CALL FOR PAPERS | Mitochondria in Cardiovascular Physiology and Disease

Impaired mitochondrial function in chronically ischemic human heart

Nis Stride,1 Steen Larsen,1 Martin Hey-Mogensen,1 Christina N. Hansen,1 Clara Prats,1 Daniel Steinbrüchel,2 Lars Køber,3 and Flemming Dela1

1Xlab, Center for Healthy Aging, Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; 2Department of Cardiothoracic Surgery, University of Copenhagen, Copenhagen, Denmark; and 3Department of Cardiology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

Submitted 31 December 2012; accepted in final form 26 March 2013

Stride N, Larsen S, Hey-Mogensen M, Hansen CN, Prats C, Steinbrüchel D, Kober L, Dela F. Impaired mitochondrial function in chronically ischemic human heart. Am J Physiol Heart Circ Physiol 304: H1407–H1414, 2013. First published March 29, 2013; doi:10.1152/ajpheart.00991.2012.—Chronic ischemic heart disease is associated with myocardial hypoperfusion. The resulting hypoxia potentially inflicts damage upon the mitochondria, leading to a compromised energetic state. Furthermore, ischemic damage may cause excessive production of reactive oxygen species (ROS), producing mitochondrial damage, hereby reinforcing a vicious circle. Ischemic preconditioning has been proven protective in acute ischemia, but the subject of chronic ischemic preconditioning has not been explored in humans. We hypothesized that mitochondrial respiratory capacity would be diminished in chronic ischemic regions of human myocardium but that these mitochondria would be more resistant to exposure to a hypoxic milieu ex vivo whenever ischemia and, second, that ROS generation would be higher in ischemic myocardium. The aim of this study was to test mitochondrial respiratory capacity during hyperoxia and hypoxia, to investigate ROS production, and finally to assess myocardial antioxidant levels. Mitochondrial respiration in biopsies from ischemic and nonischemic regions from the left ventricle of the same heart was compared in nine human subjects. Maximal oxidative phosphorylation capacity in fresh muscle fibers was lower in ischemic compared with nonischemic myocardium (P < 0.05), but the degree of coupling (respiratory control ratio) did not differ (P > 0.05). The presence of ex vivo hypoxia did not reveal any chronic ischemic preconditioning of the ischemic myocardial regions (P > 0.05). ROS production was higher in ischemic myocardium (P < 0.05), and the levels of antioxidant protein expression was lower. Diminished mitochondrial respiration capacity and excessive ROS production demonstrated an impaired mitochondrial function in ischemic human heart muscle. No chronic ischemic preconditioning effect was found.

Address for reprint requests and other correspondence: F. Dela, Xlab, Ctr. for Healthy Aging, Dept. of Biomedical Sciences, Faculty of Health & Medical Sciences, Univ. of Copenhagen, Blegdamsvej 3b, 2200 Copenhagen N, Denmark (e-mail: fdela@sund.ku.dk).http://www.ajpheart.org 0363-6135/13 Copyright © 2013 the American Physiological Society H1407

