Reduction in cardiac mitochondrial calcium loading capacity is observable during α-naphthylisothiocyanate-induced acute cholestasis: a clue for hepatic-derived cardiomyopathies?

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Abstract

Cardiovascular changes of still obscure origin are sometimes correlated with co-existing liver diseases, as cholestasis. The aim of this work was to examine and compare cardiac mitochondrial bioenergetics and calcium loading capacity from rats injected with a single dose of α-naphthylisothiocyanate (ANIT), a cholestasis-inducing compound. Forty-eight hours after ANIT administration, blood samples were collected and markers for hepatic disease were determined. Heart mitochondria from both control and ANIT-injected rats were isolated and subjected to biochemical characterization, including the susceptibility to the calcium-dependent permeability transition. The results showed that cardiac mitochondria from cholestatic animals did not have significant changes in respiratory parameters or in the basal levels of adenine nucleotide. The most impressive result from this work was that cardiac mitochondria from ANIT-injected animals had a lower calcium loading capacity. The prevention of this property by cyclosporin-A, a specific inhibitor of the mitochondrial permeability transition, showed that this phenomenon was reason for the reduced calcium loading capacity in ANIT-injected animals. The results suggest that, during the development of ANIT-induced cholestasis, heart mitochondria lose their default ability to buffer calcium. Our results may contribute to explain the occurrence of cardiomyopathies sometimes associated with cholestatic disease.

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1. Introduction

Liver diseases are one of the leading causes of death in the world. Cholestasis, defined as impaired bile flow, occurs in many chronic human liver diseases. During this condition, many toxic hydrophobic bile salts normally secreted by the liver into bile are now accumulated inside the hepatocyte [1]. The accumulation of toxic hydrophobic bile salts is associated with liver failure, leading ultimately to biliary fibrosis and cirrhosis. The mechanisms underlying these pathological events are not fully clear. A possible reason for hepatic dysfunction may be an increased production of reactive oxygen species [2,3] or an increased susceptibility of hepatocytes to apoptotic cell death [4]. Mitochondria can be central points in bile salts-induced hepatic dysfunction. Hepatic mitochondrial membrane perturbations were already observed during cholestasis [5], leading to indirect toxic effects on the mitochondrial respiratory complexes [6–8]. Thus, the accumulation of bile salts in the hepatocyte cytoplasm may directly affect hepatic mitochondria. Some of bile salt-induced perturbations include the triggering of the mitochondrial permeability transition (MPTP), a phenomenon linked to the formation and opening of protein pores (the MPTP) in the inner mitochondrial membrane [9]. MPTP opening can be caused by excessive mitochondrial biogenic "..."
calcium accumulation and oxidative stress generation [10], and can lead ultimately to cell death [11]. Bile salts were already related to the MPT [11,12]. In fact, Gores et al. [12] proposed that the activation of mitochondrial proteases may initiate the MPT and cell necrosis during cholestasis. In apparent contradiction, cholestasis in bile duct-ligated rats was described conferring resistance to rat liver MPT [14]. The observations are not in contradiction with the general pro-inducing role of bile salts in the MPT if we recall that some bile salts can indeed confer protection against MPT induction [15,16]. The secondary plasma accumulation of bile salts was also proposed as a causative factor for hepatic cardiomyopathy and heart dysfunction [17]. An adverse effect of bile salts in the β-adrenoceptors density and affinity, as well as in the plasma membrane fluidity, was also already described [18], although the relevance of this work is questionable by the fact that the tested concentrations exceeded the highest concentrations seen so far in the plasma. Taurocholate, a tauroconjugated primary bile acid, was found to impair rat cardiomyocyte function, hindering the normal cellular calcium dynamics [19]. It is pertinent to question if cardiac mitochondria may be affected the same way as hepatic mitochondria by the increased amount of bile salts in the plasma. More particularly, in this work, we were interested in comparing the susceptibility of heart mitochondria to the permeability transition in α-naphthylisothiocyanate (ANIT)-induced cholestatic rats to verify if it affected mitochondrial calcium loading capacity. ANIT is known to selectively produce hyperbilirubinemia, cholestasis and bile duct obstruction [20], in relation with increased inflammatory response in the mostly affected areas [21]. As far as we know, no direct effect of ANIT in the cardiac tissue is known.

The objective of this work was to compare some aspects of heart mitochondrial bioenergetics, especially concerning the induction of the calcium-sensitive MPTP, in both ANIT-injected and control rats. Our results showed that cardiac mitochondrial permeability transition was enhanced in cholestatic animals, despite non-significative alterations on bioenergetic parameters. This type of effect was not mimicked by ANIT addition to isolated heart mitochondria. We hypothesise that mitochondrial dysfunction due to enhanced permeability transition may be one of the causes leading to cholestatic derived cardiomyopathy.

