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Differential changes in respiratory capacity and ischemia tolerance of isolated mitochondria from atrophied and hypertrophied hearts

Heiko Bugger, Jörg-Michael Chemnitius, Torsten Doenst*

Department of Cardiovascular Surgery, Albert-Ludwigs University of Freiburg, 79106 Freiburg i. Br., Germany

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Abstract

In spite of opposing changes in rates of adenosine triphosphate turnover, hypertrophy and atrophy of the heart are accompanied by the same changes in gene expression, resembling a fetal genotype. Fetal hearts are characterized by increased ischemia tolerance. We assessed respiratory capacity of mitochondrial subpopulations from unloaded and pressure-overloaded hearts before and after 15 minutes of normothermic ischemia. Unloading was achieved by heterotopic rat heart transplantation and overloading by aortic banding. Respiratory chain gene expression (NADH dehydrogenase, cytochrome c oxidase [COX]) were analyzed by reverse transcriptase–polymerase chain reaction. Subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) were isolated by differential centrifugation. Citrate synthase was used as mitochondrial marker enzyme. Adenosine diphosphate–stimulated oxygen consumption (state 3) was measured with a Clark-type electrode. Unloading resulted in atrophy, overloading in hypertrophy. State 3 was reduced in atrophied hearts both in SSM and IFM (SSM: 204 ± 79 vs 804 ± 147 natoms oxygen min^{-1} mL^{-1}, $P < .001$; IFM: 468 ± 158 vs 1141 ± 296 natoms oxygen min^{-1} mL^{-1}, $P < .05$), but was unchanged in hypertrophied hearts. NADH dehydrogenase and COX expression was also decreased with atrophy and was unchanged with hypertrophy. Ischemia caused decreased recovery of citrate synthase in isolates of SSM ($P < .05$) but not of IFM. State 3 in control hearts was reduced in IFM (−41%, $P < .01$) and SSM (−19%, not significant). This ischemia-induced decrease was less pronounced in SSM (−2%) and IFM (−22%) of atrophied and IFM (−23%) of hypertrophied hearts. Subsarcolemmal mitochondria of hypertrophied hearts displayed the greatest ischemia-induced decrease of state 3 (−32%, $P < .05$). In conclusion, (1) long-term changes in workload differentially affect maximal respiratory capacity and ischemia tolerance of isolated mitochondria. The changes are not parallel to the changes in energy requirements. (2) Mitochondria of atrophied hearts appear to be more resistant against ischemia than controls.

1. Introduction

Long-term un- or overloading of hearts causes opposing changes in adenosine triphosphate (ATP) requirements demanding lesser activity or abundance of the respiratory chain or both in atrophied than in hypertrophied hearts. In contrast, the opposing changes in workload induce similar changes in gene expression resembling the fetal genotype [1]. Fetal hearts are characterized by increased ischemia tolerance. In contrast, myocardial hypertrophy was reported to be associated with decreased ischemia tolerance [2,3]. Several studies showed increased susceptibility of hypertrophied hearts to ischemia and reperfusion [4-7]. Recovery of cardiac function after ischemia is decreased, and the onset of ischemic contracture occurs earlier than in control hearts [8,9].

Little is known about ischemia tolerance of atrophied hearts. A single study reported decreased recovery of contractile function after ischemia and reperfusion of heterotopically transplanted rat hearts [10]. This finding argues against the notion that atrophied hearts are similar to fetal hearts, not only on the gene expression level, but also on the functional level because fetal hearts are characterized by an increased resistance against ischemic damage [11-15]. No study has yet evaluated the impact of unloading and/or ischemia on the respiratory capacity of isolated mitochondria.

Characterizing mitochondrial changes in the heart has to take into account the existence of 2 subpopulations [16,17]. Subsarcolemmal mitchondria (SSM) are localized beneath the sarcolemma, and interfibrillar mitochondria (IFM) are embedded between the myofibrils. Differences in the

* Corresponding author. Tel.: +49 761 270 2818; fax: +49 761 270 6136.
E-mail address: doenst@ch11.ukl.uni-freiburg.de (T. Doenst).

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biochemical properties of myocardial mitochondrial subpopulations have been discussed by several authors in prior reports [17-19]. Some authors suggested differential susceptibility of the 2 mitochondrial subpopulations to ischemia [20,21]. Thus, when assessing changes in mitochondrial function of the heart, both subpopulations have to be considered.

In this study, we isolated the 2 mitochondrial subpopulations from mechanically unloaded and pressure-overloaded hearts and assessed their maximal respiratory capacity before and after a period of ischemia. We found that (1) long-term changes in workload differentially affect maximal respiratory capacity and ischemia tolerance of isolated mitochondria. The changes do not parallel the changes in energy requirements. (2) Mitochondria of atrophied hearts exhibit a greater resistance to ischemia than normotrophic hearts. (3) We also identified a methodological shortcoming that has to be taken into account when differential centrifugation is used for isolating mitochondria in the setting of ischemia/reperfusion.

2. Methods

2.1. Animals

Male Wistar rats (250-450 g) were obtained from Charles River (Sulzfeld, Germany) and were fed ad libitum at 21°C with a light cycle of 12 hours. The use of animals and the experimental protocol were approved by the Animal Welfare Committee of the University of Freiburg, Germany.

2.2. Materials

Chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany), Delta-Pharma (Pfullingen, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Essex (München, Germany), Bayer (Leverkusen, Germany), Narkodorm-n (Neumünster, Germany), and BIO-RAD (München, Germany).

