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ORIGINAL ARTICLE

The ratio of cytochrome c oxidase subunit 4 isoform 4I1 and 4I2 mRNA is changed in permanent atrial fibrillation

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Abstract

Aims The conditions of hypoxia are suggested to induce permanent atrial fibrillation (AF). The regulation of *COX4I2* and *COX4I1* depends on oxygen availability in tissues. A role of *COX4I2* in the myocardium of AF patients is supposed for pathogenesis of AF and subsequent alterations in the electron transfer chain (ETC) under hypoxia.

Methods and results In vitro, influence of hypoxia on HeLa 53 cells was studied and elevated parts of COX 4I2 were confirmed. Myocardial biopsies were taken ex vivo from the patients' Right Atria with SR (n = 31) and AF (n = 11), respectively. RT- PCR for mRNA expresson, mitochondrial respiration by polarography and the protein content of cytochrome c oxidase (CytOx) subunit 4I1 and CytOx subunit 4I2 by ELISA were studied. Clinical data were correlated to the findings of gene expressions in parallel. Patients with permanent AF had a change in isoform 4I2/4I1 expression along with a decrease of isoform COX 4I1 expression. The 4I2/4I1 ratio of mRNA expression was increased from 0.630 to 1.058 in comparison. However, the protein content of CytOx subunit 4 was much lower in the AF group, whereas the respiration/units enzyme activity in both groups remained the same.

Conclusions This study describes a possible molecular correlate for the development of AF. Due to the known functional significance of COX 4I2, mitochondrial dysfunction can be assumed as a part of the pathogenesis of AF.

Keywords Cytochrome c oxidase subunit 4; Isoform 4I1 and isoform 4I2; Mitochondrial respiration; Permanent atrial fibrillation

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‡*The article is dedicated to our beloved colleague, friend, and co-worker Dr. Rabia Ramzan, who passed away too young.*

Introduction

Atrial fibrillation is an abnormal heart rhythm (arrhythmia) characterized by rapid and irregular beating of the atrial chambers of the heart. Persistent atrial fibrillation (AF) symptoms last longer than 7 days, and heart's rhythm cannot be regulated by itself anymore. In contrast, sinus rhythm (SR) refers to the normofrequency, regular heartbeat in humans. It originates in the sinus node in the right atrium of the heart. The excitation of the heart spreads out and subsequently generates the heart contraction. AF is a progressive disease and the most common persistent cardiac arrhythmia in adults. Prevalence in Europe has more than doubled in the last 10 years.[1] In order to advance the therapeutic options, basic initiating and maintaining mechanisms of atrial fibrillation must be understood.[2]

Atrial fibrillation occurs because of early afterdepolarizations[3] and delayed after-depolarizations[4,5] caused by comorbidities.[5] Shortened action potential duration and extended refractory time result and cause a maintenance of fibrillation[6] with undirected atrium excitation with irregular transition.[7]

Successful termination of atrial fibrillation becomes less likely with time.[8] A remodelling of atrial tissue is dependent

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on calcium metabolism.[6,8,9] The energy supply is changed. A Hypoxia is most likely caused by an increased oxygen consumption with a simultaneous insufficiently increased supply (oxygen deficit).[10,11] The altered respiration during atrial fibrillation causes an increased production of reactive oxygen radicals, which damage the myocardium.[12] The bioenergetic deficit promotes progression.[9,11,13] If hypoxia and metabolic changes could be limited, the fibrillation would lack its substrate.[11,13,14]

The impaired state of the myocardium[15] is reflected in reduced enzyme activity of cellular kinases, reduced ATP content, impaired oxygen utilization and mitochondrial dysfunction. Metabolic stress in the atrium leads to reduced calcium flow, reduced calcium storage and reduced contractility, resulting in a shortened action potential and hypocontractility.[12,14–17]

At the beginning of AF there is an increase in hypoxia indicators such as hypoxia-inducible factor 1α (HIF-1α) and lactate.[11,16] Atrial fibrillation is accompanied by oxidative stress due to a compensatory downregulation of the electron transport chain.[12] In the physiological state, reactive oxygen species (ROS) serve intracellular signalling pathways.[18] As soon as ROS production cannot be sufficiently limited, oxidative cellular damage occurs. Dependent on the mitochondrial membrane potential, from $\Delta\Psi m > 140$ mV the superoxide anion can be formed at complex I and III.[19] In the aging heart, increasing inefficiency of the respiratory chain leads to an increased incidence of ROS.[13,14,16]

The series of redox reactions inside the electron transmission chain (ETC) releases energy used by complex I, III, and IV to pump protons into the intermembrane space.[13,20] By translocating protons, the complexes create a gradient across the inner mitochondrial membrane driving complex V. The ATP synthase produces ATP, which serves to supply the cell with energy. Through various regulatory mechanisms, the respiratory chain adapts to the needs and bioenergetic state of the cell.[15,21]

Cytochrome c oxidase (CytOx) represents complex IV of the respiratory chain. It is the terminal enzyme of the respiratory chain, represents the rate-limiting step in aerobic energy production and catalyses the reduction of molecular dioxygen to water.[22–24]

Herein, the subunit 4 of CytOx[25] has been shown being a subunit in mammals with an important regulatory role. It has contact points with two of the catalytic subunits and contains an ATP binding site.[26,27] At high ATP/ADP ratios, CytOx is allosterically inhibited by phosphorylation of subunits.[23,28,29] Its subunits 4I1 and 4I2 have gained special regulatory importance.[22,24] There are posttranslational modifications.[23] The isoform 4I1 is ubiquitously expressed, while 4I2 occurs mainly in the lung and fetal muscles. The different isoforms cause an adaptation of enzyme activity to specific tissue requirements.[23] The CytOx adapts to the energy metabolism of the cell.

According to Prabu et al. the CytOx is downregulated in hypoxia through phosphorylation. This is accompanied by increased mitochondrial dysfunction and an increase in ROS production.[17,30] The hypoxia-dependent regulation of isoforms is only found in mammals.[23,26] Hüttemann et al. state that regulation takes place via a conserved segment in the promoter of the gene for subunit 4 of CytOx. There is an oxygen responsive element (ORE), which interacts with various transcription factors. While RBPJ and MNNR1 activate transcription, CXXC5 suppresses it. MNNR1 itself has an ORE. The maximum activity of the ORE was shown at 4% oxygen content and led to an induction of the isoform 4I2 of CytOx.[31] Another step of regulation is described by Fukuda et al. Hypoxia inducible factor 1-α (HIF-1α) accumulates at low oxygen levels and induces the expression of the mitochondrial protease LON by binding to two hypoxia responsive elements (HREs), which causes a degradation of CytOx 4I1. They say HIF-1α induces the expression of the gene for CytOx 4I2.[32,33] This could represent a key mechanism for AF induction and maintenance. The present work addresses the question whether atrial fibrillation goes along with upregulation of the isoform 4I2 of CytOx. Supporting this idea, in a cell culture model we detected higher mRNA expression for isoform 4I2 and less 4I1 under hypoxia. Afterwards, we studied myocardial biopsies taken from right atria of AF patients during ongoing cardiosurgical procedure and visual inspection of fibrillation to detect corresponding 4I2/4I1 isoform alterations.

