabTechnologies, Ortenberg, Germany). The data are presented as emission ratios (590/520).

**Biochemical and Molecular Experiments**

**ATP production.** For ATP production, a modification and down-scaling of the method described by Manfredi et al. (42) was used. Briefly, freshly isolated mitochondria (15 μg protein) were added to 1 ml respiration buffer and then the point zero sample was taken. To start the reaction, an appropriate substrate (GM or SR) and ADP (0.25 mM) were added, and the reaction tubes were incubated for 4 min. Fifty microliters from each sample was mixed with 450 μl of boiling tris EDTA (TE) buffer and incubated for 2 min at 100°C. After cooling on ice, 100 μl of the supernatant was added to a 96-well plate. An equal volume (100 μl) of the luciferase reagent (Roche Applied Science, Indianapolis, IN) was added to each well by the Mithras Luminometer with the injection function (Mithras LB 940; Berthold Technologies, Herts, UK). Solutions containing fixed ATP amounts were used to construct a standard curve to calculate ATP content. The data are presented as nanomoles of ATP produced per minute per milligram protein.

**Respiratory chain complex enzymatic activities.** The freeze-thawed mitochondrial preparation was suspended in potassium phosphate buffer (0.1 mol/l, pH 7.4). The respiratory chain enzyme activities were assayed according to published methods—rotenone-sensitive NADH-coenzyme Q reductase (complex I), succinate dehydrogenase (complex II), and cytochrome-c oxidase (COX, complex IV)—were determined by spectrophotometric assays. Citrate synthase, a ubiquitous mitochondrial matrix enzyme, served as an internal control for mitochondrial purity and content (57, 63).

**Mitochondrial biogenesis.** Biogenesis markers PGC-1α (8), peroxisome proliferator-activated receptor-γ (PPARγ), and PPARγ transcription factors involved in lipid metabolism (14, 50), nuclear respiratory factor 1 (NRF1) that activates genes coding OXPHOS subunit proteins (17), mitochondrial transcriptional factor A (TFAM) critical for the regulation of mitochondrial gene transcription and mitochondrial DNA replication (34) and Hadhβ transcript, involved in the oxidation of lipid acids (39), were analyzed.

Changes in mRNA were detected using quantitative real-time-PCR (qPCR). Briefly, total RNA was extracted from the left ventricle homogenate, using TRI-Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (1 μg) was reverse transcribed in a 20-μl reaction mixture containing 1 μl of oligo (dT) as primer, 10 mM dNTP, together with 200 U of Moloney murine leukemia virus RT and 40 U of ribonuclease inhibitor, according to the manufacturer’s protocol (Fermentas, Hanover, MD). For qPCR, an ABI Prism 7300 sequence detection system (Applied Biosystems) was used. The reaction volumes of 20 μl contained 10 μl of SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA), 500 nM each of the forward and reverse primers, and 5 μl of diluted cDNA. The appropriate cDNA dilution was determined using calibration curves established for each primer pair. The thermal profile for SYBR Green qPCR was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers for the qPCR were designed using Primer Express software (Applied Biosystems) (Table 1).

Changes in mitochondrial DNA (mtDNA) copy number relative to the genomic DNA were detected using qPCR. Total DNA was purified from the left ventricle by phenol:chloroform extraction, precipitated with ethanol, and then dissolved in TE pH 8.0 (18). For qPCR, ABI Prism 7300 sequence detection system as detailed above was used. In this section, primers used were β-globin for the genomic DNA (single copy gene) and NAD4 for the mitochondrial DNA.

