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MOLECULAR TOXICOLOGY

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Cholestasis induced by chronic treatment with α-naphthyl-isothiocyanate (ANIT) affects rat renal mitochondrial bioenergetics

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Abstract Chronic cholestasis is characteristic of many human liver diseases. Renal injury has been often associated with this type of disease. The aim of this study was to evaluate the effect of cholestasis on kidney mitochondrial bioenergetics following in vivo chronic administration of α-naphthyl-isothiocyanate (ANIT), a known cholestatic agent. Serum markers of renal injury, kidney morphology and endogenous adenine nucleotides were measured in ANIT-treated rats (80 mg/kg per week s.c. for 16 weeks). Changes in membrane potential and mitochondrial respiration as well as alterations in mitochondrial calcium homeostasis were monitored. Cholestatic animals shown no alterations in renal morphology when compared with control. Additionally, following chronic ANIT administration, no significant alterations in mitochondrial respiratory function have been shown. The phosphorylation capacity of cholestatic kidney mitochondria was enhanced. Associated with these parameters, mitochondria from treated animals exhibited a decreased susceptibility to disruption of mitochondrial calcium homeostasis, due to permeability transition induction. These data suggest that, despite being submitted to chronic treatment with ANIT, kidney mitochondria from cholestasis-induced rats present some defense mechanisms to circumvent this aggression. They show improved phosphorylative capacity and, moreover, a decreased susceptibility to mitochondrial permeability transition induction, probably due to adaptative mechanisms of calcium transport.

Keywords Kidney mitochondria · Bile acids · Cholestasis · Membrane potential · Respiratory activity · Permeability transition pore

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Introduction

Chronic cholestasis, defined as an impairment of bile flow, is characteristic of many human liver diseases. During cholestasis, toxic and hydrophobic bile acids accumulate in hepatocytes promoting cell injury because bile acids, particularly the lipophilic dihydroxy and monohydroxy species, are surface active substances acting as detergents and promoting damage to cell membranes (Gores et al. 1998; Rolo et al. 2000; Vore 1991). There is also clear evidence that patients with cholestatic liver disease are exposed to increased oxidative stress, associated with increased lipid peroxidation (Ljubuncic et al. 2000). Moreover, besides the cytotoxicity observed in liver cells, there is also evidence that the increased presence of reactive oxygen species (ROS) and lipid peroxides (LP) in plasma, reported in patients with liver disease, can exert damage in kidney and other extrahepatic tissues as well as the diseased liver. In fact, renal injury is well described in cholestasis (Holt et al. 1999).

Several studies suggest that accumulation of bile acids induces mitochondrial dysfunction. Mitochondria play a crucial role in energy production since about 95% cellular ATP is produced within the mitochondria through oxidative phosphorylation reactions. Moreover, several other metabolic pathways are controlled by mitochondria, including regulation of cytosolic calcium homeostasis and osmotic regulation (Bernardi et al.

1998, 2001; Crompton et al. 1999; Rolo and Palmeira 2000). Dysfunction of intracellular Ca^{2+} homeostasis usually leads to an increased permeability of the mitochondrial inner membrane, promoting membrane depolarization, substrate depletion, and equilibrium of species with molecular weight less than 1500 Da. This phenomenon, called mitochondrial permeability transition (MPT), is caused by the opening of the so-called mitochondrial permeability transition pores (MPTP), composed of several proteins of the outer and inner membranes: the adenine nucleotide translocator (ANT) of the inner membrane, the voltage-dependent anion channel (VDAC) of the outer membrane, or cyclophilin, a matrix protein (for a review see Crompton 1999; Zoratti and Szabò 1995). This proteinaceous complex is formed when mitochondria are loaded with high amounts of calcium or in oxidative stress conditions (Gores et al. 1998; Kowaltowski et al. 1996; Richter 1998).

There is ample evidence that MPTP induction is involved in the mechanism of cytotoxicity of bile acids in hepatocytes (Botla et al. 1995; Gores et al. 1998; Rolo et al. 2001a, 2001b).

