Atrial high energy phosphate content and mitochondrial enzyme activity during chronic atrial fibrillation

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Abstract

Objective: Prolonged atrial fibrillation (AF) results in (ultra)structural remodelling of atrial cardiomyocytes resembling alterations seen in ischemia-induced ventricular hibernation. The mechanisms underlying these changes are incompletely understood. In the present study we explored the hypothesis that a profound imbalance in energy status during chronic AF acts as a stimulus for structural remodelling.

Methods and Results: The content of high energy-phosphates and related compounds together with a selected number of mitochondrial enzymes, known to be altered under ischemic conditions, were determined in tissue samples taken from atria of goats in sinus rhythm (SR) and after 1, 2, 4, 8 and 16 weeks of AF maintained by burst pacing. Atrial remodelling was quantified by counting the percentage of cells with >10% myolysis. During AF structural remodelling developed progressively, after 8 weeks about 40% of the atrial myocytes were affected. The concentration of adenine nucleotides and their degradation products did not change significantly during AF. Also the activity of mitochondrial cytochrome c oxidase activity was similar during AF and SR. Mitochondrial NADH-oxidase and proton-translocating ATPase activities were not induced by AF. The tissue content of phosphocreatine decreased during the first week by 60%, but completely recovered between 8 and 16 weeks of AF.

Conclusions: The analysis of adenine nucleotides during AF provided no indication for the development of severe atrial ischemia. This notion is supported by enzyme cytochemical findings. However, AF-induced atrial remodelling was associated with a transient lowering of phosphocreatine content, suggesting an increase in energy demand during the early phase of AF. The subsequent recovery of the phosphocreatine pool indicates restoration of the balance between energy demand and supply in chronically fibrillating atria.

Keywords: Atrial function; Energy metabolism; Hibernation; Mitochondria; Remodeling; Supraventricular arrhythmia

1. Introduction

Atrial fibrillation, the most common cardiac arrhythmia, is electrocardiographically characterized by irregular and disorganized atrial activity. In patients, AF often starts as paroxysms, the duration of which gradually increases with time [1]. Chronic rapid atrial pacing in dogs and repeated induction of AF in goats have been shown to produce electrophysiological changes in the atria leading to progressive lengthening of the duration of AF, i.e. AF begets AF [2,3].

Recently, we reported a number of (ultra)structural changes in the atrial myocytes secondary to prolonged AF in the goat [4]. This structural remodelling included redistribution of nuclear chromatin, perinuclear loss of sarcomeres and sarcoplasmic reticulum, accumulation of glycogen and an increase in the number of small abnormally shaped mitochondria. Degenerative changes were not observed. Instead the atrial myocytes showed a shift towards a fetal phenotype (dedifferentiation).
The mechanisms underlying this AF-induced structural remodeling process are incompletely understood. It is interesting to note that the ultrastructural changes closely resemble the changes occurring in ventricular myocytes subjected to prolonged low-flow ischemia (chronic hibernating myocardium) [4–7]. However, it is not known whether AF induced remodelling is equally due to an imbalance in energy supply and demand. This may be a feasible explanation since White and co-workers [8,9] showed that the atrial oxygen consumption and coronary flow increased almost three-fold after induction of AF. As a result the atrial coronary flow reserve decreased markedly to 39% of its control value.

In the present study we explored the hypothesis that structural remodelling due to chronic AF is caused by a severe mismatch between energy supply and demand. During 16 weeks we measured the atrial content of phosphocreatine and adenine nucleotides together with the activity of some mitochondrial enzymes known to change during cardiac ischemia [10–13].

2. Methods

2.1. The goat model of chronic atrial fibrillation

We used the goat model of chronic atrial fibrillation as developed by Wijffels et al. [2]. Under general anesthesia (halothane (1–2%) and N₂O) an Irel-pacemaker (Medtronic®) was implanted in the neck of the animal. A bipolar screw-in electrode was inserted through the jugular vein and fixed to the right atrial appendage. One week after surgery, the pacemaker was switched on and AF was maintained by burst stimulation (2 s of bursts of 50 Hz at four times diastolic threshold). Since the duration of AF paroxysms progressively prolonged also the interval between the bursts could be gradually prolonged to one burst every half hour [2]. A total of 36 female goats of 61 ± 13 kg were included in this study. They were divided in six groups of six animals. One group, kept in sinus rhythm served as a control group, whereas in the other groups AF was maintained for 1, 2, 4, 8 and 16 weeks, respectively. Animal handling was carried out according to the Dutch Law on Animal Experimentation (WOD) and The European Directive for protection of vertebrate animals used for experimental and other scientific purposes.