**Table 1. Real-time PCR (qRT) primer sequences**

<table>
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<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>NM_031144</td>
<td>TGGCGCATCATGAGAATCTAC</td>
<td>AATTGCTGTCGAGTGAG</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>NM_031347</td>
<td>AAGGCTCAAGAGGGCAGAAT</td>
<td>ATCACAGGCCTGCTCAAT</td>
</tr>
<tr>
<td>PPARγ</td>
<td>NM_031324</td>
<td>TGGATAGCCACGGCAGGACCT</td>
<td>TCAAGCGGGAAGGATTTATG</td>
</tr>
<tr>
<td>PPARβ</td>
<td>NM_031341</td>
<td>TCATCCAGCAGTGAAGGACGG</td>
<td>AACACATAACGGCTGATCT</td>
</tr>
<tr>
<td>NRF1</td>
<td>XM_231566</td>
<td>TTATCTGCTGCTGCTGATGAG</td>
<td>CCTGATATATGCTGCTGAT</td>
</tr>
<tr>
<td>TFAM</td>
<td>NM_031326</td>
<td>AAGTATTGCTATGCTGCTGAGCT</td>
<td>GCCGACATTTGATGAG</td>
</tr>
<tr>
<td>Hadhβ</td>
<td>NM_133618</td>
<td>AACACATTGAGCAGCAGTGA</td>
<td>GCTATATACACGGCTGAT</td>
</tr>
<tr>
<td>Cox III</td>
<td>NC_001665</td>
<td>TGAGGGTTCAGCATGATCCTA</td>
<td>GAGGCTGTCAGAGCTGAT</td>
</tr>
<tr>
<td>18S</td>
<td>X01117</td>
<td>CGGCCTAACATCAGAAGGAA</td>
<td>GGCGCCTGAGAAACGCTGCC</td>
</tr>
<tr>
<td>b-Globin</td>
<td>X15160</td>
<td>ATCCATATCGAAGCTGTCAC</td>
<td>ATCGCCCTGAAAAAATG</td>
</tr>
<tr>
<td>ND4</td>
<td>NC_001665</td>
<td>CCAATCTGAGAAGCTGAAATGC</td>
<td>ATCGGCTATATCGATGAA</td>
</tr>
</tbody>
</table>
**Statistical Analysis**

One-way and two-way ANOVAs with appropriate post hoc tests (Tukey-Kramer or Dunnett’s tests) were performed using commercially available software (SigmaStat 2.03). The treatment time points (TTP; C, STHA, and LTHA) and the acute stressor (I/R) were taken as the independent categorical variables, and individual animals or hearts were considered a random sample from the population. To test whether TTP affected the response to superimposed I/R, two-way ANOVA was used. If significant interactions (with I/R and TTP as the two factors) were indicated, a multiple-comparisons test to detect whether origin of significance stems from I/R vs. basal was conducted. Additional details are specified in the figure legends. The data are expressed as means ± SE; values of $P < 0.05$ were considered statistically significant.

**RESULTS**

**Functional Performance of the Mitochondria**

Oxygen consumption and \( P/O \) ratio are affected by heat acclimation and I/R insult. To examine whether heat acclimation affects respiration, we measured \( O_2 \) consumption in isolated cardiac left ventricle (LV) mitochondria before and after subjection to I/R insult (Fig. 1). When the mitochondria were given a complex I-dependent substrate (GM), a significant decrease in \( O_2 \) consumption of the LTHA group under basal conditions, compared with normothermic rats (19.46 ± 0.35 vs. 14.8 ± 0.64 nmol \( O_2 \)·min\(^{-1} \)·mg\(^{-1} \)), was detected. The STHA mitochondria also showed a nonstatistically significant decrease (Fig. 1A). Respiration using SR as a substrate showed no difference between the C and LTHA groups. The STHA group had a minor, although nonstatistically significant, decrease (STHA group: 32.78 ± 3 vs. C: 39.48 ± 1.7 nmol \( O_2 \)·min\(^{-1} \)·mg\(^{-1} \)). Notably, the oxygen consumption rate on SR was higher than the rate obtained on GM (Fig. 1B). Ischemia reperfusion injury had no effect on the \( O_2 \) consumption of the STHA and LTHA groups. We observed a marked drop in the \( P/O \) ratio only in the insulted normothermic mitochondria. This decrease was significant compared with both the basal normothermic values and the STHA and LTHA acclimated insulted mitochondria (C I/R: 0.58 ± 0.04 vs. C basal: 2.61 ± 0.45, STHA: 2.04 ± 0.27, LTHA: 2.51 ± 0.24, $P < 0.05$) (Fig. 1C). No significant difference was found in the respiratory control indices among the groups both under normoxic and I/R conditions (Fig. 1D).