The current investigation was carried out to evaluate the effect of cholestasis on kidney mitochondria isolated from cholestasis-induced rats. In order to induce cholestasis α-naphthyl-isothiocyanate (ANIT), a cholestatic agent, was used. Our results show that, despite of being subjected to an increased oxidative stress (Ljubuncic et al. 2000), kidney mitochondria from ANIT-treated rats showed an enhanced phosphorylative activity and a higher resistance to MPTP induction.

Materials and methods

Chemicals

ANIT (α-naphthyl-isothiocyanate), which was dissolved in olive oil, and cyclosporin A, used as ethanolic solution, were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Calcium Green-5N was purchased from Molecular Probes (Eugene, OR, USA). All other reagents and chemicals used were of the highest grade of purity commercially available.

Induction and characterization of cholestasis

Female Wistar rats (12 weeks old) were from our colony (Animal Research Center Laboratory, University Hospitals, Coimbra, Portugal). The use and care of the animals for these studies were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Coimbra. Animals were kept under controlled light (12 h/12 h day/night cycle), temperature (22–24°C) and humidity (50–60%) conditions, and with free access to powdered rodent chow (diet URF1; Charles Rivers, Les Oncins, France) and water (pH 5.5). Animals were randomly separated into two groups of eight animals each. To induce cholestasis, ANIT (a weekly dose of 80 mg/kg) was administrated intraperitonially, and this treatment was repeated for 16 weeks. Control animals received an equivalent volume of the vehicle. One week after the 16th ANIT treatment, rats were anesthetized with ketamine chloride (88.5 mg/kg i.m.; Park Davies, Ann Abor, MI, USA) and chlorpromazine chloride (2.65 mg/kg i.m.; Lab. Victória, Portugal) and their weight was recorded. A blood sample (2 ml) was taken from the descending aorta (intra-cardiac) for measurement of serum markers of renal injury (urea nitrogen, creatinine and urates). Analyses were performed using commercial kits from Olympus (Tokyo, Japan) and Beckman (Fullerton, CA, USA).

Kidney morphology

Kidneys from four untreated and four treated female Wistar rats were removed quickly and immediately after the the rats had been killed, and were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Sections 4-μm thick were deparaffinized with xylene and rehydrated through decreasing concentrations of ethanol for staining with hematoxylin and eosin.

Preparation of mitochondria

Control and cholestatic rats were maintained ad libitum for at least 12 h before being killed, according to a previously established method (Gazzotti et al. 1979) with slight modifications.

The homogenization medium contained 250 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA and 0.1% defatted bovine serum albumin (BSA). EDTA, EGTA and defatted BSA were omitted from the final washing medium and pH was adjusted to 7.2. The mitochondrial pellet was washed twice, suspended in the washing medium and used immediately. Protein was determined by the Biuret method, using BSA as a standard (Gornall et al. 1949).

Membrane potential ($\Delta\Psi$) measurements

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of tetraphenylphosphonium ion (TPP^+) with a TPP^+-selective electrode prepared in our laboratory, as previously reported (Ferreira et al. 1997; Kamo et al. 1979), using a calomel electrode as a reference since accumulation and release of TPP^+ by coupled mitochondria is directly correlated to membrane potential (Kamo et al. 1979). Therefore, TPP^+ uptake was measured from the decreased TPP^+ concentration in the medium of the electrode. The potential difference between the selective and the reference electrodes was measured with an electrometer and recorded continuously. A matrix volume of 1.1 μl/mg was assumed and valinomycin was used to calibrate the baseline. Reactions were carried out at 25°C in 1 ml of the reaction media (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.2), supplemented with 3 μM TPP^+, 2 μM rotenone, 1 mg mitochondria and 5 mM succinate. To induce state 3 respiration, 200 nmol ADP was used. Oxygen consumption was measured simultaneously, as described below. In order to evaluate the induction MPT in the presence of calcium and phosphate, membrane potential was also evaluated. Reactions were carried out at 25°C, in 1 ml of reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone 1 mg mitochondria and 5 mM succinate. To induce MPT, pulses of calcium (20 μM $CaCl_2$) were added, until the loss of $\Delta\Psi$ compatible with the opening of MPTP was recorded. The opening of MPTP was also observed by adding a single pulse of calcium (50 μM $CaCl_2$).