2. Materials and methods

2.1. Materials

All reagents and chemicals were of the highest grade of purity commercially available. Calcium Green 5-N was obtained from Molecular Probes, Eugene, OR. ANIT was dissolved in olive oil before use for in vivo administration or in dimethylsulfoxide for in vitro testing in isolated heart mitochondria.

2.2. Animals

Female Wistar rats (12–15 weeks) were maintained in our local colony (Laboratory Animal Research Center, University Hospitals, Coimbra) with ad libitum access to food (URF1-Charles River, France) and water at pH 4.5. The room temperature and moisture were kept at 22–24 °C and 50–60%, respectively. The animals were subjected to a 12 h day/night cycle. In this study, the “Principles of Laboratory animal care” (NIH publication no. 85–23, revised 1996) were followed.

2.3. Induction and characterization of cholestasis

Rats were randomly divided in two groups of seven animals each. Acute cholestasis was induced in one group of animals with a non-fasting intraperitoneal injection of ANIT, 100 mg/kg. Control animals were injected with olive oil alone. Animals were kept 48 h before the experiments. Rats were then anaesthetized (i.m.) with ketamin chloride (88.5 mg/kg, Parke-Davis USA) and chlorpromazine chloride (2.65 mg/kg, Lab. Victoria, Portugal) and their weight was recorded (anesthetics were without effect in our results; data not shown). An arterial blood sample (1 ml) was taken from the descending aorta for measurement of liver injury serum markers. The analysis was conducted using commercial kits (Olimpus, Japan-Novadia and Beckmann, Eus-Izaza). Non-used injected animals survived the single ANIT administration.

2.4. Isolation of mitochondria from rat heart

Forty-eight hours after ANIT administration, one animal of each group were sacrificed and hearts excised. Rat heart mitochondria from both rats were prepared as previously described [22]. Mitochondrial protein content was determined by the biuret method calibrated with BSA.

2.5. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (ΔΨ) was estimated with a TPP+ electrode according to the equation of Kamo et al. [23], without correction for the “passive” binding contribution of TPP+ to the mitochondrial membranes (as the purpose of the experiment was to show relative changes in the potential rather than absolute values). A matrix volume of 1.1 μl/mg protein was assumed and valinomycin was used to calibrate the basal line. Reactions were carried out at 25 °C in 1 ml of the reaction media (200 mM sucrose, 10 mM TRIS–MOPS, 10 μM EGTA, 5 mM KH2PO4 and 2 μM rotenone, pH 7.4) supplemented with 1 μM TPP+, 0.25 μg oligomycin and 0.5 mg of mitochondria. Mitochondria (0.5 mg) were energised with 4 mM succinate. ΔΨ values before and after the addition of a calcium pulse were determined.
Table 1  
<table>
<thead>
<tr>
<th></th>
<th>Control animals</th>
<th>ANIT-injected animals</th>
<th>Statistical significance</th>
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<tbody>
<tr>
<td>Total proteins (g/dl)</td>
<td>7.72 ± 0.09</td>
<td>6.74 ± 0.19</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.55 ± 0.06</td>
<td>2.64 ± 0.09</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Serum alanine aminotransferase (U/l)</td>
<td>54.17 ± 3.39</td>
<td>338.14 ± 48.22</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>136.50 ± 9.67</td>
<td>284.29 ± 25.82</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Gama-glutamyltranspeptidase (U/l)</td>
<td>0.17 ± 0.17</td>
<td>3.86 ± 0.14</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.10 ± 0.00</td>
<td>5.11 ± 0.29</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.00 ± 0.00</td>
<td>3.39 ± 0.19</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Serum aspartate aminotransferase (U/l)</td>
<td>117.33 ± 18.95</td>
<td>419 ± 63.31</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>205.43 ± 12.99</td>
<td>193.14 ± 12.99</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

Plasma was obtained from blood samples collected at the day of the experiments. Biochemical analyses were performed using commercial kits, as described. Values are means ± S.E. of seven animals tested. Blood analyses were compared using an unpaired t-test. Body weight was compared with a paired t-test.