2.3. Surgical protocols

Ventricular unloading was achieved by infrarenal heterotopic heart transplantation in male Wistar rats [22,23]. Hearts were transplanted by anastomosing end to side the ascending aorta of the donor to the abdominal aorta of the recipient and the donor pulmonary artery to the recipient inferior vena cava. The graft continued to beat spontaneously. Hearts were removed after 1 week, a period known to induce significant atrophy.

Cardiac hypertrophy was induced in male Wistar rats by banding of the aortic arch [24]. Anesthetized and ventilated rats had a superior median thoracotomy to allow visualization of the aortic arch. A 3-0 silk suture ligature was tied around the transverse aorta against a 20-gauge needle, and the needle was then removed. Control rats were sham operated. Hearts were removed after 2 weeks because we demonstrated before that this time point reflects a state of compensated hypertrophy [24].

2.4. Total, global in vitro ischemia

The model of normothermic, total global in vitro ischemia was used to assess the effects of ischemia [25,26]. Hearts were explanted, washed quickly in NaCl, and sealed air-free in oxygen-impermeable plastic bags. Sealed hearts were incubated in a water bath for 15 or 45 minutes at 37°C. After the incubation, hearts were homogenized immediately for isolation of mitochondria.

2.5. Tissue homogenization

After explantation or incubation, hearts were immersed in ice-cold cardioplegic solution containing 0.18 mol/L KCl and 10 mmol/L Tris-HCl (pH 7.4). The hearts were finely minced, and 1 g of tissue was added to 6 mL of KEA isolation medium (0.18 mmol/L KCl, 10 mmol/L EDTA, 0.5% bovine serum albumin). The mixture was homogenized with an Ultra-Turrax T25 (IKA, Staufen, Germany) at 8750 rpm for 20 seconds, followed by one down-pass in a Potter-Elvehjem glass homogenizer by a motor-driven Teflon pestle at 2000 rpm. PH was adjusted to 7.4.

2.6. Isolation of mitochondria

Isolation of SSM and IFM was performed in a modification of the procedure described by Palmer et al [27]. Three milliliters of heart homogenate were centrifuged at 700*g*. The supernatant fraction was used for isolation of SSM by centrifuging it 3 times at 7000*g* for 10 minutes and resuspending each pellet with KEA. The last pellet was diluted with 500 μL KEA. For isolation of IFM the homogenate pellet was treated with Nagarse (5 mg/g wet weight) for 30 seconds and centrifuged at 7000*g* for 5 minutes. The resulting pellet was resuspended with KEA and centrifuged at 700*g* for 10 minutes. This pellet was resuspended and spun down 3 times at 7000*g* for 10 minutes. The last pellet was diluted with 500 μL KEA. Tissue homogenization and isolation of mitochondria were carried out at 4°C.

2.7. Biochemical analysis

Citrate synthase (CS) serves as a mitochondrial marker enzyme [28]. Total and free CS activity were measured and used to calculate latent CS activity ($CS_{total} - CS_{free}$), which is the fraction of total CS activity that is contained within the mitochondria and not readily accessible by substrate. The CS ratio (CSR) represents the relationship of latent to free CS activity and serves as an index of structural integrity of the mitochondrial preparation [29]. Citrate synthase activity was determined in whole heart homogenate or isolated mitochondria at 25°C using a modification of the procedure of Srere [28]. In brief, background activity was measured spectrophotometrically (λ = 412 nm) in cuvettes containing 100 μL H_2O, 775 μL 1 mmol/L 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) in 1 mol/L Tris-HCl, pH 8.5, 25 μL acetyl-coenzyme A, and 50 μL of sample. Maximal enzyme activity was measured immediately after addition of

Table 1
Primer and probe sequences used in the quantitative PCR

Gene	Primer/probe	Sequence (5′-3′)
NADH dehydrogenase subunit 2	Forward	CTATCATCCCACTTCTAGCCAACA
	Reverse	GGCTGTAGCTTGGGTTAGAAAATATT
	Probe	6-FAM-AGCCCACGATCAACTGAAGCAGCAA-MBG
COX IV	Forward	GCCTTTCCAGGGATGAGAAAG
	Reverse	TCTCAGCGAAGCTCTCGTTAAA
	Probe	6-FAM-CCAATTGTACCGCATCC-MBG
Histone 3B	Forward	GGAGGCAAGTGAGGCCTATCT
	Reverse	TGTCACTGACCTGATGCTCTAATG
	Probe	6-FAM-CATCTTCAAAAGGCCAA-MBG

50 μL 10 mmol/L oxaloacetate in 0.1 mol/L Tris-HCl, pH 8.5. Total CS activity was determined after preincubation of samples with 2.5% Triton X-100, and free CS activity was measured after preincubation in 0.9% NaCl.

2.8. Mitochondrial respiration

Oxygen consumption of isolated mitochondria was measured at 25°C with a Clark-type oxygen electrode fitted to a water-jacketed reaction chamber of 1-mL volume. Mitochondrial preparations were added to respiration medium (210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L KH_2PO_4, 10 mmol/L Tris/HCl, pH 7.4) to a final concentration of 1 U CS/mL in the reaction chamber. Maximal oxygen consumption (state 3 respiration), using glutamate as a substrate (10 mmol/L), was stimulated by addition of 20 μL adenosine diphosphate (ADP) (1 mmol/L). State 4 respiration was measured as the rate of oxygen consumption after total phosphorylation of added ADP. Results are preferably presented in natoms oxygen/U of CS because we demonstrated before that this relation is a more precise parameter to characterize respiratory capacity of isolated mitochondria than relation to milligram of protein [29,30].