Methods

Primer

The primers were commercially purchased from the company QuantiTect™. They were mixed with 1.1 mL TE buffer (TRIS, EDTA; pH = 8), aliquoted and stored at −20°C. Primer were available for beta-actin, Hs_ACTB_2_SG – Assay, Order number QT01680476; HIF-1α, Hs_HIF1A_1_SG – Assay, Order number QT00083664; VEGF-B, Hs_VEGFB_1_SG – Assay, Order number QT00013783; COX4.1, Hs_COX4I1_1_SG – Assay, Order number QT00065961; COX4.2, Hs_COX4I2_1_SG – Assay, Order number QT00044933). VEGF is a regulator of angiogenesis during both physiological and pathological states by binding to the VEGF receptor and acting as a transcription factor. VEGF-B is found in various cell types, including striated muscle and endothelial cells. In the ischemic myocardium, an increase of VEGF-B[34] also causes an increase in the transcription of antioxidative genes.[35]

Examination material

Human cervical squamous cell carcinoma cells (HeLa S3) were acquired from American Type Culture Collection (Rockville,

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Maryland, USA). Biopsies were taken intraoperatively from the right atrium during ongoing heart surgery at the Marburg University Hospital between 2014 and 2018 (Ethics Committee Agreement number: AZ 53/18).

Cell culture

The experiments in the preliminary study were performed with the human HeLa S3 tumour cell line. Liquid nitrogen frozen cells were thawed. Thawing was carried out at room temperature, after which the cells were mixed with medium. The cells were then centrifuged at 1000 rpm at 20°C and the resulting pellet resuspended with medium. The cells were plated out in a 250 mL cell culture flask and cultivated in an incubator at 37°C (atmosphere saturated with water vapours with 5% CO_2). The medium used is the Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Until the cells were confluent, the medium was changed regularly (every 2–3 days). The growth was checked with regular microscopic controls. When growth was sufficiently confluent, the cells were split and divided into 6-hole plates. For this purpose, the medium had to be removed and the cells were washed twice with PBS (Dulbecco's phosphate buffered saline solution, pH = 7.4). Subsequently, 2 mL of trypsin were added to the bottles followed by a 5- to 6-min incubation period. The cells detached by trypsin were mixed with a new medium (Dulbecco's modified Eagle's medium) and evenly distributed on the wells of the plate under microscopic control. A portion of each cell was returned to the culture flask, provided with medium and stored in the incubator. One 6-hole plate each for the control in the normal incubator (see above) and one for the hypoxia experiment in the hypoxia chamber was provided with the cells diluted 1:4 with medium.

Hypoxia chamber

Twelve hours after division of the cells on the 6-hole plates, a part of the cells was placed in the hypoxia chamber, which has a N_2/CO_2- content of 95%/5%, and incubated at 37°C. After an incubation period of 8 h the medium was removed. Without delay cells were treated with 700 µL Quiazole Lysis Reagent per well of the plate. This was done simultaneously for both the experimental cells and controls. This test was repeated twice after initial test with an identical test procedure.

RNA extraction

The first step of RNA extraction involved treating the cells with 700 µL quiazole lysis reagent. All steps refer to the miRNeasy™ Mini Kit, which was used for the RNA extraction. The cells lysed in 2 mL reaction vessels were mixed with 140 µL chloroform, shaken manually for 15 s and incubated for 2–3 min at room temperature. The cells had to be centrifuged at 4°C at 12 000× *g* for 15 min. All further steps were performed at room temperature. The upper part of the three resulting phases was taken off, placed in a new vessel and mixed with one and a half times the amount of 100% ethanol. Afterwards 700 µL of each sample were added to a miRNeasy column in a 2 mL collection tube and centrifuged at 8000× *g* for 15 s. After the identical procedure was performed with the rest of the samples, 700 µL RWTTM buffer (Qiagen, Hilden, Germany) was added to all columns and centrifuged at 8000 ×*g* for 15 s. With 500 µL RPETM buffer (Qiagen, Hilden, Germany) the previous step was repeated. With additional 500 µL RPETM buffer the samples were now centrifuged at 8000 ×*g* for 2 min. The columns were placed in a new reaction vessel and centrifuged at full speed for 1 min. The columns then had to be placed in a new 1.5 mL collection reaction vessel. Now 40 µL RNAse-free water was added to the column and the tube was centrifuged at 8000 ×*g* for 1 min. By centrifugation, the RNA was extracted from the column into the reaction tube. The RNA was immediately stored on ice.

RNA measurement

A spectrophotometer (NanoDrop™ spectrophotometer, Thermo Scientific™) was used to measure the samples to determine the exact RNA content. For the measurement, 2 µL of each sample were used. The absorption at the wavelengths 260 nm/280 nm and 230 nm/260 nm was determined. The determination of the absorbance excludes impurities and thus allows an exact determination of the RNA/µl content per sample. The completed and measured RNA samples were now frozen at −80°C or directly used for cDNA synthesis.

cDNA synthesis

The cDNA synthesis was performed using the BioRad iScript™ cDNA Synthesis Kit. The RNA amount of each sample was averaged to an amount of 1 µg RNA per sample. For this purpose, the measurement of RNA was used to achieve the desired amount of RNA by diluting the RNA with RNAse free water. Thus, 1 µg RNA per sample could be used. Thus, 15 µL H_2O with RNA were used per reaction vessel. The cDNA synthesis was performed in 0.2 mL reaction tubes with the cycler Primus 96 plusTM (Aviso, Jena, Germany). The samples were incubated in 200 µL reaction vessels according to the following reaction protocol: priming 5 min at 25°C, reverse transcription 20 min at 46°C, RT-inactivation 1 min at 95°C, cooling at 4°C and freezing at −22°C or used directly for real-time PCR.

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Real time-PCR

The real-time PCR[36–38] was performed with the Multicolor Detection System of BioRad™. For the RT-PCR, the cDNA produced in the previous step was used as a template. Quantitect primer assays for β-actin, VEGF-B, HIF-1α, CytOx 4I1 and CytOx 4I2 were used. First, the cDNA was diluted with H_2O in a ratio of 1:5. Subsequently, 2 μL of diluted cDNA per well were placed in a 96-well plate. Master mix was prepared for each primer pair, of which 18 μL per well was added to the cDNA provided. RT-PCR was performed using the following reaction protocol 1: activation step 15 min at 95°C, reaction protocol 2: for 40 cycles: denaturation 15 s 94°C, alignment 30 s 60°C, and polymerization 300 s 30°C. Finally, a melting curve of all samples was prepared. The melting curves showed no impurities or primer dimer formation. All data are referred to the expression of β-actin ('normalized mRNA expression').

Atrial tissue

A total of 33 patients were included in this study (*Table 1*). During routine cardiac surgery, a biopsy was taken from the wall of the right atrium while the heart-lung machine was connected. All patients included in the trial series gave an informed consent (Ethics votum AZ: 53/14), preoperatively. Biopsies were taken from the right atrium during extracorporeal circulation. Consent was also given for anonymous handling of patients' data. The study was submitted to the local ethics committee on the basis of an application, which approved the study under the local registration number: AZ: 53/14. The samples taken intraoperatively had to be immediately placed in a test tube with 3–5 mL RNA storage solution for fixation. Without delay, the specimen was divided and filled into three to four cryoreaction vessels and stored at −80°C.