2.6. Mitochondrial oxygen consumption

Oxygen consumption of isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder simultaneously with ∆Ψ measurement. Reactions were carried out, at 25 °C, in 1 ml of the reaction media composed by 200 mM sucrose, 10 mM TRIS-MOPS, 10 μM EGTA, 5 mM KH2PO4 and 2 μM rotenone, pH 7.4. Mitochondria were suspended at a concentration of 0.5 mg/ml in the respiratory medium. State 4 respiration was measured in the presence of 4 mM succinate. ADP (100 nmol) was added to induce state 3 respiration. The respiratory control ratio (RCR) was calculated as the ratio between state 3 and state 4 respiration. The ADP/O was calculated as the number of nmol ADP phosphorylated by natom oxygen consumed.

2.7. Extramitochondrial calcium movements

Extramitochondrial free Ca2+ was measured with the hexapotassium salt of the fluorescence probe Calcium Green 5-N [24]. Heart mitochondria (0.2 mg) were suspended in 2 ml of buffer containing 200 mM sucrose, 10 mM TRIS-MOPS, 10 μM EGTA (for complexing basal calcium), 1 mM KH2PO4, 2 μM rotenone, 4 mM succinate and 0.05 μg oligomycin. Free Ca2+ was monitored in the presence of 100 nM Calcium Green 5-N. Fluorescence was recorded continuously in a water-jacketed cuvette holder at 25 °C using a Perkin-Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively.

2.8. Quantification of adenine nucleotides

ATP and ADP were extracted using an alkaline extraction procedure and were separated by reverse-phase high performance liquid chromatography. The chromatographic apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector, controlled by computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100RP-18 (5 μm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH2PO4), pH 6.5 and methanol 1% was performed with a flow rate of 1 ml/min. The time required for each analysis was 5 min.

2.9. Statistical analysis

The results are presented as mean ± S.E. of at least five independent experiment days. Statistical analyses were performed using two-tailed unpaired t-tests. A P value < 0.05 was considered statistically significant. In some cases, we present representative recordings of six independent days, with one mitochondrial preparation from each group tested simultaneously.

3. Results

The results of biochemical analysis and body weight from both animal groups are summarised in Table 1. All

Table 2  
<table>
<thead>
<tr>
<th></th>
<th>Control animals</th>
<th>ANIT-injected animals</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔΨ (– mV)</td>
<td>215.8 ± 1.9</td>
<td>213.2 ± 2.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>State 4</td>
<td>89.95 ± 14.14</td>
<td>93.78 ± 14.30</td>
<td>n.s.</td>
</tr>
<tr>
<td>State 3</td>
<td>264.73 ± 9.29</td>
<td>221.35 ± 37.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>RCR</td>
<td>3.28 ± 0.41</td>
<td>2.63 ± 0.38</td>
<td>n.s.</td>
</tr>
<tr>
<td>ADP/O</td>
<td>1.75 ± 0.05</td>
<td>1.88 ± 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>Basal ATP/ADP</td>
<td>0.52 ± 0.07</td>
<td>0.50 ± 0.06</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

State 4 respiration was measured in the presence of 8 mM succinate (plus 4 μM rotenone). ADP (100 nmol) was added to induce state 3 respiration. The RCR was calculated as the ratio between state 3 and state 4 respiration. The ADP/O was calculated as the number of nmol ADP phosphorylated by natom oxygen consumed.

Values are means ± S.E. of five separate experiments and were compared using a two tailed unpaired t-test.
markers of hepatic liver disease were increased in the plasma of ANIT-injected animals. Total bilirubin concentration, a cholestasis index, was significantly higher in the ANIT-injected animals than in control animals. A decrease in the total protein content and serum albumin was also observed in ANIT-injected animals, as well as a decrease in body weight of 15.14 ± 4.69 g.

Heart mitochondria were isolated from both control and ANIT-injected animals and respiratory parameters were characterised, as well as the basal ATP/ADP ratio, determined 4 h after the isolation procedure (Table 2). As seen, no significant differences were obtained in the studied parameters. The ADP/O ratio (number of nmol ADP phosphorylated per natoms O consumed) was slightly but not statistically increased in ANIT-injected animals. Also, no significant differences were seen in the basal ATP/ADP charge.