2.2. Tissue processing

At the end of the experimental period, the goats were anaesthetized and heparin was given intravenously (5000 IU). Thoracotomy was performed and the right atrial appendage was excised and immediately frozen by a liquid nitrogen cooled aluminum clamp. These frozen samples were used for determination of high-energy phosphates and related compounds. The heart was then excised and fixed by retrograde perfusion with 3% glutaraldehyde in a 90 mM potassium phosphate buffer (pH 7.4). A number of small samples (4 mm³) was taken from the right and left atria and kept for at least 24 h in the glutaraldehyde solution. The tissue samples were embedded in Epon after postfixation as described below.

For the analysis of mitochondrial enzyme activity a second set of glutaraldehyde fixed atrial samples were stored after 15 min of fixation in 90 mM potassium phosphate buffer (pH 7.4) at 4°C.

2.3. Morphological evaluation

For morphological assessment, tissue blocks fixed for at least 24 h were washed with 90 mM potassium phosphate containing 7.5% sucrose, postfixed with 2% osmium tetroxide in 50 mM veronal acetate buffer for 1 h, dehydrated through graded ethanol series, and routinely embedded in the epoxy-resin Epon [14].

Light microscopic examination was performed on 2-μm thick sections stained with periodic acid Schiff (PAS) and 0.1% toluidine blue to color cytoplasmic glycogen (red) and the myofibrils (blue). The degree of myolysis was evaluated by planimetry in cells in which the nucleus was present in the plane of the section. An atrial myocyte was affected by myolysis if >10% of the cytoplasm was free from sarcomeric material. A minimum of 300 cells, from three different regions of the right atrial appendage were evaluated [5]. To assess the area of connective tissue, morphometry was carried out with a special grid. The total number of intersections counted in the connective tissue areas was expressed as the percentage of total number of intersections [5].

For the electron microscopic evaluation of structural changes in the atrial myocardium (Philips CM 100 microscope), ultrathin sections cut from each Epon-embedded sample were counterstained with uranyl acetate and lead citrate [5].

2.4. Enzyme cytochemistry

For determination of mitochondrial enzymes, 30-μm thick frozen sections were cut. Cytochrome c oxidase activity was evaluated in sections incubated for 30–60 min in 9 ml of Seligman’s medium (0.05 M sodium phosphate buffer (pH 7.4), 5 mg 3,3’-diaminobenzidine-4-hydrochloride, 20 mg catalase, 10 mg cytochrome c and 0.22 M sucrose) [15] at 37°C.

For determination of proton translocating ATPase activity the sections were incubated for 15–30 min at 37°C in a solution containing 48 mM Tris–maleate buffer, 3 mM lead citrate, 2.3 mM ATP, 5 mM MgSO₄ and 7.5% sucrose [12,16].

The procedure to determine NADH-oxidase activity present in the extramitochondrial space has been described by Briggs et al. [17]. The sections were cut and preincu-
bated for 10 min in a solution containing 0.1 M Tris-
maleate buffer (pH 7.5), 7% sucrose and 1 mM amin-
triazole. Subsequently the preparations were incubated at
37°C for 120 min in medium containing 0.1 M Tris-
maleate buffer (pH 7.5), 7% sucrose, 1 mM cerium
chloride, 0.71 mM NADH and 10 mM aminotriazole [12].
Following cytochemical incubation, the sections were
postfixed with osmium tetroxide, dehydrated in graded
series of ethanol and routinely embedded in Epon. Ul-
trathin sections, either unstained or briefly counterstained
with uranyl acetate and lead citrate, were examined by
electron microscopy. Mitochondrial enzyme activity was
evaluated from the presence of intense dark precipitates
along the mitochondrial membranes (cytochrome c
oxidase) or in the mitochondrial matrix (NADH-oxidase or
proton translocating ATPase). Qualitative changes in the
amount and intensity of the precipitate were determined on
electron microscopic images by comparing groups of goats
in sinus rhythm versus the different AF durations.