RNA extraction

Corresponding to the cell culture experiments, RNA was extracted. Six samples each were slowly thawed on ice. With the help of a scalpel the samples were coarsely crushed. Macroscopically well visible fat and connective tissue were removed and discarded to use predominantly cardiac muscle tissue. To the tissue 700 μL Quiazole Lysis Reagent was added. Separation was performed in Lysing Matrix A-Tubes, which are equipped with a ceramic bead (diameter 6.5 mm) and a RNAse and DNAse free comminution matrix. Throughout the entire RNA extraction procedure, the samples were cooled on ice. For lysis, samples were placed in the

Table 1 Patients 'characteristics of the study

Characteristics	SR (n = 23)	VHF (n = 10)	P-value
Age. years	69.3 ± 2.3	70.4 ± 2.6	$P = 1.0$ n.s.
Gender (male). % (n)	73.91 (17)	70.00 (7)	$P = 1.0$ n.s.
Co-morbidities. % (n)			
Diabetes	43 (10)	30 (3)	$P = 0.7$ n.s.
Hypertension	100 (23)	100 (10)	$P = 1.0$ n.s.
Hyperlipidaemia	87 (20)	60 (6)	$P = 0.2$ n.s.
Smoking	35 (7)	12.5 (1)	$P = 0.34$ n.s.
Chronic renal insufficiency	8.7 (2)	10 (1)	$P = 1.0$ n.s.
Coronary heart disease	96 (22)	80 (8)	$P = 0.21$ n.s.
Cardiac insufficiency	26 (6)	50 (5)	$P = 0.24$ n.s.
Chronic obstructive pulmonary disease	8.7 (2)	20 (2)	$P = 0.56$ n.s.
Heart valve disease	74 (17)	90 (9)	$P = 0.4$ n.s.
Body mass index, kg/m^2	29 ± 1.1	28 ± 1.9	$P = 0.98$ n.s.
Echocardiography			
Left ventricular ejection fraction, %	55 ± 2.3	51 ± 4.5	$P = 0.45$ n.s.
Left ventricular end-diastolic diameter, mm	50 ± 1.8	52 ± 3.7	$P = 0.42$ n.s.
Intraventricular septum, mm	12.3 ± 0.5	13 ± 1	$P = 0.5$ n.s.
Left atrial end-systolic diameter, mm	39.3 ± 1.3	45.7 ± 1.8	$P < 0.05$
Medication. % (n)			
Beta-blockers	65 (15)	89 (8)	$P = 0.68$ n.s.
Calcium channel blockers	30 (7)	33 (3)	$P = 1.0$ n.s.
ACE inhibitors (angiotensin-converting-enzyme)	77 (17)	56 (5)	$P = 0.38$ n.s.
Diuretics	52 (12)	67 (6)	$P = 0.69$ n.s.
Statines	61 (14)	56 (5)	$P = 1.0$ n.s.
Insulin	30 (7)	22 (2)	$P = 1.0$ n.s.
Oral antidiabetics	22 (5)	11 (1)	$P = 0.65$ n.s.
Type of cardiac surgery. % (n)			
Coronary artery bypass surgery	61 (14)	20 (2)	$P = 0.06$ n.s.
Bypass and valve surgery	30 (7)	10 (1)	$P = 0.38$ n.s.
Heart valve surgery	9 (2)	70 (7)	$P < 0.001$

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Tissue-Lyser of MP-Biomedicals™. In order to extract the comminution matrix, centrifugation was performed at maximum speed for 1 min (Centrifuge 5417R from Eppendorf™, 20 000× *g*, Hamburg, Germany). Subsequently, the upper, liquid phase had to be removed. Per sample 140 μL chloroform was added and the samples were shaken manually. All further steps were identical to the RNA extraction from the cells (see above).

cDNA synthesis

The cDNA synthesis was almost identical (iScript™ Synthesis Kit). 200 ng RNA was used per sample. *RT-PCR* The procedure of the RT-PCR corresponded to the procedure with cells obtained from the culture. The initial dilution of the cDNA was performed at a ratio of 1:3. The composition of the samples and the reaction protocol were identical.

ELISA for the cytochrome c oxidase IV isoform 4I1 and 4I2 detection

The commercially available test was supplied by Cloud-Clone Corp. (Fernhurst Dr., Katy, TX, USA). Principle of the kits is a Sandwich enzyme immunoassay. The microtitre plate provided in this kit has been pre-coated with antibodies (SED 286Hu, SED287Hu, species: *Homo sapiens*) specific to cytochrome C oxidase subunit IV isoform 4I1 and isoform 4I2. Standards or samples were added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to the isoforms. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate and incubated. After substrate solution was added, only those wells that contain cytochrome C oxidase subunit IV isoform 4I1 or 4I2, respectively, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of isoform 4I1 or 4I2 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Measurement of cytochrome c oxidase activity

Biopsies were homogenized (protein concentration 300 mg/mL). Mitochondrial respiration were measured polarographically at 25°C using KCl buffer (130 mM KCl, 3 mM Hepes, 0.5 mM EDTA, 2 mM potassium phosphate, 0.5% fatty acid free BSA, pH 7.4) in a total reaction volume of 0.3 mL. To the homogenates 5 mM glutamate and 1 mM malate were added as substrates. Stimulation of respiration was induced by adding 20 μM cytochrome c, following by adding 1 μM $CaCl_2$ and 3 μM Ca Cl_2. Measurements were performed by the Hansatech™ system. The oxygraph-system consists of a sensitive Clark-type polarographic oxygen electrode disc mounted within an electrode chamber and connected to the Oxygraph-electrode control unit (Hansatech Instruments Ltd, Narborough Road, Pentney, King's Lynn, Norfolk PE32 1JL, UK).

Statistics

For data evaluation, a reference gene, the so-called 'housekeeping gene', was determined according to the gene segments to be examined. With reference to the reference gene (normalization), there is a variance reduction of the results. Double determinations were also made. For further analysis of the results, the 'Δct method' was applied. In this method, the ΔCT of both PCR values of a gene to be measured was first determined with the two values of the reference gene (β-actin) of this sample. The ΔCT values of all samples were now averaged. The differences between this mean value of all samples and each of the four ΔCT values determined were determined, and thus the $\Delta\Delta CT$ value was determined. Now these calculated differences were inserted into the following formula to calculate the deviation[39]: $f(x) = x\ 2^{-\Delta\Delta CT}$. The results of these individual were related to each other, as both the individual genes have been normalized using the reference gene and the results are related to the mean of all ΔCT values. These are therefore the relative expressions of the genes studied. Statistical analysis of relative gene expression was performed using Sigma Stat™ and Sigma Plot™. Due to the non-normally distributed values (Kolmogorov–Smirnov test), the Equal Variance Test associated with the *t*-test failed. Alternatively, the Mann–Whitney rank sum test™ was applied, which indicates whether the values of the compared groups can be randomly arranged or whether there is a statistically significant difference. A $P < 0.05$ the result is considered significant. To evaluate patient characteristics, the Fisher's exact test™ was used to determine the significance of binary variables. Information such as age, BMI, and echocardiographic parameters were examined for significance using the *t*-test or, in the case of abnormally distributed values, the Mann–Whitney rank sum test™.

Results

Cell culture

To demonstrate the extent to which HIF-1α and VEGF are induced by hypoxia and correlate with the expression of CytOx4, isoform 1 and isoform 2, an experiment was performed in the hypoxia chamber using HeLa cells under controlled conditions (95% nitrogen, 5% carbon dioxide, 37°C, 8 h duration). It was shown that mRNA expression for

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HIF-1α is lower after 8 h of hypoxia treatment ($P = 0.003$). In addition, there is an increase in the expression of CytOx4, isoform 2 upon hypoxia treatment ($P = 0.037$) (*Figure 1*).