The maintenance of mitochondrial ∆Ψ levels after calcium addition indicated mitochondrial tolerance to calcium addition. A representative recording of the differences between both groups regarding mitochondrial ∆Ψ fluctuations is pictured in Fig. 1, left panel. After the addition of one calcium pulse, mitochondria from control and ANIT-injected animals suffered a depolarisation motivated by the entry of positive charges inside the mitochondrial matrix. Control heart mitochondria were able to repolarise to normal values after calcium accumulation; in opposition, their counterparts from ANIT-injected animals were not able to repolarise and ∆Ψ was eventually lost. This complete loss of mitochondrial ∆Ψ was related to MPTP induction, as

Fig. 1. Left panel: Typical recording of mitochondrial electric potential measured with a TPP⁺-selective electrode. Calcium (600 μM) was added in order to induce the MPTP. The ∆Ψ was calculated as described. Mitochondrial energization was achieved with 4 mM succinate. Trace A: Heart mitochondria from cholestatic rats in the presence of 0.5 μM cyclosporin-A. Trace B: Heart mitochondria from control rats. Trace C: Heart mitochondria from cholestatic rats. Mitochondrial ∆Ψ was measured simultaneously with oxygen consumption. The traces are representative of five different experiment days, with one sample of each group tested. Right panel: Statistical comparison between mitochondrial ∆Ψ developed by succinate-energized mitochondria from control and ANIT-injected animals before (black bars) and 3 min after calcium addition (white bars), in the presence and absence of 1 μM cyclosporin-A (CyA). *P<0.05 vs. control + calcium + cyclosporin-A, **P<0.001 vs. ANIT (without calcium) vs. ANIT + calcium + cyclosporin-A and vs. control + calcium, n=5.

Fig. 2. Left panel: Extramitochondrial calcium movements measured with the fluorescent calcium-sensitive probe Calcium Green 5-N. Extramitochondrial calcium was determined as described in Materials and methods. Trace A: Heart mitochondria from cholestatic rats. Trace B: Heart mitochondria from control rats. The recording shown here was typical of six different experiment days, with one sample of each group tested. Right panel: Extramitochondrial calcium concentration 1600 s after calcium addition for the two groups in the presence and absence of cyclosporin-A (CyA). *P<0.001 vs. control and vs. ANIT + cyclosporin-A, #P<0.001 vs. control + cyclosporin-A, n=6.
cyclosporin-A, the specific MPTP inhibitor [9,10], was able to prevent the ΔΨ loss in mitochondria from ANIT-injected animals. Cyclosporin-A was also able to slightly increase the ΔΨ repolarisation in cardiac mitochondria from the control group (Fig. 1, right panel). It is noticeable that no significant differences between the ΔΨ developed by both mitochondrial groups were found in the absence of calcium (Fig. 1, right panel).

In concordance with ΔΨ results, energised heart mitochondria from ANIT-injected rats were not capable to accumulate calcium (using 200–300 nmol calcium pulses per mg protein) the same way as control mitochondria did (Fig. 2, left and right panel). In the presence of cyclosporin-A, both mitochondrial populations were able to accumulate the same calcium amount (right panel). As before, cyclosporin-A also increased calcium accumulation in the control group (Fig. 2, right panel).

A strong possibility for the observed results would be a possible direct toxic effect of ANIT in the cardiac mitochondrial population. We added ANIT to a suspension of isolated heart mitochondria and tested their capacity to sustain ΔΨ values after calcium pulses. In fact, ANIT only showed toxic effects for concentrations near 1 μmol/mg protein (500 μM in the reaction buffer, totally outside the range involved in our in vivo assays) (data not shown). Even for those concentrations, the addition of EGTA and the presence of cyclosporin-A were unable to prevent ANIT toxic effects (data not shown).

4. Discussion

In our study, a single dose of ANIT caused an increase in the serum levels of hepatic enzymes, a known marker for cholestatic disease [25]. Serum bilirubin was also increased, indicative of hepatocellular damage with or without simple biliary obstruction. The reduction of the total serum protein content and more particularly of serum albumin was also an indication for liver damage [25]. Taken together, the results indicated that the single injection of ANIT was causative of cholestatic liver disease. This condition also contributed for the observed decrease in animal weight (see Table 1).

Our study was the first one to relate cardiac mitochondrial dysfunctions with hepatic cholestasis disease. We compared some values typically used in mitochondrial bioenergetics in succinate-energised mitochondria, as that substrate is normally used for MPTP assays. As seen in Table 2, no statistical differences were observed in the mitochondrial bioenergetic parameters in both groups, although the ADP/O value was slightly higher in the ANIT-group. It is not the first time that a disease condition is shown to increase the ADP/O value, as it was already observed in GK rats, an animal model for type II diabetes [26]. Nevertheless, this increase in the efficiency of cardiac mitochondrial phosphorylative system could act as an adaptation response to the disease condition. The unchanged RCR and the ATP/ADP value may also be another evidence of adaptation.