Mitochondrial respiration

Oxygen consumption of isolated mitochondria was determined polarographically at 25°C with a Clark oxygen electrode, connected to a suitable recorder in a closed chamber with magnetic stirring. Mitochondria (1 mg) and respiratory substrate (succinate,

5 mM) and rotenone (2 μM) were added to the standard reaction medium (1 ml). To induce state 3 respiration, 200 nmol ADP was used. The respiratory control ratio (RCR) and ADP/O ratios were calculated according to Chance and Williams (1956).

Measurement of mitochondrial permeability transition

Mitochondrial swelling was estimated by changes in light scattering, as monitored spectrophotometrically at 540 nm (Palmeira and Madeira 1997). The reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone, 0.5 μg/ml oligomycin and 0.6 mg mitochondria, was stirred continuously and the temperature maintained at 25°C. The experiments were started by the addition of 5 mM succinate. Calcium (60 μM $CaCl_2$) was added before any of the other compounds.

Measurement of mitochondrial calcium fluxes

The uptake and subsequent release of calcium by isolated kidney mitochondria was determined using a calcium-sensitive fluorescent dye, Calcium Green-5N (Rajdev and Reynolds 1993). The reactions were carried out at 25°C in 2 ml reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone, 0.6 mg mitochondria, 0.4 μM oligomycin and 100 nM Calcium Green-5N, which was stirred continuously in a water-jacketed cuvette holder. Fluorescence (excitation 505 nm, emission 531 nm) was monitored continuously for 50 s prior to the addition of calcium ($CaCl_2$) to a final concentration of 12.5 μM. Fluorescence was monitored continuously for an additional 4 min, and stopped by addition of excess EGTA to obtain the basal line. Calcium fluxes are expressed as relative fluorescence units (RFU).

Quantification of malondialdehyde (MDA)

The assay procedure used for MDA determination by high-performance liquid chromatography was that of Wong et al. (1987). Liquid chromatography was performed using a Gilson HPLC apparatus with a reverse phase column (RP18 Spherisorb, S5 OD2). The samples were eluted from the column at a flow rate of 1 ml/min and detection was performed at 532 nm. The MDA content of the samples was calculated from a standard curve prepared using the thiobarbituric acid –MDA complex and was expressed as micromolar concentration.

Statistics

The results are presented as mean ± SEM of n experiments and statistical significance between cholestatic rats and their control group was determined using paired Student's t-test. $P < 0.05$ was considered significant.

Results

Characterization of animals

In our studies we used ANIT-treated rats as a model of cholestasis since this compound produces cholestasis in a reproducible and dose-dependent manner (Goldfarb et al. 1962; Vore 1991). Nevertheless, about 13 days after a single dose of ANIT, normal hepatobiliary function is observed (Kossor et al. 1993). Thus, in order to analyze the effect of chronic cholestatic injury, treatments with ANIT have to be repeated weekly.

After the 16th treatment with ANIT (or vehicle), serum levels of urea nitrogen and creatinine were similar in cholestatic and control rats (Table 1). Moreover, ANIT-treated rats presented significantly increased levels of uric acid. These results indicate that kidney of cholestatic rats suffered no injury due to ANIT treatment. We also observed that ANIT-treated rats showed decreased body weight, probably due to the induced cholestasis.

We demonstrated the increased oxidative stress during cholestasis in kidney mitochondria from treated rats (see MDA data in Table 1), with MDA levels increasing from 3.2 μM in controls to 5.1 μM in cholestatic rats.

Effect of cholestasis on kidney morphology

Kidneys from four ANIT-treated rats and from four control Wistar rats were rapidly removed and fixed in order to observe changes in morphology induced by cholestasis (see Fig. 1). Our results showed that renal structures (glomeruli, tubules, vessels and interstitium) were preserved in ANIT-treated rats.