2.9. Quantitative real-time polymerase chain reaction

Total cardiac muscle RNA was isolated from frozen tissue samples using the Qiagen RNeasy midi kit (Qiagen, Hilden, Germany). Synthesis of complementary DNA and TaqMan quantitative real-time reverse transcriptase–polymerase chain reaction (PCR) were performed using the AmpliTaq Gold RT-PCR Kit (Applied Biosystems, Darmstadt, Germany). Primers and probes for NADH dehydrogenase subunit 2, cytochrome c oxidase (COX) IV, and His3B were designed using Primer Express 1.3 software (Table 1). For each set of primers, a basic local alignment search tool search revealed that sequence homology was obtained only for the target gene. Polymerase chain reaction amplification was performed in triplicates in a total reaction volume of 25 μL. The reaction mixture consisted of 5 μL diluted template, 0.025 U/μL *Taq* polymerase, 0.01 U/μL AmpErase, 5.5 mmol/L $MgCl_2$, 200 μmol/L dNTP mix, 1× TaqMan buffer A, 200 nmol/L forward and reverse primers, and 100 nmol/L probe. For each PCR, amplification was allowed to proceed for 40 cycles, each consisting of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. A series of 5 dilutions was analyzed for each target gene and allowed us to construct linear standard curves from which the concentrations of the test sample were calculated. Results were normalized to histone 3B transcription as a housekeeping gene product, which was not different among all samples.

2.10. Statistical analysis

Data are presented as mean ± SEM. Data were analyzed using a 1-way analysis of variance or a Student *t* test where appropriate. Post hoc comparisons among the groups were performed using Tukey test. Differences among groups were considered statistically significant at $P < .05$.

3. Results

Activity of CS was determined in control hearts and hearts subjected to 15 or 45 minutes of total, global in vitro ischemia. Total CS activity was unchanged in homogenates after 15 or 45 minutes of ischemia (control, 118 ± 6 U/g wet weight; 15-minute ischemia, 110 ± 5 U/g wet weight; 45-minute ischemia, 112 ± 4 U/g wet weight). Fig. 1 shows recovery of CS activity after isolation of SSM and IFM from whole heart homogenates of control hearts subjected to either 15 or 45 minutes of ischemia. With increasing duration of ischemia, recovery of CS activity decreased

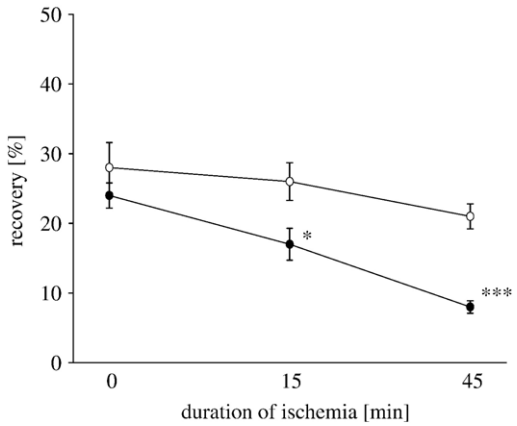

Fig. 1. Recovery of CS activity from isolates of SSM (●) and IFM (○) from homogenate of control hearts subjected to 0, 15, or 45 minutes of ischemia (n = 5). *$P < .05$; ***$P < .001$ vs 0 minute.

significantly in SSM but was less affected in IFM, suggesting loss of SSM during isolation.

Fig. 2 shows state 3 respiration of SSM and IFM of hearts subjected to 15 or 45 minutes of ischemia. The respiratory activity was related to CS as mitochondrial marker enzyme to account for differences in mitochondrial recovery. After 15 minutes of ischemia, state 3 was already reduced in both subpopulations, although the difference was not significant in SSM. After 45 minutes of ischemia, state 3 respiration was almost completely abolished in both subpopulations. The remaining experiments were therefore conducted with 15 minutes of ischemia.

Table 2 shows body weights, heart weights, and heart-to-body weight ratios of control, atrophied, and hypertrophied hearts. The heart-to-body weight ratio decreased with unloading (1 week) and increased with pressure overload (2 weeks).

When we measured total CS activity of homogenates, it was reduced in atrophied hearts (59 ± 5 vs 118 ± 6 U/g wet weight; $P < .001$), and unexpectedly also in hypertrophied hearts (98 ± 6 U/g wet weight; $P < .05$). Table 3 shows

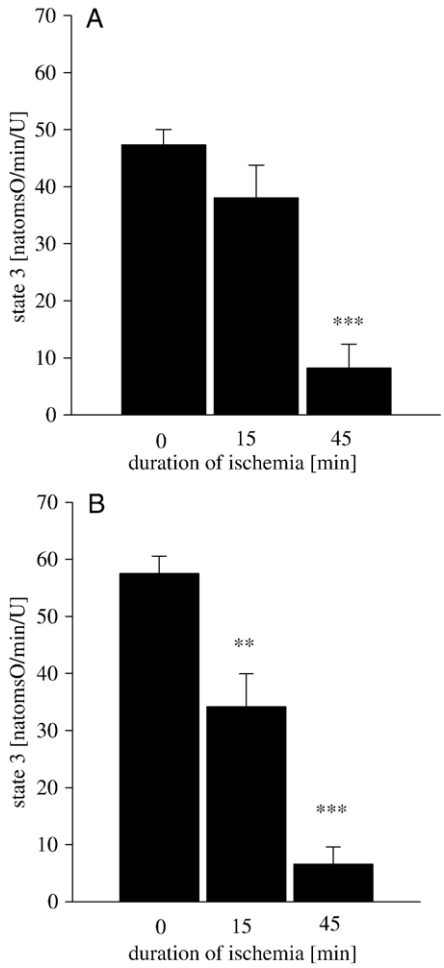

Fig. 2. State 3 respiration (natoms oxygen min^{-1} U^{-1}) of SSM (A) and IFM (B) from homogenates of control hearts subjected to 0, 15, or 45 minutes of total, global, normothermic in vitro ischemia (n = 5). $^{**}P < .01$; $^{***}P < .001$ vs 0 minute.