2.5. High energy phosphates and related compounds

Frozen tissue samples of the right atrial appendage were
freeze-dried overnight with a GT2 freeze-dryer (Lebold
Heraeus, Köln, Germany). After freeze-drying, adherent
blood and connective tissue were removed. Dry samples of
approximately 10 mg were used for determination of
adenine nucleotides and related compounds by high-per-
formance liquid chromatography (HPLC) using a modified
method of Wijnants and Van Belle [18,19]. Tissue phos-
phocreatine and creatine content were also determined by
HPLC [20].

2.6. Statistical analysis

Myolytic cell changes and difference in content of
high-energy phosphates and related compounds were tested
for statistical significance by means of the Wilcoxon–
Mann–Whitney rank test (StatXact program). P values of
less than or equal to 0.05 were considered to be statistically
significant.

3. Results

3.1. AF-induced (ultra)structural remodelling

During sustained AF atrial myocytes showed a variety
of structural changes, including disappearance of sarco-
meres (myolysis) and accumulation of glycogen (Fig. 1).
The percentage of myocytes showing structural changes
increased progressively during AF. Whereas during normal
sinus rhythm less than 10% of the atrial myocytes showed
a slight degree of myolysis, after 16 weeks of AF more
than 50% of the myocytes had lost >10% of their sarcomeres (Fig. 5a). This progressive increase was

3.2. Enzyme cytochemistry

3.2.1. Cytochrome c oxidase activity

In atrial myocytes from goats in sinus rhythm, cyto-
chrome c oxidase activity is confined to the mitochondrial
cristae as can be seen from the dark precipitate along the
inner mitochondrial membrane (Fig. 2a,b). After 1 week of
AF, the cytochrome c oxidase activity of the mitochondria
was still normal (Fig. 2c,d). After 1 week of AF the
number of abnormal mitochondria (smaller size, reoriented
cristae) had increased. However, also in these mitochondria
the cytochrome c oxidase activity was still normal (Fig.
2c,d). Even after 16 weeks of AF both the normal and
structurally altered mitochondria showed their dark label-
ing of cytochrome C oxidase activity (Fig. 2e,f).

3.2.2. Proton-translocating ATPase and NADH-oxidase
activity

In goats in normal sinus rhythm the activity of proton-
translocating ATPase and NADH-oxidase in the mito-
ochondrial matrix of atrial myocytes was below the level of
detection. Also during 16 weeks of AF, the proton-trans-
locating ATPase and NADH-oxidase activity remained
undetectable both in normal and abnormal mitochondria
(Figs. 3 and 4). In contrast cardiomyocytes from ischemic
myocardium in the dog showed an intense NADH-oxidase
and proton translocating ATPase reaction product (Figs. 3c
and 4c).

3.3. The content of high-energy phosphates and related
compounds

The phosphocreatine content of atrial myocytes (33.6
μmol/g dry weight during SR) was decreased by 60%
after 1 week of AF (P<0.02). This level remained low
during the first 8 weeks of AF but returned to normal
values between 8 and 16 weeks of AF (Table 1 and Fig.
Fig. 1. Light microscopy of atrial myocardium stained with periodic acid Schiff (PAS) and toluidine blue (TB). (a) PAS positive material (glycogen) is almost absent within the cardiomyocytes from goats with sinus rhythm. The sarcomeres stained with TB are present throughout the cytoplasm of the cardiomyocytes (×820). (b) Section of a goat with 2 weeks of atrial fibrillation showing a mild degree of myolysis and glycogen accumulation (PAS positive) (×820). (c) Section of myocardium from a goat with 16 weeks of atrial fibrillation. Most cardiomyocytes show severe myolysis and abundant plaques of glycogen (×820).

The creatine content did not change significantly during AF (Fig. 5c). The sum of phosphocreatine and creatine showed a downward trend from 56.8 to 38.3 µmol/g dry weight after 1 week of AF (P<0.18) (Table 1). The total creatine pool had returned to normal levels at week 16 (Table 1 and Fig. 5c). Also, the tissue content of the adenine nucleotides (ATP, ADP and AMP) showed a tendency to decrease in fibrillating atria, but these changes did not reach statistical significance (Table 1). The concentration of the degradation products of adenine nucleotides (adenosine, inosine, hypoxanthine, and xanthine) were very low and did not differ significantly from control tissue (data not shown). The content of GTP, GDP and NAD did not change significantly during AF (Table 1). Neither did the level of connective tissue. The unaltered NAD and connective tissue contents (see above) strongly suggest that the number of myocytes did not change during atrial fibrillation.