Patients' data

Tissue samples from the right atrium were obtained from a total of 42 patients in the course of cardiac surgery ($n = 31$ with sinus rhythm, $n = 11$ with atrial fibrillation). The clinical characteristics of the patient population have been summarized in *Table 1*. There were no significant differences comparing the examination and control groups with respect to age, body mass index (BMI), and most echocardiographic parameters. Differences were seen with respect to left atrial end-systolic diameters and the proportion of valve surgery. All patients with a history of paroxysmal atrial fibrillation were not enrolled. Also, patients with a significantly elevated preoperative CRP (>100 mg/dL) were excluded, as inflammatory signalling pathways through TNF-α could interfere with cellular energy balance and distort the results.[40–42]

Detection of mRNA of HIF-1α, VEGF, cytochrome c oxidase 4I1 and 4I2 intraoperatively obtained biopsies of the right atrium

To assess the acute nature of tissue hypoxia under cell culture conditions, the mRNA of the signalling proteins HIF-1α and VEGF was determined. The expressions of HIF-1α and VEGF in the groups of patients with sinus rhythm and with atrial fibrillation did not show significant differences (*Figure* S1A–D).

Further comparison of mRNA expressions for HIF-1α and VEGF resulted in no differences between low and high grade coronary heart disease (0–1 vessel CHD vs. 2–3 vessel CHD) to the corresponding SR and AF groups (*Figure* S1B,C).

Assuming that VEGF/HIF -1α values of < 1 indicate acute hypoxia and VEGF/HIF -1α values of >1 indicate chronic hypoxia, a comparison of the expression of CytOx isoforms was performed. No differences were found (*Figure* S1D). However, comparing the expressions of CytOx4 isoforms (ratio 4I2/4I1) between the groups with atrial fibrillation and with sinus rhythm, there were a clear difference to each other (*Figure 2*). In case of diabetes mellitus and higher grade coronary heart disease there was no effect on the result (*Figure* S2A,B). Preoperatively reported angina pectoris symptomatology and the common Statine therapy of CIHD patients had also no influence. In these calculations, known diabetes mellitus was not considered. When included, we found no differences, too (*Figure* S2C–F).

Linear regression of COX 4I2 expressions in relation to left atrial end-diastolic diameter was performed in both the sinus rhythm and atrial fibrillation patient groups. An opposite trend is visible ($r_{sinusrhythm} = 0.503$, $r_{atrial\ fibrillation} = -0.544$), but statistical analysis is compromised by the lower 'power' (*Figure* S3).

Figure 1 The normalized mRNA expressions of HIF-1α, VEGF, CytOx4, isoform 4I1, and CytOx4, isoform 4I2 and of cell culture in room air ($n = 10$) and after 8-h hypoxia treatment ($n = 11$) were compared (*$P < 0.05$). Significance of expression of HIF ($P = 0.003$) and expression of CytOx4, isoform 4I2, ($P = 0.037$) by Mann–Whitney *U* test. (VEGF $P = 0.307$, Cox4I1 $P = 0.504$).

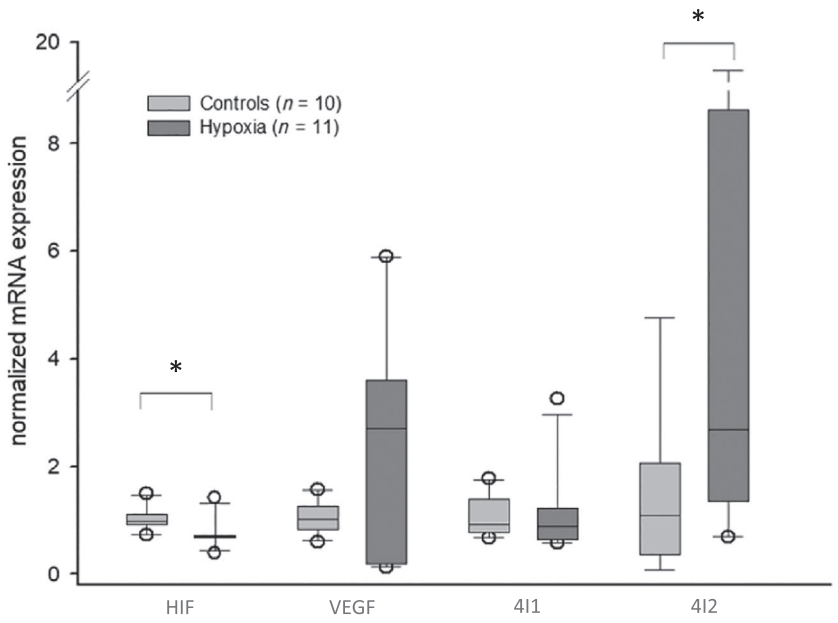

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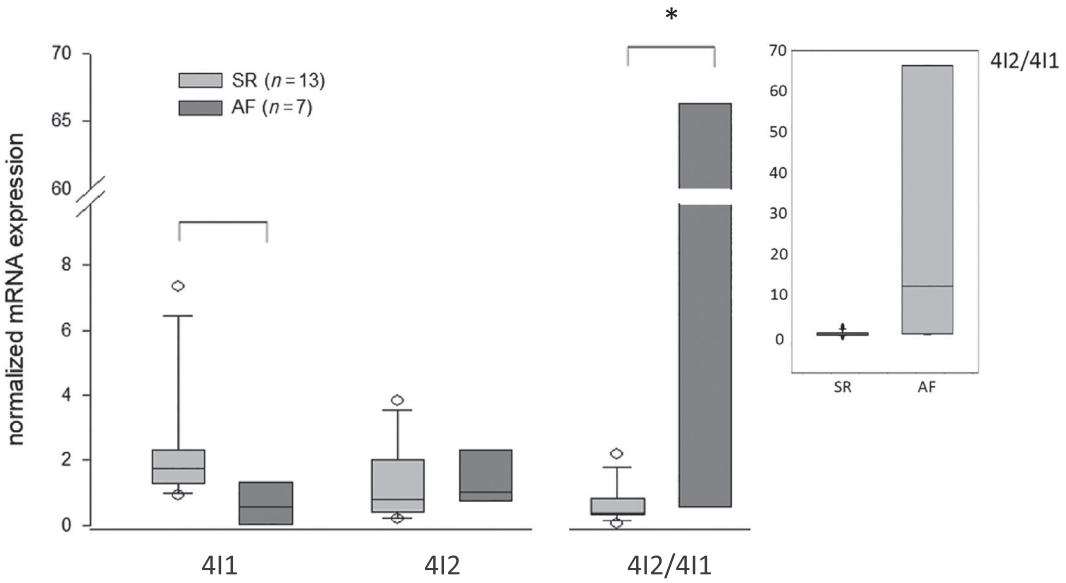
Figure 2 Normalized mRNA expressions of CytOx 4, isoform 4I1, and CytOx4, isoform 4I2, and their quotients of the two isoforms of the group of patients with sinus rhythm (n = 13) and those with AF (n = 7) were compared (*$P < 0.05$). Patients with diabetes mellitus were excluded. According to Mann–Whitney U test significant differences of CytOx4I1 expression (P = 0.006) and quotient 4I2/4I1 (P = 0.007) (COX 4I2 P = 0.267). For each RT-PCR determination of the mRNA expression of IF 4I2 and IF 4I1 in a tissue sample, the quotient of the ratio 4I2/4I1 was selectively determined.