**ATP Production Is Affected by Heat Acclimation and I/R Insult**

To further evaluate the efficiency of mitochondrial respiration, we measured ATP production. When GM was given as a substrate, I/R resulted in a significant decrease in ATP production capacity in both the normothermic mitochondria (96.3 ± 1.1 vs. 83.2 ± 2.6 nmol·min\(^{-1} \)·mg\(^{-1} \), $P < 0.05$) and the STHA mitochondria (98.43 ± 1.9 vs. 86.5 ± 4.1 nmol·min\(^{-1} \)·mg\(^{-1} \), $P < 0.05$) (Fig. 2A). The LTHA mitochondrial function was not impaired vs. the normoxic phase. In contrast, when SR was given as a substrate, only STHA mitochondria under basal conditions, demonstrated a drop in their capacity to produce ATP. I/R treatment had no further effect on the SR-fueled groups (Fig. 2B).

![Fig. 1. Respiratory function of isolated mitochondria from the left ventricle of hearts of control (C), short-term acclimation (STHA), and long-term heat acclimation (LTHA) rats under basal-normoxic (B) conditions and post I/R insult (I/R). A: oxygen consumption of mitochondria on glutamate with malate (GM) was significantly lower under basal conditions in the LTHA group. B: oxygen consumption of mitochondria on SR was not significantly different between the groups under both normoxic and I/R insult conditions. C: \( P/O \) ratio of mitochondria on GM was significantly lower in the C-I/R insulted mitochondria vs. that of STHA and LTHA groups. D: RCI on GM. Values are expressed as means ± SE; $n = 6$. *Significant difference from C (Tukey-Kramer, $P < 0.03$–0.006). ^Significant difference from basal levels within the group (Tukey-Kramer, $P < 0.001$). C, control; STHA, heat acclimation for 2 days; LTHA, heat acclimation for 30 days; B, basal state; I/R, ischemic reperfusion insult.](http://ajpregu.physiology.org/).
Mitochondrial Membrane Potential and ATP Production in AC Mitochondria Are Preserved Compared with Control Mitochondria in the In Vitro Hypoxia Model

An in vitro hypoxia/reoxygenation model was used to track the dynamic profile of the hypoxia/reoxygenation response in the same mitochondria. Prolonged hypoxic periods (30 and 45 min) and subsequent 20 and 40 min of reoxygenation caused the most significant effects (Fig. 5). Upon reoxygenation, only the LTHA group maintained, and even elevated, the capacity to produce ATP (LTHA-20 min and C-20 min of reoxygenation yielded 118.1 ± 5.4% vs. 92.7 ± 6.4%, respectively, P < 0.005). Our data also show that following 30 min of hypoxia, membrane integrity was significantly decreased only in the normothermic mitochondria (Fig. 5B). Hypoxia of 45 min masked any differences among groups regarding ATP production (Fig. 5C). The ΔΨm decreased significantly vs. the matched basal state in C and LTHA mitochondria (P < 0.015) but recovered in the LTHA group when reoxygenation progressed (Fig. 5D).

I/R Insult Superimposed on Heat Acclimation Affects Mitochondrial Membrane Potential

Changes in mitochondrial membrane potential were assessed by monitoring changes in the fluorescence properties of JC-1. No difference in the basal state of the three treatment groups was observed (Fig. 4). Following I/R insult, a 25% decrease in membrane ΔΨm occurred in the normothermic group only, compared with its basal state (0.24 ± 0.009 vs. 0.18 ± 0.01, respectively, P < 0.002) Fig. 4.
Does Ca\textsuperscript{2+} Overload Affect AC Mitochondrial Respiratory Function and Membrane Integrity?

Cellular and mitochondrial calcium levels are presented in Fig. 6. Fig. 6A shows that the percentage of $[\text{Ca}^{2+}]_{\text{in}}$ relative to total cardiomyocyte $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_{\text{Total}}$) increases with heat acclimation. Fig. 6B shows $[\text{Ca}^{2+}]_{\text{in}}$ levels during hypoxia/reoxygenation episodes. The rate of rise of $[\text{Ca}^{2+}]_{\text{in}}$ in C mitochondria was markedly greater than in LTHA cardiomyocytes ($P < 0.05$) and more pronounced during the post ATP-depletion rigor (T2 state, Fig. 6B), suggesting that the benefits of LTHA in mitochondria already manifest under basal conditions. The higher $[\text{Ca}^{2+}]_{\text{in}}$ in LTHA mitochondria seems to be maintained throughout the hypoxia/reoxygenation protocol (Fig. 6B). LTHA cardiomyocytes survived a profoundly longer time than the C cardiomyocytes (e.g., time, in minutes, to rigor contracture under hypoxic conditions: C, 32.6 ± 2.85; LTHA, 50 ± 7.3, $P < 0.05$; survival following reoxygenation: C, 13.7 ± 2.79; LTHA, 23.0 ± 9.4, $P < 0.05$). When Ca\textsuperscript{2+} overload occurred, O\textsubscript{2} consumption, RCI, and $\Delta \Psi_{m}$ were significantly lower in C than in the short- and the long-term acclimated groups (Fig. 6, C–E). ATP production was not affected by this stress in either group (data not shown).