4. Discussion

We have recently shown that sustained AF results in structural remodelling of the atrial myocardium [4]. Similar changes were observed during chronic low flow ischemia in the ventricle, the so-called hibernating myocardium [4,5]. Although, the cellular changes in hibernating ventricular myocytes are most likely caused by energy depletion during low flow ischemia, in case of atrial fibrillation the stimulus for structural remodelling is unknown. An enhanced lactate production was previously found in patients with chronic AF. This led to the suggestion that atrial ischemia occurs during AF [21]. However, in a later study enhanced lactate production by the fibrillating atria could not be confirmed [22]. Jayachandran et al. [23] showed in dogs that after 4 weeks of pacing-induced chronic AF the atrial coronary blood flow was reduced to 78%. It is unknown whether this reduction in atrial blood flow is associated with atrial ischemia.

In the current study we used cytochemical techniques to monitor alterations in the activity of mitochondrial enzymes (NADH-oxidase, proton-translocating ATPase and cytochrome c oxidase). These enzymes, are known markers of ischemia-induced uncoupling of the oxidative phosphorylation [12]. To explore whether chronic AF leads to an impairment of the energy status we further analyzed changes in atrial high energy phosphates and related compounds during 16 weeks of AF.
AF is associated with ischemia-induced changes upon atrial cardiomyocytes. This notion is further supported by the fact that cytochrome c oxidase activity was not diminished in hibernating atrial myocytes. Also other signs of severe ischemia such as swelling of mitochondria, depletion of glycogen stores, cellular edema, nuclear pyknosis and formation of contraction bands were absent. Based on enzyme cytochemistry and ultrastructural analysis we conclude that during the whole course of 16 weeks of AF, atrial ischemia does most likely not occur.

4.2. High-energy phosphates and related compounds

In ischemic myocardium, phosphocreatine and adenine nucleotides are known to deplete [25–27]. According to Jennings et al. [28] during profound ischemia the following changes occur: (1) the content of phosphocreatine and ATP are <10% of control, (2) the adenine nucleotide pool is depleted and relatively high levels of AMP, hypoxanthine and xanthine are present, (3) anaerobic glycolysis is
virtually absent and the glycogen stores are substantially reduced.

Biochemical analysis of homogenates of atrial samples from our goats in chronic atrial fibrillation showed no depletion of high energy phosphates and accumulation of degradation products as AMP, inosine or hypoxanthine. Light microscopy indicated certainly no depletion of glycogen. The only statistically significant change was the temporary lowering of the phosphocreatine content. It is of interest to note that Murakami et al. [29] detected a decrease in the phosphocreatine/ATP ratio in the absence of hypoxia as a result of an increased work load due to dobutamine infusion. Extending this finding to our observations on AF may indicate that the decline in phosphocreatine reflects an enhanced demand of high energy phosphates rather than an impaired energy production due to ischemia. Recent studies of Leistad and co-workers [30,31] indicate that the drop in the atrial phosphocreatine level occurs already within 25 min of AF. The fact that the tissue content of this high-energy phosphate stabilizes, shows that soon after the onset of AF a temporary shift in the metabolic balance occurs resulting in a situation with a lower phosphocreatine content. The stable phosphocreatine level up to 8 weeks of AF points towards a new steady

Table 1
High energy phosphates and related compounds in atrial tissue of goats with normal sinus rhythm and suffering from chronic atrial fibrillation