Table 2
Body weight, heart weight, and heart-to-body weight ratio of rats with control, unloaded, and overloaded hearts (n = 10-15)

	Controls	Unloaded	Overloaded
Body weight (g)	377 ± 5	436 ± 17	266 ± 9
Heart weight (g)	1.00 ± 0.03	0.94 ± 0.07	1.04 ± 0.05
$\frac{\text{Heart weight [g]}}{\text{body weight [kg]}}$	2.64 ± 0.04	2.10 ± 0.17*	3.97 ± 0.26**

* $P < .05$ vs controls.
** $P < .001$ vs controls.

total, latent, and free CS activity and CSRs of SSM and IFM from homogenates of control, atrophied, and hypertrophied hearts with and without 15 minutes of ischemia. As observed in homogenates, SSM and IFM from atrophied hearts showed significantly decreased CS activity compared with controls. Subsarcolemmal and interfibrillar mitochondria from hypertrophied hearts showed only little or no decrease of CS activity compared with controls. High CSR values of SSM or IFM of control, atrophied, and hypertrophied hearts indicate similar structural integrity of the isolates (CS_{latent} as percentage of CS_{total}: SSM, 93%-97%; IFM, 85%-90%).

Fig. 3A and B shows state 3 respiration of SSM and IFM from control, atrophied, and hypertrophied hearts. Because CS activity was significantly different among the groups, we related state 3 to equal volumes of mitochondrial isolate. This was possible because equal amounts of heart tissue were used, the procedure was standardized, and the percentage of recovery of mitochondrial material (as assessed by CS activity) was not different among the groups. Maximal oxygen consumption was significantly reduced in both subpopulations of atrophied hearts and was unchanged in mitochondrial preparations of hypertrophied hearts. Fig. 3C and D shows state 3 respiration of the same experiments as shown in Fig. 3A and B but related to total CS activity. Again, maximal oxygen consumption was significantly reduced in both subpopulations of atrophied hearts, despite the observed decrease in CS activity, suggesting a selective down-regulation of respiratory chain activity. State 3 respiration was unchanged in mitochondrial preparations from hypertrophied hearts.

Table 4 shows state 4 respiration, respiratory control ratios (RCRs), and ADP/oxygen (ADP/O) ratios. State 4 respiration was lowest in control hearts and the RCRs were the highest. Ischemia led to an increase in state 4 and to a decrease in RCR. Unloading and overloading led to an increase in state 4 respiration and a decrease in RCR compared with nonischemic controls. Ischemia did not affect state 4 respiration of unloaded hearts. The decrease in RCR was less pronounced than in controls. Hypertrophied hearts showed a decrease in state 4 but unchanged RCR values after ischemia. The ADP/O ratios were not different among all groups.

Fig. 4 shows the relative expression of complex I (NADH dehydrogenase subunit 2; A and C) and complex

Table 3
Total, free, and latent CS activity and CSR of SSM (A) and IFM (B) from homogenates of control, atrophied, and hypertrophied hearts without and after 15 minutes of ischemia (n = 5-7)

	Total (U/mL)	Latent (U/mL)	Free (U/mL)	CSR
(A) SSM				
Control	18.7 ± 0.5	16.9 ± 0.5	1.8 ± 0.2	9.5 ± 1.0
Control + ischemia	13.5 ± 1.4**	11.4 ± 1.0**	2.0 ± 0.4	6.3 ± 0.8*
Atrophy	8.0 ± 1.0$^{\Phi\Phi\Phi}$	7.0 ± 0.8$^{\Phi\Phi\Phi}$	1.0 ± 0.1	7.2 ± 0.3
Atrophy + ischemia	6.2 ± 0.9	5.3 ± 0.8	0.9 ± 0.0	5.7 ± 0.2**
Hypertrophy	16.1 ± 1.0	13.6 ± 0.8$^{\Phi}$	2.5 ± 0.3	5.9 ± 0.6$^{\Phi\Phi}$
Hypertrophy + ischemia	14.8 ± 0.8	13.3 ± 0.8	1.5 ± 0.1	9.2 ± 0.3***
(B) IFM				
Control	20.4 ± 1.6	19.6 ± 1.5	0.9 ± 0.1	24.8 ± 3.4
Control + ischemia	18.4 ± 1.1	17.6 ± 1.1	0.8 ± 0.0	21.9 ± 1.2
Atrophy	14.0 ± 1.1$^{\Phi\Phi}$	13.1 ± 1.0$^{\Phi\Phi}$	0.9 ± 0.1	15.0 ± 0.8$^{\Phi}$
Atrophy + ischemia	13.8 ± 0.9	12.9 ± 0.9	0.9 ± 0.0	14.4 ± 1.2
Hypertrophy	16.5 ± 0.6$^{\Phi}$	15.6 ± 0.6$^{\Phi}$	0.9 ± 0.1	17.0 ± 1.1$^{\Phi}$
Hypertrophy + ischemia	21.6 ± 1.5**	20.5 ± 1.4**	1.2 ± 0.1	17.8 ± 1.0

$^{\Phi}$ $P < .05$, $^{\Phi\Phi}$ $P < .01$, $^{\Phi\Phi\Phi}$ $P < .001$, all vs control.
* $P < .05$, ** $P < .01$, *** $P < .001$, all vs without ischemia.

IV (cytochrome *c* oxidase IV; B and D) of the respiratory chain in atrophied and hypertrophied hearts compared with controls. The expression pattern paralleled the pattern of state 3 respiration, that is, unloading was associated with a down-regulation of respiratory chain genes (although not quite significant for complex IV), whereas overloading showed no changes in expression of these genes.