CHRONIC ISCHEMIC HEART DISEASE (IHD) is caused by progressive atherosclerosis of the coronary arteries, leading to regional hypoperfusion of the myocardium. This results in local hypoxia that potentially limits ATP production from the processes of oxidative phosphorylation (OXPHOS) in mitochondria (4, 8, 12). Furthermore, acute ischemia and reperfusion causes mitochondrial dysfunction and subsequent excessive production of reactive oxygen species (ROS) by the electron transport chain (ETC) (23). Blockade of complex I or III in the ETC decreases ROS production during episodes of ischemia and helps protect the mitochondria against ischemic damage (2, 9), whereas blockade at a step distal of complex III [site of cytochrome c oxidase (COX)] has been shown to increase ROS generation (9). Traditionally (5), complexes I and III are regarded as the primary sources of ROS (within the ETC), but recently complex II has also been shown to produce ROS, at least in skeletal muscle (31). The deleterious actions of ROS include damage to the mitochondrial membrane constituents (lipids and proteins) and mtDNA (28), opening of mitochondrial permeability transition pores, resulting in mitochondrial depolarization, cytochrome c loss, and apoptosis (11). These processes damage myocardial structure and may ultimately lead to cardiomyopathy with a compromised cardiac function. The majority of studies in this field have explored the effect of acute episodes of ischemia and reperfusion, whereas the effect of chronic ischemia has only been marginally investigated in animals and to our knowledge not at all in humans. The mitochondrial effects of chronic ischemia have been assessed in swine (16, 21) and rats (4, 12), and an impaired energetic state has been found, along with mitochondrial structural damage and a compromised OXPHOS capacity in the affected myocardial regions. However, one study reported a preserved OXPHOS capacity but decreased respiratory control ratio (RCR) interpreted as mild uncoupling of the mitochondria (21). Chronic ischemia is thought to produce regions of viable “hibernating” cardiac myocytes with the potential to regain some degree of mitochondrial and contractile function upon reestablishment of sufficient oxygen provision, i.e., via coronary angioplasty bypass grafting (17). In two of the animal studies mentioned above, a protective “chronic preconditioning effect” was reported. Chronic ischemic mitochondria became more resistant to subsequent episodes of acute ischemia (16, 21), as illustrated by a less dramatic decrease in OXPHOS capacity and higher levels of ATP upon an experimental ischemic insult. These animal studies have provided a “proof of concept,” but whether this effect of chronic ischemic priming of the mitochondria in the myocardium is applicable in the human myocardium is not known.

We hypothesized that OXPHOS capacity would be reduced in the ischemic regions of the heart compared with the well-perfused regions, because of alterations in the kinetic properties of COX which may lead to impaired electron handling and excessive ROS generation within the ETC, upstream of COX.

Second, we hypothesized that mitochondria within the ischemic regions would be chronically preconditioned and that these would be more resistant to exposure to a hypoxic milieu ex
vivo. We tested these hypotheses in paired biopsies from human hearts, comparing ischemic and nonischemic myocardium in nine patients undergoing coronary artery bypass grafting (CABG) surgery.

METHODS

Ethical approval. The protocol was approved by The Danish National Committee on Biomedical Research Ethics (prot. 2008-7041-132), and informed consent was obtained from all participants. The study was conducted in agreement with The Declaration of Helsinki.

Study group. Nine patients undergoing elective CABG, with or without additional valve surgery, were included in the study. The patients were all diagnosed with chronic IHD upon referral established by coronary angiography. Demographic data and data on medication status were obtained from the medical file. Echocardiography and coronary angiography were performed in all patients as a part of diagnostic work-up, and these analyses were used in assessment of left ventricular ejection fraction and angiographic data (Table 1).

Surgical procedure. Patients were premedicated with oral triazolam (0.125–0.250 mg), and anesthesia was induced with midazolam (0.06–0.1 mg/kg), fentanyl (10–15 μg/kg), and pancuronium (0.1 mg/kg), supplemented with propofol (0.5–1.0 mg/kg) if necessary. After endotracheal intubation, patients were mechanically normovolemitated and a central venous catheter was inserted. Anesthesia was maintained with sevoflurane, propofol, and repeated doses of fentanyl when needed. Venous blood samples were drawn peroperatively before cardiopulmonary bypass for determination of serum creatinine. NYHA, New York Heart Association class; CCS, Canadian Cardiovascular Society class (I–IV); HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 1. Patient demographics, medication and plasma biochemistry

<table>
<thead>
<tr>
<th>Demographics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
</tr>
<tr>
<td>Age, yr</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>Sex, men/women</td>
<td>5/4</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.5 ± 1.5</td>
</tr>
<tr>
<td>NYHA, score 1–4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>CCS Class</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>51.8 ± 3.2</td>
</tr>
<tr>
<td>Diabetes mellitus type 2, n</td>
<td>4</td>
</tr>
<tr>
<td>Number of affected vessels, 1/2/3</td>
<td>1</td>
</tr>
<tr>
<td>Medication, n</td>
<td>5</td>
</tr>
<tr>
<td>Ace inhibitor</td>
<td>2</td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>2</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>3</td>
</tr>
<tr>
<td>Loop diuretics</td>
<td>2</td>
</tr>
<tr>
<td>Statins</td>
<td>4</td>
</tr>
</tbody>
</table>