Heat Acclimation and I/R Affect Transcription Levels of Genes Involved in Mitochondrial Biogenesis

To further elaborate the improved mitochondrial function achieved by heat acclimation, we examined the transcription levels of a number of genes involved in mitochondrial biogenesis (Fig. 7). During the basal state of each experimental group, the PGC-1\textalpha levels were significantly upregulated after both STHA and LTHA compared with the normothermic C group (1.64 ± 0.07, 1.38 ± 0.1 vs. 1.05 ± 0.04, respectively; $P < 0.001$, $P < 0.026$). PPAR\textgamma and NRF were upregulated significantly vs. normothermic C in STHA ($P < 0.01$, $P < 0.037$, respectively), but with progression of acclimation, the LTHA levels of these transcripts were significantly lower than in the STHA group ($P < 0.004$). The LTHA levels of PPAR\textgamma were significantly downregulated compared with the C group ($P < 0.02$). Neither TFAM nor Hadhb demonstrated significant changes from the C group, although TFAM, similar to NRF, tended to be elevated vs. both C and LTHA groups. Ischemia/reperfusion insult resulted in a significant drop of PGC-1\textalpha and NRF in all experimental groups (C: $P < 0.002$, STHA: $P < 0.001$, LTHA: $P < 0.001$), whereas PPAR\textgamma, PPAR\textdelta, and Hadhb were upregulated significantly vs. the matched basal condition in C and LTHA (PPAR\textgamma: C, $P < 0.001$ and LTHA, $P < 0.001$; PPAR\textdelta: C, $P < 0.001$ and LTHA, $P < 0.001$; Hadhb: C, $P < 0.005$ and LTHA: $P < 0.023$), with no change in the level of these transcripts in the STHA phase. In contrast,
Heat acclimation enhances mito-performance during insults

Fig. 6. Calcium ([Ca$^{2+}$]$_{m}$) levels and the effect of [Ca$^{2+}$]$_{m}$ overload on mitochondrial respiratory function. A: total and mitochondrial [Ca$^{2+}$]$_{m}$ levels in C, STHA, and LTHA cardiomyocytes, using Indo-1 fluorescence (405/495 nm emission ratio) as a measure. Basal [Ca$^{2+}$]$_{m}$ showed a progressive significant increase (% total) over the course of heat acclimation (P < 0.05). B: [Ca$^{2+}$]$_{m}$ in C and LTHA cardiomyocytes subjected to hypoxia: [Ca$^{2+}$]$_{m}$ upon ATP-depletion rigor (T1) and following (in sequence) with postrigor hypoxia, allowing recovery of 50% (T2) of the studied cardiomyocytes when reoxygenized are presented. For Ca$^{2+}$/H$_{9004}$/H$_{9023}$, see Fig. 1 caption.

The TFAM levels were profoundly elevated (vs. matched basal state) in all experimental groups (C: P < 0.01; STHA: P < 0.009; LTHA: P < 0.002).

Short-Term Heat Acclimation Reduces mtDNA Copy Number

We found that the mtDNA copy number relative to the genomic DNA was significantly reduced in the STHA hearts compared with C (P < 0.03). In the LTHA state, however, the ratio was similar to that observed in the C state. The data are presented in Fig. 8 (C: 0.91 ± 0.17; STHA: 0.41 ± 0.15; LTHA: 0.71 ± 0.13).

To investigate the differences in mtDNA copy number that originated in the mitochondria, we also measured the levels of CS (mtDNA independent) in homogenates. We found a significant increase in the STHA group (P < 0.004) vs. both the C and the LTHA (activity units per milligram protein: C, 1,105 ± 80; STHA, 1,552 ± 119; and LTHA, 1,228 ± 70), indicating that the mtDNA copy number in STHA is decreased per se relative to mitochondrial content.