Subjecting control, atrophied, and hypertrophied hearts to 15 minutes of ischemia did not change total CS activity of whole heart homogenates (control, 110 ± 5 U/g wet weight; atrophy, 64 ± 7 U/g wet weight; hypertrophy, 100 ± 5 U/g wet weight). As demonstrated in Table 3, recovery of total CS activity in SSM was decreased after ischemia in all groups. The decrease reached significance in the control group only.

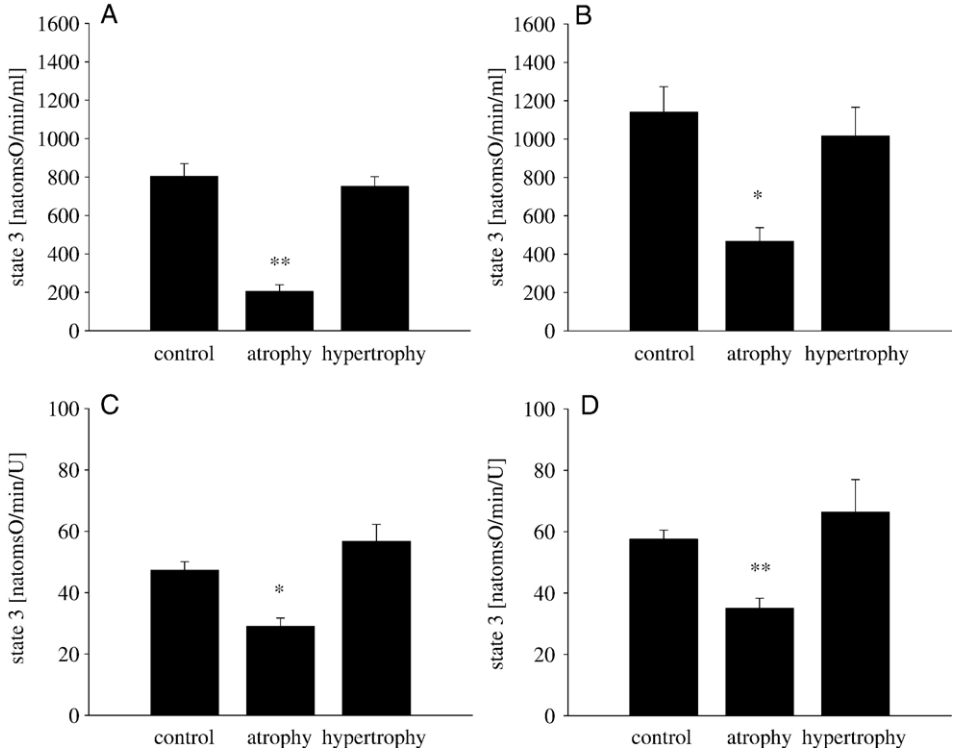

Fig. 3. State 3 respiration of subsarcolemmal (A, C) and interfibrillar mitochondria (B, D) from homogenates of control, atrophied, and hypertrophied hearts related to either 1 mL of mitochondrial isolate (A, B) or CS activity (C, D) (n = 5-7). *$P < .05$, **$P < .01$; all vs control.

Table 4
State 4 respiration (natoms oxygen min^{-1} U CS $^{-1}$), RCR, and ADP/O ratio of SSM and IFM from homogenates of control, atrophied, and hypertrophied hearts without and after 15 minutes of ischemia (n = 5-7)

	SSM			IFM		
	State 4	RCR	ADP/O	State 4	RCR	ADP/O
Control	3.9 ± 0.6	12.8 ± 1.4	2.4 ± 0.1	5.6 ± 0.8	10.8 ± 0.9	2.1 ± 0.1
Control + ischemia	25.1 ± 4.8**	2.1 ± 0.4***	2.1 ± 0.2	20.1 ± 3.1**	1.9 ± 0.4***	2.1 ± 0.2
Atrophy	7.6 ± 0.9†	3.9 ± 0.3††	1.9 ± 0.1	9.7 ± 1.0	3.6 ± 0.1††	1.7 ± 0.1
Atrophy + ischemia	10.4 ± 1.3	2.9 ± 0.3*	2.1 ± 0.1	13.0 ± 0.9*	2.2 ± 0.2***	2.2 ± 0.3
Hypertrophy	10.4 ± 1.0††	5.7 ± 0.6††	2.1 ± 0.1	11.9 ± 1.2	5.8 ± 0.9†	2.1 ± 0.1
Hypertrophy + ischemia	6.1 ± 0.3*	4.6 ± 0.6	2.2 ± 0.1	7.0 ± 0.9*	6.0 ± 1.1	2.1 ± 0.1

* $P < .05$ vs without ischemia.
** $P < .01$ vs without ischemia.
*** $P < .001$ vs without ischemia.
† $P < .01$ vs control.
†† $P < .001$ vs control.

Recovery of CS activity after ischemia was unaffected in IFM of control and atrophied hearts, but was increased in hypertrophied hearts (+31%, $P < .05$).