Plasma biochemistry

<table>
<thead>
<tr>
<th>Cholesterol, mmol/l</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>HDL</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>LDL</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Nonesterified fatty acids, μmol/l</td>
<td>689 ± 104</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>Creatinum, mmol/l</td>
<td>72 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE and are fasting plasma concentrations, except for creatinum. NYHA, New York Heart Association class; CCS, Canadian Cardiovascular Society class (I–IV); HDL, high-density lipoprotein; LDL, low-density lipoprotein. Biopsies were taken immediately after completed cardioplegia infusion. Transmural 1 millimeter True-cut tissue specimens were obtained from both ischemic and nonischemic areas of the myocardium of the left ventricle near the endocardial surface. The tissue defect was closed by a single stitch prolene 5-0. Selection of target areas was guided from the coronary angiogram, and ischemic biopsies were taken in the proximity of a stenosed artery without significant collateral perfusion, whereas the nonischemic biopsies were taken from areas supplied from a persistent coronary artery.

Tissue preparation. Myocardium was instantly submerged into ice-cold buffer solution [BIOPS (29)] containing (in mmol/l) 2.77 Ca(K)₂EGTA, 7.23 K₂EGTA, 20 imidazole, 20 taurine, 6.56 MgCl₂, 5.77 ATP, 15 phosphocreatine, 0.5 dithiothreitol, and 50 4-morpholineethanesulfonic acid at pH 7.1. Samples were quickly transferred to the laboratory where connective tissue was removed and myocardium was snap frozen in liquid N₂ for biochemistry analyses in a subset of seven patients (because of limited tissue availability in 2 patients). Time from biopsy to freezing was 10–15 min.

High-resolution respirometry. A part (2–4 mg) of the biopsy was prepared for measurements of respiration via high-resolution respirometry using the permeabilized fiber technique (18) in BIOPS on ice. The fibers were first carefully separated by mechanical dissection with sharp forceps using a stereomicroscope at low magnification. The tissue was then permeabilized by gentle agitation for 30 min at 0°C in BIOPS, supplemented with 50 μg/ml saponin and then washed in ice-cold respiration medium [Mir05 (29)] 2 × 10 min during gentle agitation. Mir05 contains of 110 mmol/l sucrose, 60 mmol/l K-lactobionate, 0.5 mmol/l EGTA, 1 g/l BSA essentially fatty acid free, 3 mmol/l MgCl₂, 20 mmol/l taurine, 10 mmol/l KH₂PO₄, and 20 mmol/l K-HEPES at pH 7.1. The O₂ solubility of Mir05 was set at 10.5 μM/kPa. Respiration was measured at 37°C (Orboros, Oxygen; Innsbruck, Austria). Data acquisition and analyses were performed using DatLab software (Orboros). The absolute respiratory rates (oxygen fluxes) were expressed as picoamoles oxygen per milligram of wet weight per second. RCR was calculated as the ratio between the respiratory states 3 and 2. Two respirometry protocols were performed on tissue from ischemic and nonischemic regions in parallel.

Protocol A was carried out both at hyperoxia ([O₂], 450–200 μmol/l) and hypoxia ([O₂], 90–60 μmol/l). Baseline was obtained after addition of tissue in absence of substrates. State 2 respiration was reached with addition of malate and glutamate (GM₅) at 5 and 10 mmol/l, respectively. ADP addition (5.0 mmol/l) stimulated state 3 respiration (GM₆). The integrity of the outer mitochondrial membrane was tested by addition of cytochrome c (10 μmol/l). An increase in oxygen consumption rate was interpreted as a sign of outer mitochondrial membrane disruption. With succinate (10 mmol/l) complex I and II, sustained respiration was assessed. Isolated complex-II contribution was determined after inhibition of complex I with rotenone (0.5 μmol/l) and complex IV (COX) capacity was measured with N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD) + ascorbate (0.5 + 2 mmol/l) after inhibition of complex III with antimycin A (2.5 μmol/l). The oxygen consumption ascribed to autoxidation of this substrate was subtracted.