Discussion

Using I/R or Ca$^{2+}$ overload as a stress trigger, we demonstrated the adaptive kinetics of mitochondrial respiratory functions during the process of mammalian heat acclimation and their contribution to HACT. Complementing our previous work (3), here, we show that adaptations at the outer membrane of the mitochondria precede the enzymatic changes in the respiratory complexes and mitochondrial respiration. The preservation of the mitochondrial outer membrane permeability alone, however, was insufficient to confer cardioprotection via HACT mechanisms (3). The combination of membranal adaptations and enhancement of the respiratory complexes is required to achieve HACT. Upregulation in the expression of genes associated with mitochondrial biogenesis and metabolic cascades, which were already noted after STHA, implies that the “switching on” of processes regulating acclimated mitochondrial quality occurs early on during STHA (2 days of acclimation), but for the impact of acclimation on mitochondrial respiratory function to be complete, the LTHA phase, namely 30 days, is required.

Mitochondria Respiration Functional Performance Under Basal and I/R Insult

Under basal conditions, the O$_{2}$ consumption of isolated mitochondria via complex I, was significantly lower in the LTHA phase, whereas the membrane potential, P/O ratio, and ATP production were similar to control values. This result suggests an increased efficiency of the heat-acclimated mitochondria, as a workable adaptation to reduce heat production. The ablation of complex I by rotenone and using succinate as a substrate revealed unaltered O$_{2}$ consumption and ATP production upon LTHA and, in contrast, a profound decrease in ATP production upon STHA, implying that complex II-dependent respiration is susceptible to the initial acclimatory thermal stress, thus confirming prior observations (using 3-nitropropionic acid, a SDH inhibitor) that SDH is a likely site of mitochondrial oxygen consumption. In both STHA and LTHA groups, a slight increase in oxygen consumption was measured, whereas C mitochondria demonstrated a significant decrease from the basal state (STHA and LTHA vs. C, P < 0.01 and 0.04, respectively). D effect of 100 µM calcium supplement on mitochondrial oxygen consumption. In all experimental groups (C: P < 0.01; STHA: P < 0.009; LTHA: P < 0.002).
membrane potential, and ATP production were highly preserved compared with the basal state. This observation was not true for STHA mitochondria, which despite their ability to maintain intact membrane potential, were inefficient ATP producers. The normothermic group of mitochondria was the most susceptible to I/R and was unable to maintain 

\[ \Delta \psi_m \], a key event in the I/R-induced damage (5, 33, 51, 56), P/O ratio dropped significantly, and ATP production also significantly decreased. The results presented here highlight the vulnerability of complex I to stress and the advantages of LTHA. With respect to complex II-dependent respiration, no I/R effect was observed, suggesting that Complex II is less sensitive to that stress and that the minor decrease in activity was below the threshold required to impair respiration and ATP production.

Notably, the observation of slightly low RCI values (<3) in the heat-treated (STHA, LTHA) groups, both under basal and following I/R insult, may be as a result of mitochondrial preparation. However, because the control RCI value was 3, these data imply that heat, rather than I/R, affects coupling. To our knowledge, RCI values for chronically heat-treated animals have not been previously reported.

In the in vitro experimental hypoxia/reoxygenation model, isolated mitochondria, which were subjected to the sequential hypoxia/reoxygenation protocol (Fig. 5) and fueled by GM, demonstrated similar STHA-LTHA \( \Delta \psi_m \) and ATP production acclimatory kinetics, supporting the conclusion stated above. However, enhanced performance conferred by LTHA (in the two parameters studied) was detected only after subjection to hypoxia for 30 min. The benefits of LTHA declined following hypoxia of 45 min, indicating the upper limits of resolution among treatments of this preparation.