Fig. 5A shows state 3 respiration of SSM and IFM of control, atrophied, and hypertrophied hearts before and after 15 minutes of ischemia, related to total CS activity. As shown above, state 3 respiration after ischemia of control hearts was slightly reduced in SSM (not significant), but was significantly reduced in IFM. In atrophied hearts, state 3 respiration was not affected by ischemia in SSM and only slightly reduced in IFM (not significant). State 3 respiration of hypertrophied hearts was significantly decreased in SSM and only slightly affected in IFM. The percent changes of the mean values displayed on top of the plots indicate that isolated mitochondria from atrophied hearts show the smallest changes after 15 minutes of ischemia. Subsarcolemmal mitochondria of hypertrophied hearts showed the most pronounced decrease in maximal oxygen consumption

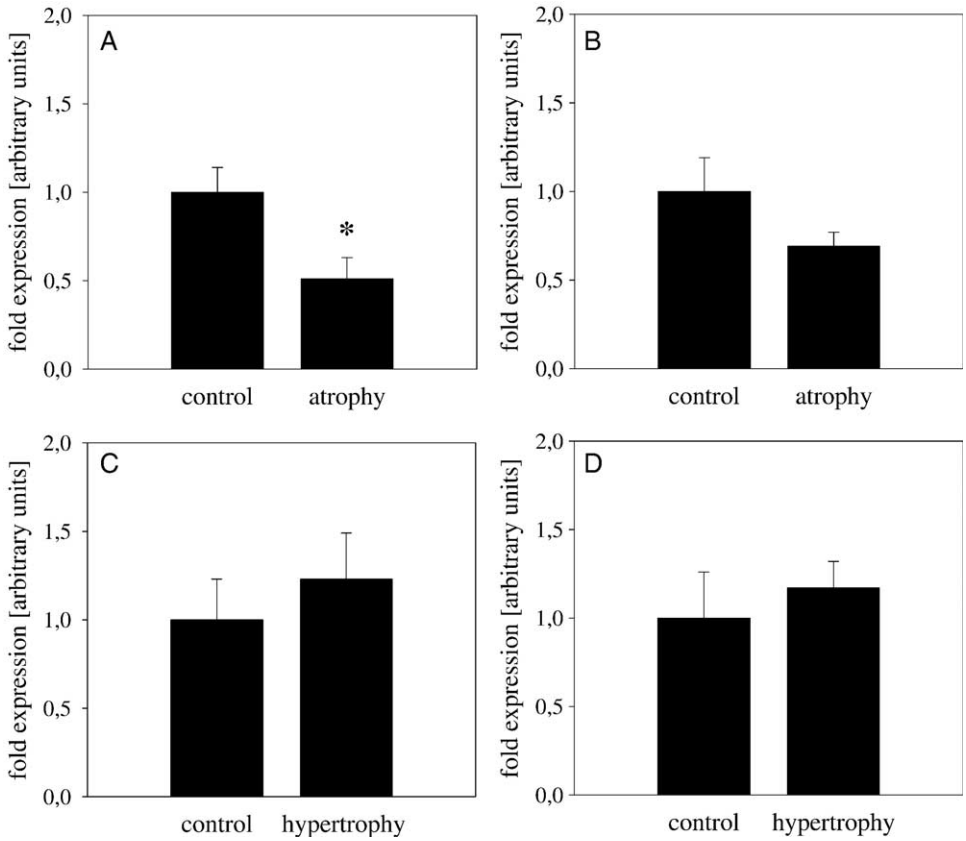

Fig. 4. Expression of NADH dehydrogenase subunit2 (A, C) and cytochrome-*c* oxidase IV (B, D) in atrophied (A, B) and hypertrophied hearts (C, D). *$P < .05$ vs control.