Effect of cholestasis on kidney mitochondria respiratory indices

Respiratory parameters of kidney mitochondria isolated from ANIT-treated and control rats, in the presence of succinate as respiratory substrate, were evaluated. We observed no impairment of mitochondrial respiratory indices in cholestasis-induced rats (Table 2).

In fact, no significant differences in state 4 respiration, were found, while state 3 respiration (determined in the presence of 200 nmol ADP), RCR (defined as the ratio between state 3 and state 4 respiration), and ADP/O ratio, (defined as the amount of oxygen required to completely phosphorylate externally added ADP) were significantly increased in ANIT-treated rats.

The effect of induced cholestasis on the membrane potential of isolated kidney mitochondria was also

Table 1 Characterization of the control rats and those treated with α-naphthyl-isothiocyanate (ANIT). Data for body weight and serum markers of kidney injury are given as means ± SEM of the number of experiments indicated. Analyses were performed using commercial kits from Olympus Tokyo, Japan and Beckman, Fullerton, CA, USA. For further details see Materials and methods section

Parameter	Control	ANIT
Body weight (g)	309.8 ± 10.93 (n = 8)	273.13 ± 6.79* (n = 8)
Ureanitrogen (mg/dl)	20.50 ± 0.50 (n = 4)	19.00 ± 0.91 (n = 4)
Creatinine (mg/dl)	0.70 ± 0.00 (n = 4)	0.73 ± 0.03 (n = 4)
Uric acid (mg/dl)	0.425 ± 0.048 (n = 4)	0.950 ± 0.087** (n = 4)
Malondialdehyde (μM)	3.2 ± 0.5 (n = 4)	5.1 ± 0.2** (n = 4)

*$P < 0.05$, **$P < 0.01$ compared with corresponding values in controls

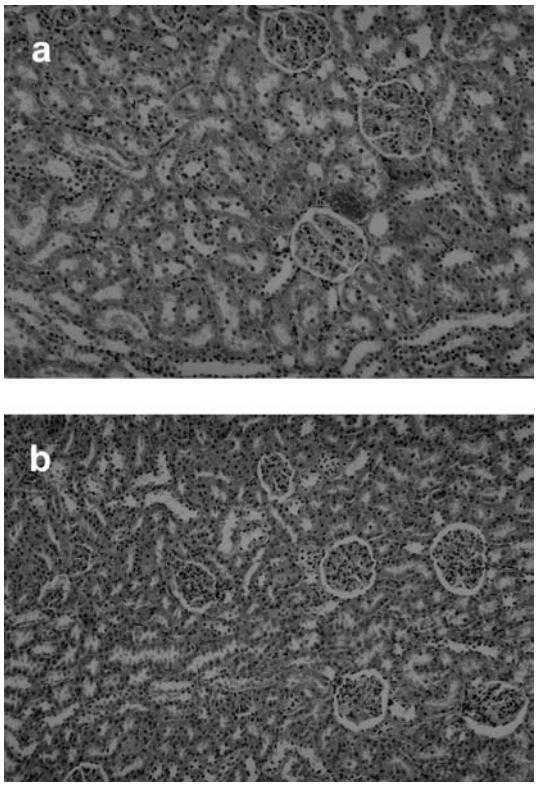


Fig. 1A, B Kidney morphology. Renal tissue of **A** untreated Wistar rat, and **B** rat treated with α-naphthyl-isothiocyanate (ANIT-) rat. Treatment with ANIT induced no morphological changes on kidney (**A** H&E ×160, **B** H&E ×100)

evaluated (see Table 3). We observed that membrane potential developing after addition of succinate was significantly greater in ANIT-treated rats than in controls. Furthermore, the repolarization rate was increased and the lag phase that precedes repolarization was lengthened in cholestatic kidney mitochondria, which is indicative that the phosphorylative system activity is enhanced in cholestatic rats compared to that in controls.