The significantly improved ATP production in GM respiring mitochondria at the end of the I/R protocol in LTHA using the Langendorff apparatus (Fig. 2A) is incongruous with the results obtained in the in vitro hypoxia protocol, in which the ATP production was only slightly increased vs. the C group (Fig. 5A). In view of the basic differences between the two models used—isolated mitochondria subjected to hypoxia/reoxygenation vs. isolated heart, subjected to ischemia/reperfusion protocol—our data suggest that within the whole heart tissue, during I/R insult, the mitochondria are exposed to a harsher

Fig. 7. Transcript levels of selected genes involved in mitochondrial biogenesis in the left ventricle of C, STHA, and LTHA rats under basal conditions and post I/R insult; n = 4. *Significant difference from C (Tukey-Kramer, \( P < 0.037-0.001 \)). **Significant difference from basal (B) within the group (Tukey-Kramer, \( P < 0.026-0.001 \)). #Significant difference from LTHA (Tukey-Kramer, \( P < 0.023-0.001 \)). For abbreviations, see Fig. 1 caption.

Fig. 8. Mitochondrial DNA of C, STHA, and LTHA at basal levels presented as mitochondrial vs. nuclear genomic sequence. *Significant difference from C (Tukey-Kramer, \( P < 0.031 \)); n = 4. For abbreviations, see Fig. 1 caption.
environment than the milder conditions in the in vitro hypoxia model, and thus, the benefits of LTHA vs. nonacclimated or STHA under more stressful conditions are emphasized. In addition to mitochondrial adaptation per se, we cannot rule out the role of the protective extra-mitochondrial environment in LTHA (3).

Several published works (13, 37) have addressed the issue of a reduction in mitochondrial complex activity post I/R insult. Liu et al. (37) found significant levels of damage to complex I proteins, whereas Chen et al. (13) showed damage to complex II activity. Hence, to further elaborate the contribution of the different components of the respiratory chain to HACT, we studied the biochemical performance of complexes I, II, and IV post-I/R insult. Under our experimental set-up with 30-min global ischemia and 40-min reperfusion and in agreement with our physiological results (as discussed in Figs. 2A and 4), complex I was the most susceptible. The post I/R mitochondria of nonacclimated rats decreased the activity of this complex by 50%, whereas with acclimation, adaptive changes ultimately accounted for a 15% elevation in activity of this complex in the LTHA mitochondria. Complex II was enhanced in LTHA mitochondria by almost 30%. Complex IV, cytochrome-c oxidase, was enhanced in both C and LTHA mitochondria but was attenuated in STHA mitochondria (Fig. 3). The latter finding agrees with decreased ATP production when mitochondria were fueled by SR in that acclimation phase under both basal and I/R conditions.

Collectively, our data show that post I/R metabolic suppression (decreased ATP production) in nonacclimated and STHA is primarily due to the sensitivity of complex I to such stress (Fig. 3). The maintained/enhanced function of the LTHA mitochondria is linked to improvement of both complex I and complex II performance. Interestingly, chronic heat-affected complex II similarly to that seen in hibernating rodents, when suppressed metabolism during torpor vs. interbout euthermia is linked to succinate-fueled (but not to glutamate or malate-fueled) skeletal and liver mitochondria (6), suggesting that this complex is inherently affected by environmental thermal stress.

Studying complex III activity is beyond the scope of the current study. Previous studies from our laboratory (Canaana H, unpublished data) (15), however, have demonstrated that in cardiomyocytes, both heat stress and hypoxia induce a lower amount of reactive oxygen species (ROS) in AC mitochondria. Similar to the current investigation and the study by Assayag et al. (3), this adaptation was shown during LTHA, during which antimycin (a complex III inhibitor) increased ROS production by 25%, 45%, and 18% in C, STHA, and LTHA, respectively, suggesting that complex III follows the same acclimatory kinetics.