ELISA for the cytochrome c oxidase IV isoform 4I1 and 4I2 detection

In *Figure 3*, the results of the protein determinations of isoforms 4I1 and 4I2 are shown in each patient group. First of all, it is impressive that in the case of atrial fibrillation, protein content of subunit IV of cytochrome c oxidase (complex IV of the ETC) is obviously reduced overall. This finding is consistent with former results in the study of myocardial insufficiency.[15] In the first group (SR), a difference of 4I1 content was detected at 12.87 ± 1.091 ng/mL and 4I2 at 8.13 ± 1.52 ng/mL, respectively (normality test/Shapiro–Wilk passed, Student's t-test, P = 0.00474). In the group with AF, 4I1 was detected at 3.27 ± 0.09 ng/mL and increased 4I2 at 3.46 ± 0.18 ng/mL (Shapiro–Wilk passed, Student's t-test, P = 0.011, and Welch's t-test, P = 0.0229, respectively). Thus, we find both isoforms significantly decreased in the case of atrial fibrillation and, in addition, the ratio of isoforms (4I2/4I1) is changed from 0.63 to 1.058. In the experimental approach with HeLa cells, hypoxia caused a shift of the ratio from 0.87 to 2.78. Therefore, a disturbed tissue respiration must be considered as causative in the context of the pathogenesis of AF.

Measurement of tissue respiration

Tissue respiration in the presence of 5 mM glutamine and 5 mM malate was studied in the myocardial tissue samples of patients with sinus rhythm (A) and atrial fibrillation (B), respectively: Values for sinus rhythm were 4.05 ± 2.25 μmol/L/min and 3.72 ± 1.73 μmol/L/min and after 1 μM $CaCl_2$ and 3 μM $CaCl_2$ 2.59 ± 1.06 μmol/L/min and 1.06 ± 0.95 μmol/L/min, respectively. In the group of atrial fibrillation, the data were heterogeneous distributed, calculation were performed according the Krustal–Wallis one-way analysis of variance on ranks: The corresponding values were G1, median 4.17; 25% percentile 0.781; 75% percentile 11.34, G2, median 7.95; 25% percentile 3.37; 75% 12.08 μmol/L/min, G3, median 4.70; 25% percentile 2.47, 75% percentile 10.76, G4 median 4.62; 25% percentile 3.95; 75% percentile 10.2 μmol/L/min. The protein stem concentrations were 68.71 ± 0.76 mg/mL and 93.85 ± 13.91 mg/mL, respectively. Equal amounts of homogenate were used in the experimental settings. *Figure 4A,B* shows that tissue respirations in both groups was not increased by cytochrome c (electron transfer directly to complex IV). However, no inhibitory effect of a higher calcium concentration (3 μM) on respiration is found in the samples under the conditions of atrial fibrillation (B) (see Gellerich et al.[43]). The observation indexes respiration under stress to increased ATP production and to increased ROS production.[44]

Discussion

According Grover et al., fibrillating myocardium shows not only increased coronary perfusion but also increased lactate

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Figure 3 Protein estimation with the ELISA procedure (*$P < 0.05$): The subunit IV of cytochrome c oxidase (complex IV of the ETC) is in case of AF obviously reduced overall. This finding is consistent with former results in the study of myocardial insufficiency.[15] It also shows that myocardial isoform 4I2 is also increased in comparison to other tissues under physiological conditions. Moreover, we find both isoforms significantly decreased in the case of atrial fibrillation and, in addition, the ratio of isoforms (4I2/4I1) is changed from 0.63 to 1.058.

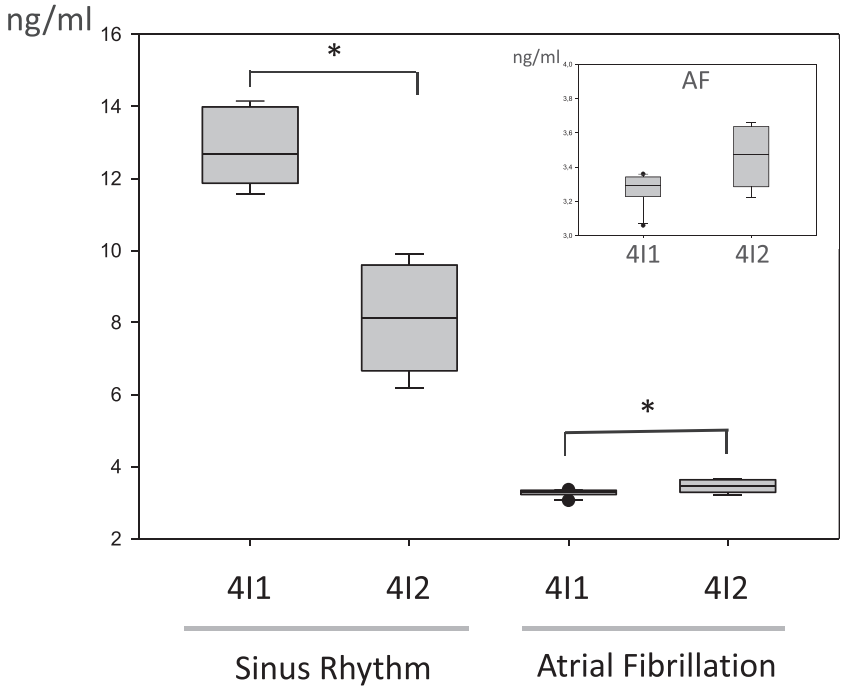
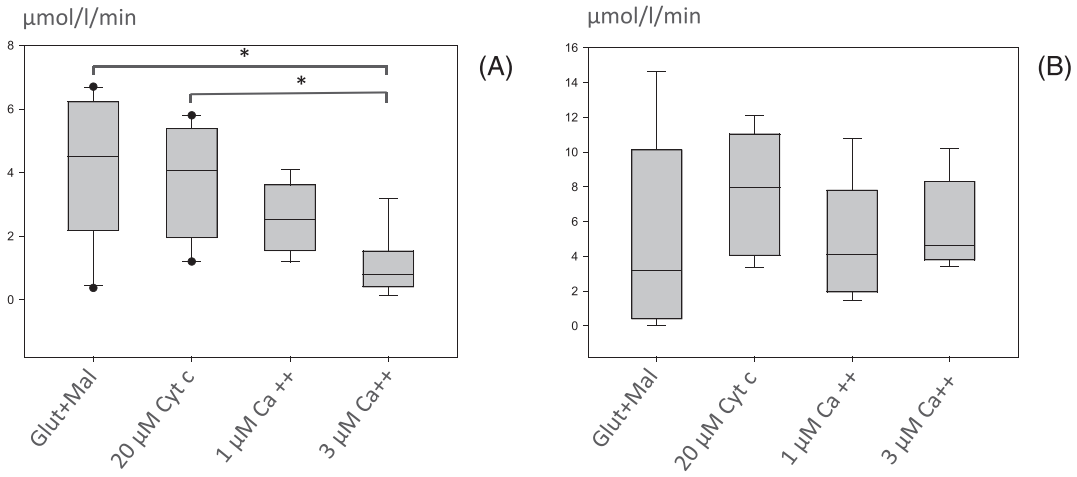