Protocol B was performed at hyperoxic conditions and was identical to protocol A until reaching state GM₆. Hereafter complex IV was subsequently inhibited via stepwise additions of Na-azide in concentrations of 0.05, 0.25, 0.5, 1, 2, 5, 10, 20, 25 mmol/l. All protocols were carried out in duplicate. Furthermore, data from respirometric measurements at hyperoxic conditions in protocol A are based on fourfold measures up to state GM₆ because the addition of substrates was similar in protocols A and B until this step.

The high oxygen tension within protocols A and B was chosen to avoid limitations to OXPHOS because of poor O₂ availability and diffusion artifacts. This oxygen concentration is widely used in high-resolution respirometry (18). The oxygen concentration in the hypoxic experiment within protocol A (60–90 μmol/l) converts into
partial pressures of oxygen around 45–70 mmHg, the low level of which corresponds with in vivo measurements in human left ventricle in patients after CABG surgery (3). The data on mitochondrial respiration are shown as mass-specific oxygen flux (i.e., per mg tissue) and normalized to mitochondrial content, as estimated by citrate synthase activity (CS) content (19).

ROS generation. Hydrogen peroxide (H$_2$O$_2$) release (measured as fluorescence/min, and expressed as arbitrary units) was measured in the saponin skinned fibers with Amplex Red (Molecular Probes) as a trap of H$_2$O$_2$, catalyzed by horseradish peroxidase. Superoxide release is converted to H$_2$O$_2$ by addition of exogenous superoxide dismutase (SOD). H$_2$O$_2$ reacts with Amplex Red with a 1:1 yield generating the fluorescent compound resorufin which is stable once formed. Fluorescence was continuously measured with a spectrofluorometer equipped with temperature control and stirring (wavelengths, excitation of 560 nm and emission 590 nm) (SAFAS Xenius, Monaco). Skinned fibers (≈1 mg) were added to the measurement buffer consisting of 0.05 mmol/l Mir05 + Amplex Red, 12 U/ml horseradish peroxidase, and 90 U/ml SOD, and sequential substrate addition was performed (final concentrations identical to respirometry protocol): malate + glutamate, ADP, oligomycin (2 μg/ml), succinate, rotenone, and antimycin A. H$_2$O$_2$ release was determined in tissue from both the ischemic and nonischemic region in parallel and in double measurements. The ROS data were normalized to mitochondrial content by CS activity a surrogate measure of mitochondrial content.

Western blot analyses. Approximately 10 mg of frozen myocardium (n = 7) was homogenized in ice-cold phosphate buffer (0.3 mol/l). Triton X-100 was added immediately after to a final concentration of 0.1%. Phosphatase inhibitors were added to final concentrations of (in mmol/l) 20 β-glycerophosphate (Sigma), 10 pyrophosphate (Sigma), 2 Na-ortovanadate (Sigma), and 2.5 PMSF. Mini-Complete Protease inhibitor tablet was added according to the manufactures recommendation (Roche). Samples were incubated for 5 min at room temperature and subsequently centrifuged for 10 min at 13,000 g. The pellet was discarded. From each sample 5–15 μg of total protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with the following antibodies (manufacturer, order number; source; clonality): ETC complexes I–V (Mitosciences; MS601; mouse; mono), glutathion peroxidase 1 (GPX1) (Cell Signaling; 3286; rabbit; poly), Vascular endothelial growth factor (VEGF) (AbCam; Ab46154; rabbit; poly), coenzyme Q-binding protein COQ10 homolog B (COQ10B) (AbCam Coenzyme Q10 homolog B antibody; Ab41997;rabbit; poly), Catalase (RnDSystems; AF3398, goat), Manganese superoxide dismutase (MnSOD) (Millipore; 06-984, rabbit, poly). COQ10B is not coenzyme Q10, but it is essential for the function of coenzyme Q10 in regard to mitochondrial respiration (1).