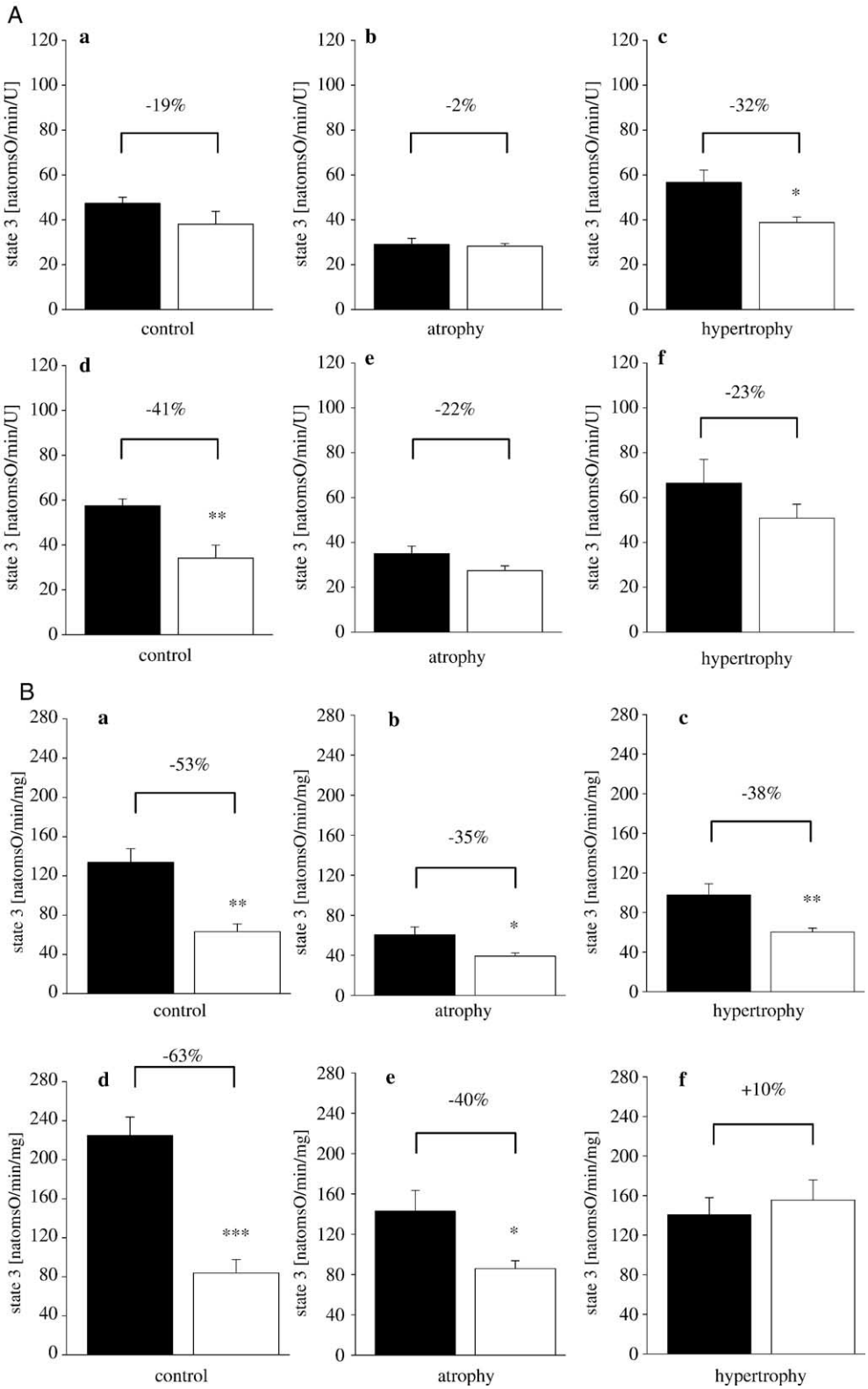

Fig. 5. A, State 3 respiration (natoms oxygen min^{-1} U^{-1}) of SSM (a-c) and IFM (d-f) isolated from homogenates of control, atrophied, and hypertrophied hearts before ischemia (■) and after 15 minutes of ischemia (□) (n = 5-7). B, State 3 respiration (natoms oxygen min^{-1} mg^{-1}) of SSM (a-c) and IFM (d-f) isolated from homogenates of control, atrophied, and hypertrophied hearts before ischemia (■) and after 15 minutes of ischemia (□) (n = 5-7). *$P < .05$, **$P < .01$, *** $P < .001$; all vs before ischemia (■).

Table 5
Protein content (mg/mL) of SSM and IFM of control, atrophied, and hypertrophied hearts without and after 15 minutes of ischemia (n = 5-7)

	SSM	IFM
Control	6.1 ± 0.4	5.0 ± 0.3
Control + ischemia	6.9 ± 0.8	7.1 ± 0.6**
Atrophy	3.4 ± 0.4†††	3.5 ± 0.6†
Atrophy + ischemia	3.8 ± 0.4	4.2 ± 0.4
Hypertrophy	8.1 ± 0.8†	7.2 ± 0.7††
Hypertrophy + ischemia	6.2 ± 1.1	5.4 ± 1.0*

* $P < .05$ vs without ischemia.
** $P < .01$ vs without ischemia.
† $P < .05$ vs control.
†† $P < .01$ vs control.
††† $P < .001$ vs control.

after 15 minutes of ischemia. In contrast, IFM of hypertrophied hearts showed a smaller decrease after 15 minutes of ischemia than IFM of control hearts after ischemia.

Because differences in CS activity among the groups could mask or exaggerate differences in state 3 respiration, we also related state 3 to mitochondrial protein content (Fig. 5B). Although isolated mitochondrial subpopulations can be contaminated by nonmitochondrial proteins, and despite the fact that protein contents were significantly different among groups (Table 5), the pattern of state 3 respiration without ischemia or after ischemia was similar (ie, less decrease in state 3 of atrophied hearts after ischemia).

4. Discussion

There are 3 major findings of this study. (1) Long-term changes in workload of the heart differentially affect maximal respiratory capacity and ischemia tolerance of isolated mitochondria. The changes are not parallel to the changes in energy requirements. (2) Mitochondria of atrophied hearts exhibit a greater resistance to ischemia than control hearts. (3) We also identified a methodological shortcoming that has to be taken into account when differential centrifugation is used for isolating mitochondria in the setting of ischemia reperfusion.

Because of increased energy demand with overloading and the decreased energy demand with unloading we expected to find parallel changes in the maximal respiratory capacity of the ATP-producing mitochondria. Although this is the case in atrophy, long-term pressure overload did not result in increased maximal respiratory capacity of the mitochondria. This finding is unexpected. The increase in energy demand in our model is significant. Hearts are exposed to a pressure gradient of more than 100 mm Hg (unpublished observations, 2005), that is, a total systolic pressure of about twice the normal value. Thus, to provide the heart with the required ATP for muscle contraction the mitochondria are likely to exploit a greater fraction of their maximal respiratory capacity in vivo. This conclusion is supported by our finding that the expression of subunits of complexes I and IV of the respiratory chain paralleled state 3 respiration, both in atrophy and hypertrophy. In other words, the results suggest that the amount of respiratory chain enzymes is not increased in hypertrophy. If this conclusion is correct, it is tempting to speculate that a lack of increased respiratory capacity may be involved in the patho-mechanism of pathologic hypertrophy and possibly in the onset of heart failure. Because the maximal respiratory capacity is very difficult to assess in vivo, this hypothesis cannot be easily tested, but it gives rise to a host of questions that will have to be addressed in this context. One of them is affecting the ischemia tolerance of these hearts and the impact of ischemia on respiratory capacity.

Myocardial ischemia affects respiratory function of isolated mitochondria. Here, we demonstrate decreased state 3 respiration of IFM, but not SSM, after 15 minutes of ischemia and nearly deleted state 3 after 45 minutes of ischemia in both subpopulations. Although 15 minutes of ischemia allow full recovery of contractile function after reperfusion, elongation of the ischemic period leads to decreases of functional recovery [31]. Twenty minutes of ischemia may already result in irreversible cell damage or cell death [32]. These results are supported by the severe depression of ADP-stimulated respiratory activity in isolated mitochondria after 45 minutes of in vitro ischemia. We therefore chose a period of 15 minutes of ischemia to compare the respiratory capacity of isolated mitochondria of mechanically unloaded or overloaded hearts to controls.