No significant differences between ANIT-treated and control rats were observed in the membrane potential decrease due to the addition of ADP (ΔADP) nor in the recovery of membrane potential after the complete phosphorylation of added ADP ('repolarization').

Effect of cholestasis on calcium-induced mitochondrial permeability transition pore (MPTP)

Calcium accumulation capacity of kidney mitochondria from ANIT-treated and control rats was determined by observing the effect of addition of small pulses of calcium in the mitochondrial $\Delta\Psi$, measured with a TPP^+-selective electrode. Our results show that kidney mitochondria from ANIT-treated rats presented the capacity of accumulating a large amount of calcium because it could carry a large number of pulses before the loss of $\Delta\Psi$, compatible with the opening of MPTP (Fig. 2). Similar results were observed when we tested the effect of a larger single calcium pulse: kidney mitochondria from ANIT-treated rats were able to retain

Table 2 Effect of cholestasis on kidney respiratory indices. Cholestasis was induced in rats with α-naphthyl-isothiocyanate (ANIT). Mitochondria (1 mg protein) were incubated in 1 ml of the respiratory standard medium. The respiratory control ratio (RCR, defined as the ratio between state 3 and state 4 respiration) and ADP/O (defined as the amount of oxygen required to completely phosphorylate externally added ADP) were determined accordingly to Chance and Williams (1956).Values are given as means ± SEM of the number of independent experiments indicated, performed with at least three different mitochondrial preparations

Condition	State 4 (nmol O/mg per min)	State 3 (nmol O/mg per min)	RCR	ADP/O (nmol ADP per nmol O)
Control	60.7 ± 1.38 (n = 7)	173.2 ± 8.01 (n = 7)	2.87 ± 0.18 (n = 7)	0.95 ± 0.19 (n = 6)
ANIT	62.2 ± 1.90 (n = 7)	217.4 ± 14.34* (n = 7)	3.52 ± 0.30* (n = 7)	1.60 ± 0.15* (n = 6)

Statistics: *$P < 0.05$, compared with corresponding values in controls

Table 3 Effect of cholestasis on membrane potential. Cholestasis was induced in rats with α-naphthyl-isothiocyanate (ANIT). The membrane potential was measured in kidney mitochondria preparations after the addition of 5 mM succinate, as the respiratory substrate (Energization) and after the addition of 200 nmol ADP (ΔADP). Repolarization corresponds to the recovery of membrane potential after the complete phosphorylation of the added ADP. Repolarization rate was determined as the time required to the complete phosphorylation of the added ADP; data are presented as the ratio between the values obtained and the mean of the repolarization rate in non-cholestatic rats. Lag phase reflects the time required to phosphorylate the added ADP. In all experiments, the baseline was determined by addition of 10 ng/ml of valinomicin at the end of experiments, in order to completely abolish membrane potential. Values are given as mean ± SEM of the number of independent experiments indicated, performed with at least three different mitochondrial preparations

Condition	Energization (mV)	ΔADP (mV)	Repolarization (mV)	Repolarization rate (% control)	Lag phase (s)
Control	214.2 ± 4.39 (n = 7)	34.9 ± 3.40 (n = 7)	202.8 ± 4.25 (n = 7)	100.0 ± 21.17 (n = 7)	54.0 ± 2.96 (n = 7)
ANIT	218.7 ± 3.17* (n = 7)	36.0 ± 1.44 (n = 7)	206.88 ± 6.98 (n = 7)	144.86 ± 20.47** (n = 7)	33.0 ± 1.06*** (n = 7)

Statistics: *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, compared with corresponding values in controls