Secondary antibodies labeled with horseradish peroxidase (P0447, P0448, and P0449, Dako) were used for detection and visualized by enhanced chemiluminescence (RPN2209, GE Healthcare) using a CCD system (LAS4000, software version 4.00.0003, GE Healthcare). The bands were quantified using ImageQuant TL software (v. 7.0.1.0, GE Healthcare). Ischemic and nonischemic samples were loaded in an alternating pattern to compensate for regional variation in transfer efficiency. Protein expression was measured relative to an external control, included in three lanes on all gels. Representative blots are shown in Fig. 1.

Enzyme activities. Maximal CS and hydroxy-acyl-dehydrogenase (HAD) activity was measured in a reaction coupled to conversion of NAD$^+$ to NADH. NADH production was assessed spectrophotometrically by determination of NADH changes at 340 nm at 37°C, pH 7.0, in myocardial lysates (1 μg/μl protein), using an automatic analyzer (Hitachi automatic analyzer 912; Boehringer, Mannheim, Germany). CS activity was measured with acetyl-CoA and oxaloacetate as substrate and 3-hydroxyacyl-CoA-dehydrogenase (HAD) was measured with acetoacetyl-CoA as substrate. Enzyme activity was measured in duplicates.

Hydroxyproline assay. Hydroxyproline content was determined from the Western blot homogenate. Aliquots equal to 75 μg of total protein were dried at 100°C on a heating block. Samples were hydrolyzed with HCl (6.0 mol/l) for 20 h at 100°C and hereafter dried and washed three times. The dry, acid-free samples were dissolved in 150 μl buffer containing (in %) 0.6 acetic acid, 6 sodium acetate, 2.5 citric acid, and 1.7 sodium hydroxide (pH 6.0) and sonicated for 30 min. Chloramine T (75 μl; 0.05 mol/l) was added, and samples were incubated 20 min at room temperature. Perchloric acid (75 μl; 15.4%) and 4-dimethyl amino benzaldehyde (15%) in 1-propanol was added, and samples were incubated for 20 min at 60°C. The absorbance was measured spectrophotometrically at 560 nm and quantified via a standard curve made from known concentrations of 1-hydroxyproline (13). The calculation of collagen content was made from the assumption that hydroxyprolin constitutes 15% of total collagen in cardiac tissue.

Plasma cholesterol, lipids, and glucose. NEFA concentration was measured using a commercially available kit (NEFA-C test; Wako Chemical, Neuss, Germany). Fasting plasma triacylglycerol and glucose and nonfasting creatinin values were obtained from the medical file within 1 wk before surgery. These were analyzed on a Roche Modular system P, (Roche, Basel, Switzerland) using standard applications.

Statistics. All comparisons on mitochondrial function (respirometry and ROS production) were performed using a two-way ANOVA for repeated measurements followed by a post hoc test (Holm-Sidak method). For other comparisons, paired student t-tests was applied. A P value < 0.05 was considered significant in two-tailed testing. Data are expressed as means ± SE unless otherwise stated.
RESULTS

Myocardial OXPHOS capacity in ischemic vs. nonischemic region. Parallel mitochondrial respiration measurements revealed lower OXPHOS capacity per mitochondrion (CS activity) in the myocardium from ischemic regions compared with nonischemic regions during hyperoxic conditions ($P < 0.05$, Fig. 2). Representative trace is shown in Fig. 3. The difference was significant with combined complex I- and II-linked respiration. Inhibition of complex I with rotenone to assess electron transport through complex II alone also showed a significant difference in oxygen consumption between ischemic and nonischemic tissue ($P > 0.05$, Fig. 2). To mimic the effect of acute myocardial ischemia, parallel measurements were carried out in hypoxic conditions, thereby introducing an oxygen limitation to mitochondrial respiration. At this low oxygen concentration, OXPHOS capacity was clearly limited and regional ischemic conditions inside the permeabilized muscle fibers were assumed to be present. This ex vivo hypoxia resulted in diminished differences between ischemic and nonischemic myocardium so that the significant differences between the ischemic and the nonischemic regions disappeared (Fig. 2). The ratio between hypoxic and hyperoxic oxygen consumption did not differ between regions (Fig. 2), showing no evidence of less ex vivo ischemic susceptibility in mitochondria from chronically in vivo preconditioned myocardium. Mitochondrial RCR, indicating the tightness of the coupling between respiration and phosphorylation, was not different between the hyperoxic and the hypoxic condition neither in the ischemic (2.9 ± 0.1 and 3.2 ± 0.3) nor in the nonischemic (2.5 ± 0.1 and 2.8 ± 0.3) tissue, respectively. Furthermore, the RCR was similar between ischemic and nonischemic tissue.