Figure 4 Tissue respiration in the presence of 5 mM glutamine and 5 mM malate was studied in the myocardial tissue samples of patients with sinus rhythm (A) and atrial fibrillation (B), respectively: To address the question of whether altered expression of the 4I2/4I1 isoforms of CytOx also signify a corresponding functional correlate, mitochondrial respiration of the respective myocardial samples (SR (A) vs. AF (B)) was tested (*$P < 0.05$). The well-known standard technique according to Chance and Williams[78,79] was applied.[80] Stimulation of mitochondrial respiration (see Methods section) was performed with the substrates glutamate and malate. Although the numerical values for respiration (μmol/L/min) were higher in the AF group, these values did not reach statistical significance. Stimulation of respiration with 20 μM cytochrome c by increased electron transfer to the CytOx or administration of 1 μM Ca^{++} also did not increase respiration in either group (see Gellerich et al.[43,44]). Interestingly, an inhibitory effect could be achieved by a higher Ca^{++} concentration (3 μM) in the SR groups (*$P < 0.05$), but not in the AF group (respiration values in the AF group n.s., correlating experiments (3 μM Ca^{++}) across both groups SR vs. AF *$P < 0.05$). As calcium binding to cytochrome c oxidase has a regulatory effect on its activity,[81] it has to be discussed whether the binding sites were influenced by conformational changes at CytOx or whether an altered redox potential might play a role.[82]



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and increased myocardial oxygen utilization.[45] In a cell culture with HeLa cells we followed the thesis of Fukuda et al.[32] and were able to detect and prove an increased expression of the COX 4I2 isoform after an induced 8-h hypoxia/anoxia. This change is accompanied by reduced HIF-1α and a tendency to increased VEGF-B expression (*Figure 1*). This observation is in agreement with the results of Sommer et al.[46] who assigned COX 4I2 an essential role for acute oxygen sensing by triggering mitochondrial hyperplasia and release of superoxides or after conversion to hydrogen peroxides.

The purpose of this small experimental study was to demonstrate the mechanical approach of hypoxic induction of isoform 4I2 in principle only, especially because no measurement of the partial pressure of oxygen in the myocardial tissue is available during the subsequent intraoperative biopsy collection. An exchange of the subunit 4 isoforms could cause a change in the oxygen affinity of cytochrome c oxidase[47], which could explain the results of Grover et al.[45] The observation made in this experiment that a reduction in HIF 1 expression occurs in the hypoxia group may be related to the fact that HIF-1 represents the initial response to hypoxia, but HIF-2 represents a more chronic response.[48] The reduction of HIF 1 would thus signal the transition to chronification and would therefore be comprehensible. This HIF switch and its importance in the respiratory chain remains an interesting challenge and could not be pursued in this study.

The VEGF expression gene is a primary target gene of HIF-1α, which is induced in hypoxic cells along with other angiogenesis-stimulating factors. HIF-1α has been shown to bind to a hypoxia responsive element (HRE) of the VEGF gene.[49–51] The VEGF family consists of VEGF types A, -B, -C, -D and placental growth factor (PlGF). These different signalling proteins play important roles in angiogenesis. In the present work, we focused on VEGF-B, which mediates its function by binding to VEGF receptor 1 (VEGFR-1). The angiogenic function of VEGF-B has been described mainly in ischemic myocardium. Therefore, it was used in this work as a parameter to detect incipient angiogenesis in terms of tissue response to chronic hypoxia.[34,52] Furthermore, VEGF-B has antioxidant properties and can effectively reduce oxidative stress.[35] VEGF receptors (VEGFR-1, -2, and -3) are also regulated by HIF-1α. Hypoxic stress results in selective increases in these receptors induced by HIF-1α. Most importantly, VEGFR-2 is expressed in skeletal and cardiac muscle and lung.[53] HIF-1α is thus a modulator of angiogenesis via upregulation of several genes, including VEGF and VEGFRs.[54]

Cytochrome c oxidase isoforms 4I1 and 4I2

The activity of CytOx (complex IV) is considered as 'rate-limiting step' for regulation of mitochondrial ATP production. Isoform switch is assumed being a central regulator of the respiratory chain under varying oxygen availability.[25] Isoform 4I1 is ubiquitously expressed and allosterically regulated by ATP. Isoform 4I2 is expressed more abundantly under hypoxic conditions and is ATP-independent and the enzymatic activity of CytOx is beyond allosteric control. Therefore, it seems reasonable that AF is associated with functional hypoxia.[10,11] The switch from isoform 4I1 to isoform 4I2 results in altered enzymatic kinetics. Due to its independence from regulation by high ATP/ADP ratios, CytOx exhibits less restricted activity and hyperbolic kinetics even under hypoxic conditions.[25] Thus, under hypoxic conditions, oxidative stress occurs as complexes I and III.[55] On the other hand, under hypoxic conditions, although CytOx function is maintained, formation of subunit 4I2 is increased and ATP-independent enzyme kinetics results in elevated mitochondrial membrane potential accompanied by increased ROS formation.[19,25]

According to current knowledge, in mammals, subunit 4 is an important molecular structure that responds to environmental conditions and has an important regulatory position.[23,26,28,29] The ubiquitously occurring isoform 4I1 is expressed at high ATP/ADP ratios and exhibits significantly lower turnover rates due to allosteric inhibition in the presence of ATP.[24] While the ATP-binding site of isoform 4I1 appears to be conserved among all vertebrates, there is still disagreement about the evolution of the gene with respect to the properties of isoform 4I2.[56] Hüttemann et al. first found isoform 4I2 in tuna.[57] A high level was found in human lung tissue, but only to a lesser extent in other tissues.[24] Binding of HIF-1α to regions in the promoter gene results in increased transcription of the subunit 4I2 gene and LON, a protease that degrades subunit 4I1. Electron transfer and energy production become twofold more effective.[26,32] However, the hypothesis of regulation by HIF-1α and HREs is controversial, as Hüttemann et al. suggest a conserved oxygen responsive element is responsible for the increase in expression upon hypoxia, to which various transcription factors bind, independently of HIF-1α.[58] In mammals, the peptide sequence of isoform 4I2 contains two cysteine groups. These are thought to form a disulfide bond, prevent a conformational change, and thus may abolish the allosteric inhibition by ATP present in isoform 4I1.[25,26,56] According to Schiffer et al., increased levels of isoform 4I2 in skeletal muscle result in low oxygen consumption, less ROS production, and low resting metabolic rate and energy consumption.[59]

The switch from isoform 1 to isoform 2 in CytOx subunit 4 results not only in independence from ATP/ADP concentrations but also in a higher turnover rate of the enzyme by changing the H^+/e^- - stoichiometry and thus plays a central role in adaptation in hypoxia.[32] Whether ROS production is limited or enhanced by the switch is controversial. According to Hüttemann et al., the increased enzyme kinetics leads to an increase in membrane potential and thus to increased oxidative stress.[19,25] In contrast, according to Fukuda et al. there is a limitation of ROS production by the 4I2 under

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hypoxic conditions.[35] Recently, Reguera et al. and Cutanova et al. pointed out the importance of isoform imbalance for oxygen affinity of CytOx and ETC dysfunction.[47,60] So CytOx isoform 4I2 gene expression might be involved pathogenesis of AF by changing the oxygen affinity.