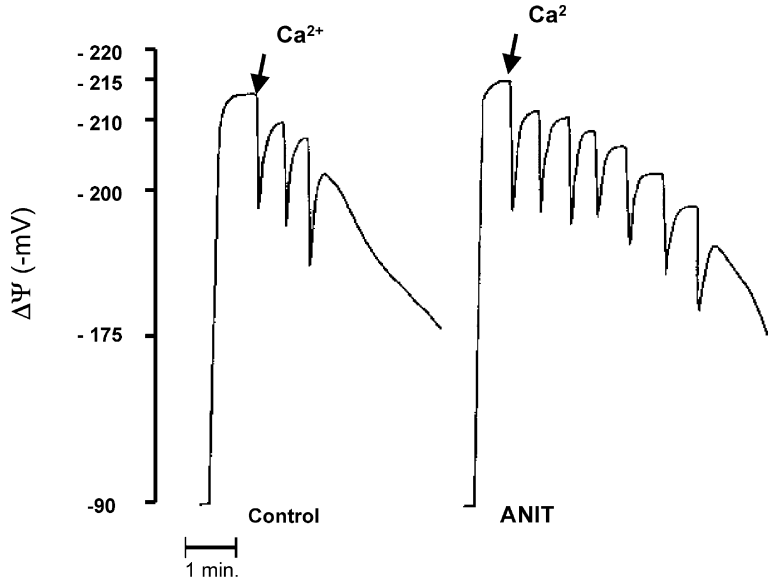


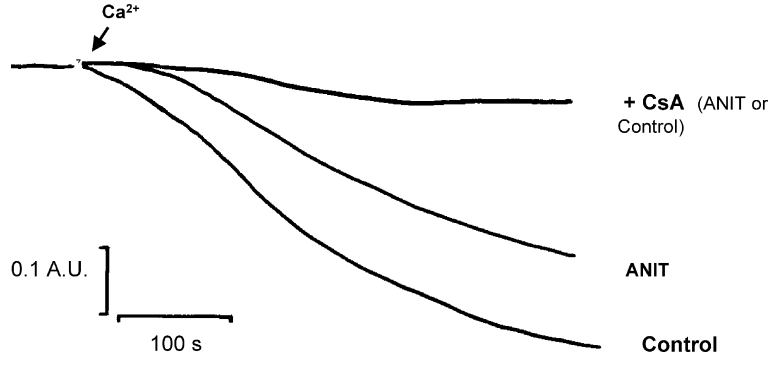
Fig. 2 Mitochondrial membrane potential ($\Delta\Psi$) of kidney mitochondria from α-naphthyl-isothiocyanate (ANIT)-treated and control rats, measured with a tetraphenylphosphonium ion (TPP^+)-selective electrode. Calcium pulses (20 μM soluble salt) were added in order to induce mitochondrial permeability transition pores (MPTP). The $\Delta\Psi$ was calculated without correction for TPP^+-passive binding to mitochondrial membranes. Reactions were carried out in 1 ml of reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone, 1 mg mitochondria. Energization was achieved with 5 mM succinate. The traces are representative of experiments performed with four different mitochondrial preparations

calcium longer than those from untreated Wistar rats (data not shown). Indeed, we observed that in the presence of cyclosporin A this great decline of $\Delta\Psi$ was inhibited, even in the presence of larger amounts of calcium (data not shown).

Large amplitude swelling occurs when mitochondria undergo the permeability transition (Al-Nasser and Crompton 1986; Bernardi and Petronilli 1996; Oliveira et al. 2001). Therefore, the effect of cholestasis on Ca^{2+}/P_i-induced mitochondrial swelling was studied by measuring the changes in the suspension absorbance at 540 nm in a sucrose-based media. Our results showed that kidney mitochondria isolated from ANIT-treated rats presented a decreased amplitude of swelling after the addition of 30 μM calcium (see Fig. 3) compared with that in controls. We observed that in the presence of cyclosporin A, this decrease in absorbance was prevented, clearly indicating the observed swelling was due to MPTP opening (Fig. 3).

The final evidence arises from determinations of extramitochondrial calcium movements using fluorescent calcium-sensitive probe Calcium Green-5N. The results confirm, again, the previous data (Fig. 4). In fact, kidney mitochondria from ANIT-treated rats were able to retain calcium longer than those from control Wistar rats, indicating that ANIT-treated rats mitochondria are less susceptible to the induction of MPT in a calcium-phosphate system.