The integrity of the outer mitochondrial membrane was tested by addition of exogenous cytochrome c. The elicited stimulation of respiration is an indicator of the extent of outer mitochondrial membrane disruption since a surplus of cytochrome c is normally present in the mitochondrial intramembranous space. Exogenous cytochrome c increased complex I-linked respiration by 11 ± 5 and 6 ± 3% in ischemic and nonischemic myocardium ($P < 0.05$), respectively. The capacity of complex IV of the respiratory chain, COX, was evaluated.

Fig. 2. Mitochondrial respiration in human ischemic and nonischemic myocardium. Baseline, no substrate or ADP added; complex I (CI), glutamate + malate + ADP; complex I + II (CI + II), complex I substrates + succinate; complex II (CII), inhibition of complex I with rotenone; residual oxygen consumption (ROX), oxygen flux after inhibition of the electron flux by inhibition of complex III with antimycin A. Respiratory rates per milligram tissue during hyperoxia (A) and hypoxia (B) are shown. C and D: respiratory rates corrected for citrate synthase (CS) activity as biomarker for mitochondrial content during hyperoxia (C) and hypoxia (D). E: ratio in respiratory rates between hyperoxia and hypoxia. Two-way analysis of variance for repeated measures shows a main effect of ischemia ($P < 0.05$) in all conditions. During hyperoxia, both with (C) and without (A) CS correction, a significant ($P < 0.001$) interaction occurred between ischemia/nonischemia and the substrate-inhibitor protocol, allowing for post hoc (Holm-Sidak method) analysis: *$P < 0.05$, significant difference between ischemia and nonischemia. The ratio between respiration during hypoxia and during hyperoxia is displayed in E, and it shows that hypoxia affected ischemic and nonischemic regions similarly. Flux rates were always lower during hypoxia compared with hyperoxia. Data are means ± SE.
separately. Electrons for cytochrome c oxidation were provided by ascorbate + TMPD. The results revealed similar COX respiratory capacity in the ischemic and nonischemic regions under both hyperoxic and hypoxic conditions (Fig. 4). A gradual inhibition of COX by sequential Na-azide addition resulted in identical inhibition curves (Fig. 4).

Mitochondrial density and myocardial collagen content. CS activity tended \((P = 0.052)\) to be lower in the ischemic compared with the nonischemic myocardium \((44 \pm 7 \text{ and } 58 \pm 2 \mu \text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}, \text{respectively})\), indicating a slightly lower mitochondrial density in this region. HAD activity \((48 \pm 7 \text{ and } 64 \pm 2 \mu \text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}, \text{respectively})\) and protein content of complex V (Fig. 5) were significantly \((P < 0.05)\) lower in the ischemic compared with the nonischemic regions. Complex I-IV content was comparable between the regions. The collagen content in ischemic and nonischemic myocardium was 15.7 \(\pm\) 1.4 and 12.6 \(\pm\) 0.9 ng/\(\mu\)g protein, respectively. This difference was not significant \((P = 0.12)\).

Taken together these observations argue in favor of a modestly depressed mitochondrial content in the ischemic region.