Patients' selection and characterization

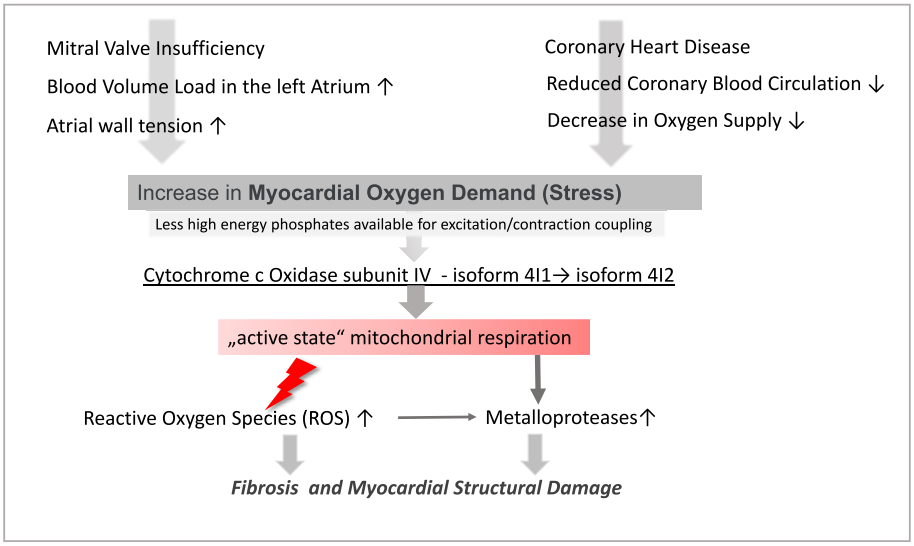
Under surgeon's visual control of atrial movement (AF or SR, respectively) before cannulation of the right Atrium small myocardial biopsies were taken and immediately snap frozen. Enrolment was performed randomly beyond the indications for heart valve replacement or CABG surgery. According to the known increase of COX 4I1 dependency on glucose transporter 4 (Glut4) in a first step all patients with Diabetes mellitus type 2 were excluded.[61] So clinical status of the investigated people is characterized by having no differences in secondary diseases and medication according to the case histories (see *Table 1*).

In agreement with the results in cell culture, we also found an increased COX 4I2/4I1 ratio in the examined myocardial samples as an expression of hypoxia (*Figure 2*), but the HIF-1α- and VEGF B expressions do not show any difference, so that this finding in the patient groups can be interpreted as indicating a more chronic hypoxic state. When the patients' data were regrouped (cluster analysis), there were also no differences in HIF-1α- and VEGF B expression depending on the extent of coronary artery disease (CIHD, 0–1 vessel vs. 2–3 vessel). Even excluding the presence of diabetes mellitus or high-grade CIHD or status of acute or chronic hypoxia, these expression patterns did not differ. Data of echocardiography revealed consistent left ventricular ejection fractions, end-diastolic diameter and intraventricular septum size, but a bigger left atrial end-systolic diameter in the group of AF, although with weak statistical power (*Figure S3*). Nevertheless, in these special cases, according to Laplace's law, an increased wall stress can be assumed.

Atrial fibrillation and oxidative stress

Initiation and maintenance of AF is associated with structural, electrochemical, and metabolic changes. A link between mitochondrial dysfunction and AF was previously shown several times.[12,62] Emelyanova et al. describe a selective reduction in respiratory chain activity in AF, which is associated with increased generation of ROS, leading to oxidative stress.[12] Progression of AF may result from the increased oxidative stress. The existing dysfunctional mitochondrial respiration in cardiac surgery patients showed an increased postoperative risk of developing atrial fibrillation.[62] This is corresponding to Takano et al., who found impaired oxygen uptake and hemodynamics in hearts of patients with atrial fibrillation during heavy exercise.[63] Serum analyses of patients who developed postop-

Figure 5 A suggested two-way model for the pathophysiology of AF: Mitral valve insufficiency result in congestive loading of the atria with subsequent increased wall tension and higher oxygen consumption. Increased blood volume load increases myocardial oxygen demand because of further excitation/contraction coupling requires more ATP consumption. Maximum oxygen supply fails to cover the metabolic needs. The resulting relative hypoxia induces a 'heart in stress' and increased isoform 4I2 gene expression and action of LON proteases[83] for 4I1 degradation. In case of coronary heart disease (right side), the scenario resembles the same conditions. Because of a reduced coronary reserve of blood circulation, the oxygen supply is decreased. The change in the 4I2/4I1 ratio is an hypoxic sensor but also induces an 'active state' mitochondrial respiration[17] with a production of reactive oxygen species (ROS) doing harm to the myocardium and a subsequent stimulation of metalloproteinases involved in fibrosis and myocardial structural damage.



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erative AF showed increased expression of genes encoding inflammatory markers, such as transforming growth factor-β (TGF-β), tumour necrosis factor-α-induced protein 6 (TNFAIP6), and nuclear factor-κB (NF- κB). The cause of mitochondrial dysfunction in AF may be an inflammatory process. In addition, data showed an increase in markers of oxidative stress and apoptosis, as well as a restriction in fatty acid metabolism.[64] Many inflammatory markers, such as CRP, interleukin-6 (IL-6), TNF-α, interleukin 8 (IL-8), have been shown to be increased in association with AF.[16] Inflammatory processes thus condition oxidative stress and impaired fatty acid oxidation, both resulting in mitochondrial dysfunction.[64,65] This dysfunction leads to ATP depletion and thus energy deficit.

Atrial dilation

Atrial stretch is one of the most important risk factors for the development of atrial fibrillation. Chronic stretching of the Atria causes alteration of signalling pathways although stretching of the Atrial myocardium results more pronounced responses than stretching of the ventricular myocardium.[66] Kalifa et al. showed that increased intra-atrial pressure results in increased generation of excitation waves on pulmonary veins.[67] Likewise, a shortening of the refractory period caused by stretch has been described as causative of increased vulnerability to AF.[68] The structural changes in the myocardium caused by pressure and volume stress are induced by an angiotensin II-dependent and independent mechanism.[9] Mechanical stress activates extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK), with only ERK being activated by angiotensin II.[69] ERK in turn activates profibrotic signalling pathways and leads to structural remodelling.[70] Cardiomyocyte energy metabolism was also shown to be impaired by stretch-induced AF.[71] Likelihood appears high when endoluminal atrial stretch results in increased pressure on the myocardial wall suppressing physiologic blood circulation for reduced oxygen supply. The relative hypoxia results in an expression of subunit 4I2. However, the question remains open whether the changes are a consequence or at least the cause of AF.

Conclusions

Under the experimental condition of a cell culture with HeLa cells, we could detect and confirm the mRNA expression of COX 4I2 in response to hypoxia (*Figure 1*). Using myocardial biopsies of atrial tissue, we detected an altered ratio of mRNA expression of the isoforms 4I2/4I1 (*Figure 2*). Corresponding protein concentrations in subunit IV of cytochrome c oxidase, the 'pacemaker' enzyme of the respiratory chain, were identified (*Figure S3*). The functional activity of the enzyme appears to be the same (*Figure 4*), but no respiratory control by calcium ions could be detected as under physiological conditions.