**AC Mitochondrial Performance During Ca2+ Overload**

Mitochondrial Ca2+ was measured using Indo 1, according to Miyata et al. (47). Ca2+ level of nonacclimated mitochondria resembled those values obtained by previous authors (e.g., 47). The very high level of [Ca2+] observed in LTHA but not in C and STHA is, consequently, a result of prolonged heat adaptation. Considering that Ca2+ overload causes the damage from I/R (54) and our finding that LTHA mitochondria contain a higher percentage of cellular Ca2+ (Fig. 6A), we tested the direct effect of Ca2+ overload on O2 consumption, membrane potential, and ATP production on mitochondria fueled by GM. In congruence with the physiological experiments of this investigation (Figs. 4 and 5) and Assayag et al. (3), we demonstrated that membrane adaptation is an early adaptive signature of heat acclimation. Only nonacclimated mitochondria could not maintain ΔΨm following Ca2+ overload. Concomitantly, O2 consumption and RCI dropped significantly in this group (P < 0.048, P < 0.014) (Fig. 6). One explanation for the disruption of ΔΨm is mitochondrial matrix Ca2+ overload and an opening of mitochondrial permeability transition pores (PTM) (51, 56). Although this issue was not studied, our finding of elevated mitochondrial BclXL [vs. decreased BAD, (3)], during both STHA and LTHA could explain the differences in ΔΨm observed between C and the acclimated groups (for discussion, see Heat Acclimation Kinetics). The maintenance of high, but relatively stable, [Ca2+]m during hypoxia/reoxygenation sessions promotes the hypothesis that the LTHA mitochondria contribute to HACT by stabilizing Ca2+ levels to allow continued respiration when the oxygen demand/oxygen supply balance is impaired. Interestingly, on the basis of data of mitochondrial 45Ca2+ uptake and respiratory function of mitochondria in the muskrat (Ondatra zibethicus), a rodent inhabiting fresh water streams, known for its marked hypoxic tolerance, McKean (43) concluded that an increased ability to sequester calcium by the mitochondria without causing them damage favors recovery from hypoxia, ischemia, or acidosis.

The hypothesis that an increase in [Ca2+]m can activate TCA dehydrogenases in the matrix to enhance respiration was proposed long ago (64). Recently, Haumann et al. (27) suggested an alternative option, namely that CaCl2 might induce a large increase in respiration due to changes in the buffering capacity of the mitochondrial matrix. Levi et al. (35), using NMR spectroscopy, demonstrated such an increase during global ischemia in LTHA hearts. The current, as well as previous investigations (15), strengthen the theory that heat acclimation decreases the Ca2+ sensitivity of various mitochondrial enzymes. This hypothesis agrees with our earlier data demonstrating the decreased Ca2+ sensitivity of the contractile machinery of LTHA hearts (15) and transformation from fast (V1) to the predominantly slow (V3) myosin isofrom with lower ATPase activity.

In our set-up, the enhanced Ca2+ signal alone did not affect ATP production, and this observation has already been published by Territo et al. (64).

**Mitochondria Biogenesis**

Remodeling of the mitochondria, comprising biogenesis and mitophagy (degradation of the mitochondria’s own components) processes, is linked to a variety of cardioprotective and neuroprotective mechanisms (24). Hence, to further understand the machinery leading to enhanced mitochondrial function in LTHA hearts, we focused on the possible involvement of mitochondrial biogenesis by studying mitochondrial biogenesis markers. Our results indicate that the expression of PGC-1α, PPARγ, PPARδ, NRF, TFAM, and mitochondrial biogenesis regulatory proteins linked with the encoding of mitochondrial electron transfer component (e.g., parallel upregulation of PGC-1α and NRF-1 and nuclear genes encoding mitochondrial
electron transfer chain proteins and activity) increases during STHA, thus preceding the physiological compensatory responses of the respiratory chain elements seen during LTHA. According to our findings, the latter response is not due to increased mtDNA content but rather to the adaptive upregulation of respiratory chain activity. Mild heat stress (38), ischemic or hypoxic cardiac preconditioning (44), and exercise (38), all physiological conditions requiring increased energy demands and linked to enhanced cardioprotection via cross-tolerance mechanisms, demonstrated similar initial upregulated mitogenesis.

Interestingly, whereas the general machinery of the STHA phenotype presented increased mitochondrial biogenesis (increased Pgc-1α, NRF, TFAM mRNA), a significant decrease in mtDNA (Fig. 9) implying cardiac mitophagy during that acclimation phase was also observed. These findings fit with a recent review by Gustafsson and Gottlieb (26) on mitochondrial quality control, suggesting that mitochondrial quality depends on a balance between biogenesis and mitophagy, during which the latter results in a selective elimination of damaged mitochondria, leaving the mitochondrial population with enhanced ischemic/anoxic tolerance. Mitophagy is beyond the scope of the current investigation. However, a literature search revealed that an important regulator of mitochondrial biogenesis is the histone deacetylase sirtuin 1 (Sirt1). Sirt1 regulates autophagy (32), suggesting that mitochondrial removal and synthesis are linked (24). Indeed, STHA is characterized by cellular metabolic impairments and an increased level of ROS, both providing an autophagy signal (30).