ROS \((\text{H}_2\text{O}_2)\) generative capacity and expression of antioxidants and other proteins. H\(_2\)O\(_2\) formation (normalized to CS activity) in the saponin skinned fibers showed markedly higher rates in the myocardium from ischemic regions (Fig. 6). This difference was introduced after stimulation of complex-I respiration by addition of ADP (state 3), and it remained significantly higher throughout almost all subsequent steps. This greater potential for ROS generation in the ischemic tissue was not compensated by a higher level of antioxidant protein expression. On the contrary, catalase was significantly \((P < 0.05)\) reduced in ischemic tissue. COQ10B content tended to be lower in ischemic compared with nonischemic tissue, whereas GPX, MnSOD, and VEGF contents did not differ \((P = 0.05)\).

DIFFERENCES FROM NONISCHEMIC TISSUE

The respirometry data were expressed relative to a valid marker of mitochondrial content \((19)\), CS activity, and therefore the difference in mitochondrial OXPHOS capacity between the ischemic and nonischemic region cannot be ascribed to differences in mitochondrial density, i.e., a quantitative difference. In contrast, the present data indicate that chronic ischemia induces qualitative mitochondrial changes contributing to decreased OXPHOS capacity in the affected myocardial regions. A substitution of cardiomyocytes with extracellular matrix proteins such as collagen I and III would imply that the mitochondrial density per tissue mass would decrease. This has previously been reported in noninfarcted myocardium from patients deceased from myocardial infarction compared with myocardium from deceased patients without IHD \((33)\) and is furthermore a well-described feature of remodeling in heart failure \((10)\). We found a higher, although nonsignificant, collagen content in ischemic myocardium. This together with the nonsignificant decrease in CS activity and the decreased HAD activity and complex-V content could be interpreted as a slightly lower mitochondrial density in the ischemic compared with the nonischemic myocardium. However, the magnitude of changes in CS activity does not match the changes in, e.g., complex I + II-linked respiration, and the difference in respiratory OXPHOS capacity corrected for CS...
activity between the ischemic and the nonischemic tissue was quite marked (Fig. 2).

There was excessive ROS generation capacity in the chronically ischemic myocardium after normalization to mitochondrial content (CS activity) (Fig. 6). This was primarily seen after addition of ADP where ROS production would be expected to decrease (24), but this was only the case in the nonischemic myocardium (Fig. 6). With the final addition of antimycin A an increase in total ROS was expected. We did observe a 13\%/100610 and 18\%/100610% increase in ischemic and nonischemic tissue, but this increase was not significant (Fig. 6). We suspect that the differences in ROS production between the two sampling sites from the left ventricle are most likely due to complex-I ROS production. However, the measurements of ROS were done in permeabilized fibers that leave the mitochondria in situ in the cellular compartment. Therefore, nonmitochondrial ROS production rates are also part of the measurements, and some researchers believe that the nonmitochondrial part of the total ROS production can be quite substantial (6). Distinction between mitochondrial and nonmitochondrial ROS production was not possible in the present study because this would require measurements in isolated mitochondria which, in turn, requires sample sizes of \~50–75 mg.

Previous investigations of ischemic ROS production has mainly been undertaken in animal models of acute ischemia where increased H2O2 production in the ischemic mitochondria were reported (7, 8, 27, 30), whereas a decreased H2O2 production was found in a study on chronic ischemic swine mitochondria (21).

The protein expression of MnSOD, which mediates the processing of the highly reactive superoxide to the less-reactive H2O2, was 17% lower in the ischemic tissue (\(P < 0.05\)), and even though not statistically significant, there seemed to be a trend toward reduced expression of all antioxidants in chronically ischemic myocardium (Fig. 7). Animal studies in MnSOD knockout (MnSOD\(^{-/-}\)) mice have reported decreased CS production.
and complex-I/II activity in cardiac mitochondria, and oxidative damage to mtDNA was observed even in heterozygote (MnSOD<sup>+/−</sup>) mice (22), illustrating that lower antioxidant levels do in fact result in mitochondrial malfunction. H<sub>2</sub>O<sub>2</sub> can be removed by glutathion peroxidase (GPX); consequently, the levels of GPX could influence ROS measurements. However, GPX protein expression was not different in the two regions, and therefore the excess ROS generation could not be explained by lower GPX capacity in the ischemic mitochondria.