Increased mRNA expression of COX 4I2 was already shown to be increased in hypoxia.[25]

The lower HIF1α expression after hypoxia can be interpreted to mean that acute, induced hypoxia causes a short-lived increase, but that this increase is reversed after induction of a cascade of hypoxia proteins or as a transition to the expression of HIF-2. Comparison of myocardial tissue samples from patients with AF and sinus rhythm did not reveal any difference in mRNA expression between HIF-1α and VEGF-B. As both groups of patients showed cardiac disease, it was postulated in this case as it is more likely a chronic hypoxic state, in which hypoxic-induced reactive gene expression and neoangiogenesis occur in parallel. Nevertheless, it could be shown that a bigger COX 4I2/4I1 relation was also detectable in the ex vivo studies. Thus, an altered mitochondrial respiration and a change in energy metabolism in AF can be assumed. Weather an increased expression of isoform 4I2, also induced by HIF-1α is thought to ensure more efficient energy production by CytOx[32] or not remains. According to Emelyanova et al., the functional activity of cytochrome c oxidase does not change in AF.[12] Functional activity is thus maintained despite hypoxia. But, as we have found, a regulative effect of calcium could no longer be detected in the AF group.

Increased mechanical stress on the atria (stretching) is thought to result in impaired myocardial blood flow. It has been already shown that myocardial blood flow is impaired in patients with AF and atrial strain both before and after successful ablation.[72] During AF, coronary reserve is reduced, especially of the subendocardial layer.[73] The linear regression in our study suggests that isoform 4I2 expression increases with larger atrial diameter in AF. However, a larger number of patients is needed to illustrate this trend.

During acute onset of angina pectoris symptoms an increase of reactive oxygen species (ROS) in the serum of patients is found,[74] as evidenced by a marked reduction of serum antioxidant capacity. Initiation of neovascularization is mediated by ROS, this induces an increase in VEGF.[75–77] In a further study, ROS release in serum of patients with AF should be investigated in dependence on a COX 4I2/4I1 mRNA expression to detect myocardial mitochondrial dysfunction. At last but not least the role of Diabetes for 4I2/4iI1 mRNA expression remains unclear.

With the introduction of a changed COX 4I2/4I1 ratio, a molecular correlate between sinus rhythm and atrial fibrillation became apparent. The different 4I2/4I1 quotient is also obviously due to a reduction of isoform 4I1. However, this observation does not contradict the assumption of increased 4I2 expression because there is a degradation of COX 4–1 and, in addition, an induction of isoform 4I2. The degradation of isoform 4I1 occurs through a HIF-1α-induced mitochondrial LON protease, whereas there is an increased expression of isoform 4I2, also induced by HIF-1α. These results argue against a housekeeping function of isoform 4I1 in human tissues.[25]

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In *Figure 5*, we have suggested a two way- model for the pathophysiology of AF with special relevance of 4I2/4I1 switch and ROS production doing further harm to the myocardial tissue and sustains chronicity of AF.

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Conflict of interest

None declared.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. A-D. Normalized mRNA expressions of HIF-1α and VEGF **(A)** in the group of patients with *sinus rhythm* (n = 13) and those with *atrial fibrillation* (n = 7) were compared (*$P < 0.05$). Patients with diabetes mellitus were excluded. No significance according to Mann–Whitney U test (HIF *P = 0.874*, VEGF *P = 0.874*). **(B)** Normalized mRNA expressions of HIF-1α and VEGF of the patient group with *0–1-vessel CHD* (n = 11) and *2–3-vessel CHD* (n = 14) were compared. Again, patients with diabetes mellitus were excluded. No difference were found (HIF *P = 0.180*, VEGF *P = 0.763*). Testing the expressions *of HIF-1α and VEGF* in the group of patients with *sinus rhythm* (n = 4) and those with *atrial fibrillation* (n = 4) without patients with diabetes mellitus and the patients *with 2- or 3-vessel CHD* **(C)** resulted also in no difference (HIF *P = 0.921*, VEGF *P = 0.306*). Finally, the comparison between expressions of *CytOx 4, isoform 4I1 and CytOx4 isoform 4I2*, of the patient group with *acute hypoxia* (VEGF/HIF < 1) (n = 11) and *chronic hypoxia* (VEGF/HIF > 1) (n = 14) had no significance according to Mann–Whitney U test. (Cox4I1 *P = 0.978*, Cox4I2 *P = 0.119*).

Figure S2. A–E. Normalized mRNA expressions of CytOx 4, isoform 1, and CytOx4, isoform 2, of the patient group with 0–1-vessel CIHD (n = 11) and that with 2–3-vessel CHD (n = 14) were compared **(A)** (*$P < 0.05$). Patients with diabetes mellitus were excluded. No significance according to Mann–Whitney U test (Cox4I1 *P = 0.529*, Cox4I2 *P = 0.978*). Normalized mRNA expressions of CytOx4, isoform 1, and CytOx4, isoform 2, of the patient group with sinus rhythm (n = 4) and that with atrial fibrillation (n = 4) were contrasted. Patients with diabetes mellitus *and patients with 2- or 3-vessel CAD* were excluded **(B)**. No significance by Mann–Whitney U test. (Cox4I1 *P = 0.152*, Cox4I2 *P = 0.209*). **(C)** Normalized mRNA expressions of HIF-1α, VEGF, CytOx4, isoform1, and CytOx4, isoform 2, of the patient group without preoperative angina pectoris symptomatology (n = 13) and that with angina pectoris symptomatology (n = 11) were compared. Significance of expression of VEGF (*P = 0.024*) by Mann–Whitney-U-Test. (HIF *P = 0.354*, Cox4I1 *P = 0.183*, Cox4I2 *P = 0.202*). **(D)** Normalized mRNA expressions of HIF-1α, VEGF, CytOx4, isoform1, and CytOx4, isoform 2, of the patient group with (n = 13) and without (11) receiving preoperative statins, but no Diabetes mellitus, were compared. Although a trend suggests differences in expression of 4I2 (*P = 0,072*) no differences were found (HIF *P = 0,871*, VEGF *P = 0,772*, COX4I1 *P = 0,271*). (E) The normalized mRNA expressions of HIF-1α, VEGF, CytOx4, isoform1, and CytOx4, isoform 2, of the patient group without diabetes mellitus (n = 25) and that with diabetes mellitus (n = 16) were compared. No significance according to Mann–Whitney U test (HIF *P = 0.329*, VEGF *P = 0.267*, COX4I1 *P = 0.204*, COX4I2 *P = 0.329*). **(F)** The normalized mRNA expressions of HIF-1α, VEGF-B, CytOx 4, isoform 1, and CytOx4, isoform 2 of the patient group with sinus rhythm (n = 9) and that with atrial fibrillation (n = 3) of patients with diabetes mellitus were compared. No significant differences by Mann–Whitney U test, but a trend of higher CytOx 4I1 expression (*P = 0.064*). (HIF *P = 0.276*, VEGF *P = 0.460*, COX 4I2 *P = 0.683*) is assumed.

Figure S3. Plot of linear regressions of the different subgroups with sinus rhythm and atrial fibrillation. The expression of isoform 2 of CytOx was plotted as a function of left atrial end-diastolic diameter, which was determined preoperatively by echocardiography. In the group with sinus rhythm, we find a correlation of *R = 0.253* with a power of *0.109*. In the group with atrial fibrillation, we find a correlation of R = *0.296* with a power of *0.063*, so a trend is seen, only.

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