The mitochondrial permeability transition is a condition often related to mitochondrial dysfunction. In this work, we primarily investigated the differences between heart mitochondria from ANIT-injected and control rats in the susceptibility to the calcium-dependent permeability transition. A rather large calcium concentration (500–600 μM) was used in our experiments. In our hands, a high resistance to in vitro calcium-induced MPTP characterizes isolated cardiac mitochondria in the absence of strong pro-oxidants (as it is the case here). The results allowed us to conclude that heart mitochondria from ANIT-injected animals were more susceptible to the induction of the permeability transition. This fact disturbed the default mitochondrial calcium loading capacity in ANIT-injected animals. Importantly, the equivalent inhibitory effect of cyclosporin-A, the specific MPT-inhibitor, in either mitochondrial ΔΨ and calcium movements, allowed us to conclude that an enhanced MPT is the solo reason for the loss of calcium loading capacity in heart mitochondria isolated from cholestatic rats. We also observed some protection afforded by cyclosporin-A in the control group. This clearly demonstrated that a small sub-set of calcium-susceptible mitochondria existed in that group. Nevertheless, the apparent number of those susceptible mitochondria was bigger in the cholestatic group.

Presently, we are not able to determine the main mechanism for the enhanced susceptibility to the calcium-sensitive permeability transition. Nevertheless, it is probable that the increase of bile salts in the cardiomyocyte cytoplasm may induce perturbations in mitochondrial membranes, as already described for hepatic mitochondria [5], which may prone mitochondria to suffer the MPT. Other possibility, not tested in this study, is that bile salts may directly increase mitochondrial oxidative stress and thus cause the opening of the MPTP by this pathway. This is in agreement with the protective role of antioxidants in the apoptotic effect of bile salts [13]. Nevertheless, differences in the endogenous calcium content did not seem to be a possible cause, as seen by experiments using a mitochondrial uncoupler in mitochondria without external calcium addition (not shown). A direct effect of ANIT in the myocardium was also excluded for several reasons. One of them was the lack of ANIT effect on the MPTP in isolated cardiac mitochondria in concentrations up to 500 μM (a concentration that would be unreachable in the cardiac tissue during our in vivo treatment) (data not shown). Furthermore, experiments with liver mitochondria isolated from the same animals showed an opposite result: hepatic mitochondria from ANIT-injected animals showed a higher calcium loading capacity [27]. Also, our results are also in clear contradiction with the results ones with the hepatic MPT in bile-duct ligation-induced cholestatic rats [14], although different organs may have a different response to the same
stimulus and the liver is known to resist very well to disease conditions.

It is important to notice that this defective mitochondrial capacity in buffering externally added calcium was only accompanied by slight or no changes on other mitochondrial parameters. The RCR value, which gives us the degree of coupling between the mitochondrial protonotive force and ATP synthesis, was not affected in ANIT-injected animals. Moreover, the ADP/O value was even slightly increased.

The role of the MPT in cellular dysfunction is well documented [28,29]. Cardiac dysfunction sometimes associated with cholestatic liver disease can be associated with an increased susceptibility of heart mitochondria to the permeability transition. The decrease in mitochondrial function in situ that normally accompanies an enhanced permeability transition may hinder the normal myocardial function. Although our experimental conditions may seem very artificial (specially concerning the high calcium concentrations used), it is known that microdomains may exist between mitochondria and the sarcoplasmic reticulum, creating domains where calcium concentrations felt by one single mitochondrion are much higher than in bulk cytosol [30]. Also, it is known that a higher calcium amount is necessary to induce the MPTP in vitro when succinate and rotenone are present in the reaction buffer [9,10]. The presence of the mentioned compounds creates conditions to generate a high degree of reduction of intra-mitochondrial pyridine nucleotides, which antagonises MPTP induction for lower calcium intramitochondrial concentrations [10]. It is feasible that in vivo conditions, with lower ratios between reduced and oxidised intramitochondrial pyridine nucleotides due to mitochondrial complex I activity, may allow for a MPTP induction for much lower calcium conditions, as it happens in isolated cardiac mitochondria (Oliveira, unpublished results).

We are tempted to suggest that the alteration of bile acids levels in the plasma (although not measured in this work) or other increased metabolite may be also toxic for cardiac cells and especially for cardiac mitochondria, leading ultimately to cardiovascular bioenergetic alterations. Moreover, our results also suggest that patients with hepatic diseases translated into defective cardiac mitochondrial calcium handling may not resist so well to cardiac ischemia and reperfusion episodes. As far as we know, no clinical data is available concerning this.

Our results may be very helpful in the comprehension of possible cardiac problems associated with an increase of bile salts in the plasma and opens new horizons for the treatment of cardiac problems associated with hepatic dysfunction.

Acknowledgements

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