Mechanical unloading resulted in heart atrophy, and isolated mitochondria from atrophied hearts were characterized by decreased respiratory function. However, 15 minutes of ischemia in these hearts resulted in a less pronounced decrease in state 3 rates in both SSM and IFM. Because percent changes of state 3 respiration after ischemia were smaller in each subpopulation in atrophied hearts compared with controls, resistance to ischemia-induced impairment of state 3 respiration may be higher in atrophied hearts. This conclusion is consistent with the fact that atrophied hearts are characterized by an expression pattern similar to a fetal genotype [1], and fetal hearts are more resistant to ischemic damage than adult hearts [11-15]. Therefore, we can speculate that the reexpression of a fetal genotype may be involved in the ability of atrophied hearts to withstand ischemic damage.

This speculation is further supported by our previous finding of a substrate switch in unloaded hearts from fatty acid to glucose oxidation [24], a mechanism considered cardioprotective [33]. Others have demonstrated that changes in myocardial substrate utilization can affect contractile recovery after ischemia [34-37]. Such a benefit may be the consequence of more oxygen-efficient ATP production when preferentially using glucose as a substrate instead of fatty acids or the consequence of less lactate and H^+ accumulation by improved coupling of glycolysis to glucose oxidation [38]. Because unloaded hearts also shift from fatty acids to glucose as preferred oxidative substrate, the resistance to an impairment in mitochondrial function during ischemia in

these hearts may be the consequence of one or both of the above-mentioned mechanisms.

We demonstrate a significant decrease in state 3 respiration after ischemia in SSM of hypertrophied hearts compared with SSM of control hearts, but no significant change in state 3 in IFM of hypertrophied hearts compared with controls. The RCR is lower in hypertrophied hearts, but less affected by ischemia. The results suggest a differential effect of hypertrophy on ischemia tolerance of isolated mitochondrial subpopulations. Others have suggested an increased susceptibility of hypertrophied hearts to ischemia [3-9]. However, the lesser impact of ischemia on state 3 of IFM in hypertrophied hearts may be because of specifics of the isolation procedure. It is conceivable that the hypertrophied myocardium provides better protection against the shear forces of the isolation procedure for IFM than SSM. Thus, IFM may be damaged less during the isolation procedure and the impact of ischemia may be underestimated. Although we cannot exclude this possibility completely, the stable CSR (as marker for structural integrity) in IFM and even an increased CSR in SSM after ischemia (compared with before) argue against it.

State 4 respiration was lowest in control hearts and increased several fold after ischemia. Mitochondria from unloaded hearts showed higher state 4 respiration than controls, but the ischemia-induced increase in state 4 respiration was much less pronounced compared with controls. State 4 respiration is thought to be an indicator of uncoupled mitochondrial respiration. According to this belief, ischemia would lead to significant mitochondrial uncoupling in control hearts, unloaded hearts would exhibit uncoupling compared with controls, and the ischemia-induced uncoupling in control hearts would be less pronounced in unloaded hearts. However, the unchanged ADP/O ratios would argue against this conclusion, and because of the changes in state 3 respiration in the nonischemic groups, conclusions should be drawn with caution. In addition, it has also to be considered that state 4 respiration alone is not suitable to reliably assess mitochondrial uncoupling. Measurements of ATP synthesis, membrane potential, and uncoupling protein activity, for example, are additionally used to assess mitochondrial uncoupling. Several different methods need to be used if this parameter is to be assessed reliably. Although some of those experiments are quite difficult to perform, as supported by the variety of different results in the literature in equally designed studies, investigation of uncoupling was beyond the scope of this study.

In addition to the potential impact of the animal model (hypertrophy) on the isolation procedure, another method-specific factor is likely to affect the results and therefore requires discussion. We found decreased recovery of CS activity in SSM but not IFM from control hearts after 15 or 45 minutes of ischemia. This phenomenon has been observed before and appears to be the consequence of damage by ischemia and/or the crude isolation procedure [21]. Subsarcolemmal mitochondria are located beneath the sarcolemma and therefore more exposed and less protected against extracellular shear stress than IFM. After ischemia, the susceptibility of SSM to damage may be higher than under normal conditions. Thus, structurally damaged SSM may not obey the same acceleration forces than normal SSM during differential centrifugation, which leads to a loss of CS activity during the isolation procedure. Thus, SSM may undergo a selection for structurally intact mitochondria during the isolation process of mitochondria from hearts subjected to ischemia. Therefore, respiratory rates may be higher in these preparations than in IFM, which do not show any changes in recovery of CS activity and therefore may represent the average respiratory function of all IFM. As mentioned above, IFM may be more protected from the rough isolation procedure by their location between the myofibrils and thus were less damaged than SSM. Taken together, SSM may be more susceptible to ischemia than indicated by the measurements of state 3 after differential centrifugation because of their relatively unprotected and exposed position.

We conclude that (1) long-term changes in workload differentially affect maximal respiratory capacity and ischemia tolerance of isolated mitochondria. The changes are not parallel to the changes in energy requirements, and mitochondria from hypertrophied hearts may need to exploit a greater fraction of their maximal ATP-producing capacity in vivo. (2) Mitochondria of atrophied hearts appear to exhibit a greater resistance to ischemia than normotrophic hearts. (3) A selection phenomenon of SSM has to be taken into account when this technique is used to isolate mitochondria in the setting of ischemia reperfusion.

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