I/R insult resulted in a drop of PGC-1α and NRF transcripts, together with increased transcript levels of genes coding lipid metabolic pathways in C and LTHA hearts, suggesting enhanced lipid utilization at that stage (21, 31, 55), irrespective of the acclimatory status. The only finding linked with acclimation status was the abolished upregulation of PPARγ, PPARδ (associated with lipid metabolism), and Hadhb (associated iron metabolism) on the STHA mitochondria. This response is additional evidence of the cellular impairments known to occur upon the STHA phase (3, 30), leading to aggravation of the cellular response to a novel stressor.

Previous studies on heat or cold acclimation provided evidence that adequate upregulation and downregulation of transcript expression precede profound changes in protein level and activation when acclimation progresses (25, 41). Thus, we can conclude that on early upregulation of biogenesis transcripts and mitophagy (on STHA), the evolvement of mitochondria with improved membrane integrity (Ref 3; Figs. 1, 4, and 5) displays their contribution to HACT only when acclimation homeostasis and respiratory chain adaptations have been achieved.

A limitation of this study is that assessment regarding biogenesis was based on signaling only. Mitochondrial imaging via electron microscopy or measurement of actual synthesis of the mitochondria was beyond the scope of this investigation.

Heat Acclimation Kinetics

We suggest here that the acclimation of features linked to maintaining mitochondrial membrane integrity are already seen at the early stage, i.e., STHA. Here, this adaptation is exhibited by maintenance of ΔΨm. This finding is in accord with Assayag et al. (3), who demonstrated upregulation and an increased BclXL/BAD ratio and preservation of cytochrome-c within the mitochondria, during I/R stress. Upregulation of BclXL had been previously identified as essential for the maintenance of mitochondrial membrane integrity (66), suggesting that these two STHA-mediated acclimatory adaptations are interconnected. The intervention in BclXL/BAD complex to prevent mitochondrial membrane permeability is associated with posttranslational modifications [BAD phosphorylation/dephosphorylation (11)], explaining this rapid survival response. At that acclimatory phase, concerted, metabolic-associated compensatory responses (e.g., an increase in PGC-1α, PPARδ, and TFAM transcript levels as displayed here) are likely to attenuate the cellular metabolic impairments occurring at the onset of heat acclimation. These rapid responses converge to rescue basic metabolic functions but are insufficient to contribute to HACT and thus, during STHA, the ability to cope with novel stressors (e.g., I/R, heat stress) is diminished, as previously shown (3, 15). The present study reveals the “dual nature” of STHA: although the mitochondrial membrane potential and the P/O ratio, similar to LTHA, are already protected from I/R insult, the “inner” membrane and matrix functions in complex activity and ATP production are negatively affected by this insult. The LTHA adaptations, both in the mitochondria and the cytosolic compartments, are required to create the cytoprotective environment. With respect to energy metabolism, in addition to enhanced aerobic respiration

![Fig. 9. Conceptual model of the kinetics of mitochondrial acclimation to heat. Initial rapid adaptation of mitochondrial membrane integrity is followed by long-term progressive changes in respiratory chain complex activity, which is required to achieve maximal contribution of the mitochondria to HACT. This model is based on data accumulated in this investigation and in a study by Assayag et al. (3). PTM, posttranslational modifications (phosphorylation). For other abbreviations see Fig. 1 caption.](image-url)
displayed by noninsulted respiratory parameters in the face of I/R, other known adaptations include upregulation of PDK1 (phosphate dehydrogenase kinase) and upregulated glycolysis (20), collectively enhancing both aerobic and anaerobic features. Our concept of phases in mitochondrial acclimation is summarized in Fig. 9.

Despite the limitations, the results of this study shed new light on the importance of mitochondrial respiratory chain functions (especially complex I) in HACT. Moreover, the results of study contribute to our understanding of the kinetics of acclimation of the mitochondria during exposure of the organism to chronic heat.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.A., A.S., H.C., and R.S. performed experiments; M.A., A.S., H.C., R.S., and M.H. analyzed data; M.A. drafted manuscript; M.A., A.S., G.G., and M.H. approved final version of manuscript; A.S. and M.H. edited and revised manuscript; G.G. and M.H. conceived and designed of research.

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