The composition of subunits comprising the COX has been shown to change with the influence of hypoxia in cells (14), and since the activity of COX subunit IV is regulated allosterically by the energetic level within the cell (25), we decided to study the kinetic properties of this enzyme complex. We hypothesized that the response to gradual inhibition of complex IV by increasing Na-azide concentrations, upon maximal OXPHOS stimulation with substrates for complex I and II, would reveal different kinetics and thereby place COX composition as a central element in the chain of events leading to a depressed mitochondrial OXPHOS capacity. This was not the case. Both the sensitivity (50% inhibitory concentration; data not shown) to Na-azide and the maximal inhibition ratio were nearly identical in mitochondria from ischemic and nonischemic regions (76 ± 2 and 77 ± 2%, respectively, Fig. 4). This finding could be interpreted in the direction that altered COX composition is probably not central to orchestration of mitochondrial function in chronic IHD.

We also hypothesized that a chronic ischemic preconditioning effect would be present in mitochondria from the ischemic region. This however could not be confirmed by our ex vivo hypoxia measurements. After lowering the oxygen concentration in the respiration chamber to levels presumably leading to local myocyte ischemia, we observed reductions in respiration of roughly the same proportion (~ 20%) in ischemic and nonischemic myocardium, indicating that no difference in susceptibility to hypoxia was present. This finding is partly in contrast to previous observations of reduced susceptibility to acute ischemia in porcine chronic ischemic myocardium (21), in which a protective effect of chronic ischemic preconditioning was reported, allegedly caused by mild uncoupling of the mitochondria as indicated by a lower RCR in the preconditioned tissue. In the present study we did not find a significant reduction in RCR in the ischemic region, which could explain the lack of chronic ischemic preconditioning effect in the present study, or alternatively interpreted as species-to-species variation.

Late in the course of ischemia, an increase VEGF mRNA expression have been found in the affected myocardial region (20). We evaluated the local impact of chronic ischemic conditions on the extent of protein expression of VEGF within the ischemic and nonischemic myocardial regions. VEGF appeared higher in the ischemic regions even though not significantly (Fig. 7).

**Study limitations.** In the hypoxic experiments we measured oxygen consumption at low oxygen concentrations. Since we need oxygen present for the assessment of mitochondrial respiration, there is a limit to the extent of ischemia that we can inflict upon the tissue. Therefore, we cannot rule out that an even lower experimental oxygen tension in the hypoxic protocol could potentially demask a protective effect of chronic preconditioning in previously ischemic myocardium. Furthermore, the subject of normalization is a limitation to the evaluation of intrinsic mitochondrial function. Regrettably, we did not obtain enough tissue to perform a thorough analyses of mitochondrial turnover since this would require morphological examination and separate tissue processing. Finally, it should be noted that four out the nine patients had left ventricular hypertrophy. Mitochondrial function could therefore be further deteriorated (26, 32) apart from the ischemia related dysfunc tion, but because of the limited number of patients, meaningful subgroup analyses could not be done.

In summary, we have found that complex II-linked respiration is diminished in chronically ischemic human left ventricular cardiac tissue. We have found some indications of decreased mitochondrial content in the ischemic region but not to an extent that can explain the decreased complex II-linked OXPHOS capacity. The diminished oxygen flux in the ischemic tissue was present at the maximal state 3 respiration during which electrons was passing from both complex I and complex II and converging into the Q-junction (15) and further to complex III. This may represent a “bottleneck” for electron transport through the respiratory system, which fits well with our finding of the tendency to a decreased COQ10B protein content in the ischemic muscle. Further studies on the function and abundance of subunits in complex-III protein are therefore warranted. We have further tested whether cardiac tissue that has been subjected to chronic ischemia would be less prone to a decrease in respiratory rates when exposed to acute hypoxia, but this was not the case. Thus we observed no chronic ischemic preconditioning effect in this study.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark, for assisting with blood analyses.

**GRANTS**

This work was supported by the Nordea Foundation, the Danish Council for Independent Research/Medical Sciences, and the Lundbeck Foundation.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

REFERENCES
6. Brown GC, Borutaite V. There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion 12: 1–4, 2012.