<table>
<thead>
<tr>
<th></th>
<th>Sinus rhythm (n=4)</th>
<th>1 (n=5)</th>
<th>2 (n=5)</th>
<th>4 (n=6)</th>
<th>8 (n=5)</th>
<th>16 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCr</td>
<td>33.7±8.8</td>
<td>13.8±5.6*</td>
<td>15.8±6.8*</td>
<td>17.0±8.3*</td>
<td>11.9±7.0*</td>
<td>33.3±9.6</td>
</tr>
<tr>
<td>Cr</td>
<td>23.2±10.9</td>
<td>24.5±16.9</td>
<td>23.0±12.8</td>
<td>25.0±13.0</td>
<td>22.5±14.8</td>
<td>19.7±10.4</td>
</tr>
<tr>
<td>P[Cr]+Cr</td>
<td>56.8±17.2</td>
<td>38.3±19.5</td>
<td>38.8±17.3</td>
<td>42.1±19.6</td>
<td>34.4±21.5</td>
<td>53.0±11.5</td>
</tr>
<tr>
<td>ATP</td>
<td>9.5±4.4</td>
<td>8.7±1.0</td>
<td>6.5±2.8</td>
<td>6.3±4.2</td>
<td>6.9±4.7</td>
<td>8.6±1.1</td>
</tr>
<tr>
<td>ADP</td>
<td>2.3±2.1</td>
<td>1.8±0.8</td>
<td>1.4±0.6</td>
<td>1.4±0.9</td>
<td>1.4±0.9</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>AMP</td>
<td>0.50±0.64</td>
<td>0.25±0.21</td>
<td>0.21±0.13</td>
<td>0.28±0.30</td>
<td>0.21±0.12</td>
<td>0.25±0.16</td>
</tr>
<tr>
<td>ATP+ADP+AMP</td>
<td>12.3±7.0</td>
<td>10.7±1.8</td>
<td>8.1±3.4</td>
<td>7.9±5.2</td>
<td>8.5±5.7</td>
<td>10.3±1.4</td>
</tr>
<tr>
<td>GTP</td>
<td>0.71±0.27</td>
<td>0.94±0.17</td>
<td>0.67±0.22</td>
<td>0.66±0.46</td>
<td>0.74±0.50</td>
<td>0.69±0.09</td>
</tr>
<tr>
<td>GDP</td>
<td>0.10±0.07</td>
<td>0.14±0.06</td>
<td>0.12±0.05</td>
<td>0.10±0.07</td>
<td>0.11±0.08</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>NAD</td>
<td>0.95±0.46</td>
<td>1.0±0.13</td>
<td>0.77±0.27</td>
<td>0.74±0.49</td>
<td>0.78±0.52</td>
<td>0.95±0.08</td>
</tr>
</tbody>
</table>

*PCr and Cr refer to phosphocreatine and creatine, respectively. Values are expressed as mean±S.D. Content is in μmol/g dry weight of tissue. * Significant difference vs. sinus rhythm (P<0.05).
Fig. 5. Degree of myolysis (a) and energy status (b–d) of the atrial myocardium during sinus rhythm and at 1, 2, 4, 8 and 16 weeks of AF. (a) Percentage of right atrial myocytes with >10% sarcomere free cytoplasm. (b) Atrial phosphocreatine content. (c) Atrial creatine content. (d) Atrial ATP content. Values represent mean±S.D. *P values <0.05 vs. sinus rhythm.

The state of the atrial energy metabolism. This notion was supported by the fact that atrial ATP and AMP levels remain unaltered. We have to take into account that in the here performed experimental set-up standard deviations are high, resulting from variations between individual animals, tissue heterogeneity, and small groups. However, if AF was associated with an ongoing energy supply–demand mismatch one might expect a continuous decline in atrial phosphocreatine content followed by depletion of the atrial ATP pool. This process does obviously not occur in the present preparation.

An unexpected finding was that between 8 and 16 weeks of AF the phosphocreatine content returned to control values. The temporary shift in atrial phosphocreatine content appears therefore to be biphasic. The reason for the phosphocreatine recovery is not clear. We may speculate that the delay in the recovery of the phosphocreatine is explained from the fact that between 8 and 16 weeks of AF a tapering off of the increase in the number of atrial myocytes with structural changes occurs. An explanation might be that the cellular remodelling of the atrial myocardium in itself is an energy demanding process, responsible for the lower phosphocreatine level. Alternatively, reduction of myocardial function after prolonged AF might be responsible for a lower energy demand which in turn can result in normalization of atrial phosphocreatine levels at 16 weeks of AF.

We conclude from the biochemical studies that ischemic injury is absent as can be deduced from normal ATP and other nucleotide levels. This assumption was further supported by the enzyme cytochemical studies on mitochondria enzymes. The decline in phosphocreatine after 1 week of AF indicates an initial shift in the energy status of the atrial myocardium during AF. A steady state for the next weeks most likely occurs. The recovery of phosphocreatine pool after 16 weeks of AF reflects a second metabolic shift in structurally altered atrial myocardium.

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