Fig. 3 Typical recording of mitochondrial swelling (decrease of absorbance at 540 nm) induced by calcium in kidney mitochondria from α-naphthyl-isothiocyanate (ANIT)-treated and control rats. The reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone, 0.5 μg/ml of oligomycin and 0.6 mg mitochondria was stirred continuously and the temperature maintained at 25°C. The experiments were started by the addition of 5 mM succinate. Ca^{2+} (60 μM $CaCl_2$) was added prior to all the other compounds. The traces are representative of experiments performed with four different mitochondrial preparations (*CsA* cyclosporin A, *A.U.* absorbance units)



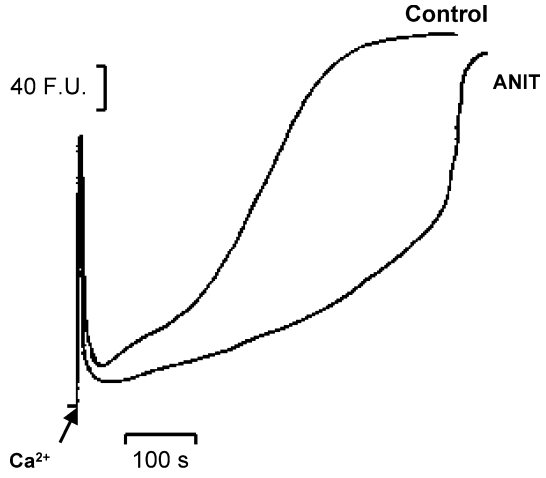


Fig. 4 Typical measurements of extramitochondrial calcium movements in kidney mitochondria from α-naphthyl-isothiocyanate (ANIT)-treated and control rats, using the fluorescent calcium-sensitive probe Calcium Green-5N. The reactions were carried out in 2 ml of reaction medium (200 mM sucrose, 10 mM Tris-MOPS pH 7.4, 1 mM KH_2PO_4 and 10 μM EGTA), supplemented with 2 μM rotenone, 0.6 mg mitochondria, 0.4 μM oligomycin, 5 mM succinate and 100 nM Calcium Green-5N. Fluorescence was monitored continuously for 50 s prior to the addition of Ca^{2+} ($CaCl_2$) to a final concentration of 12.5 μM. Fluorescence was monitored continuously for an additional 4 min, and stopped with excess EGTA to obtain the baseline. Ca^{2+} fluxes are expressed as relative fluorescence units (*F.U.*). The traces are representative of experiments performed with four different mitochondrial preparations

Discussion

Abnormalities of renal function have been correlated with cholestatic liver disease, and these malfunctions have been associated with increased oxidative stress (Holt et al. 1999; Ljubuncic et al. 2000). This oxidative stress in liver and kidney (as well as other organs) involves intra-organ generation of ROS, possibly mediated by bile acids and accumulation of degradative products of lipid peroxidation. As a result, the increased presence of ROS and these toxic products in plasma increases the possibility that they could exert their injurious actions in the kidney and other extrahepatic tissues, as well as in the diseased liver (Ljubuncic et al. 2000). However, our results indicate that despite the increased oxidative stress (as determined by MDA levels in control and cholestatic rats), kidneys from cholestasis-induced rats do not show any morphological or functional injury (see Table 1 and Fig. 1). Moreover, we observed that uric acid levels are increased in cholestasis-induced rats. These increased levels may play an important role in preventing oxidative damage because uric acid has an antioxidant function (Acworth et al. 1997; Kirschbaum 2001). Additionally, bilirubin concentration is also increased in cholestasis. Since bilirubin acts in vivo as an efficient scavenger of ROS (Tomaro and Batlle 2002), we can propose that bilirubin could play a key physiological role in protection against the oxidative damage that occurs in cholestasis.

Both under pathological and physiological conditions, ROS and mitochondria play an important role in apoptosis induction (Richter 1998; Simon et al. 2000). Moreover, it has been well described that mitochondrial function is perturbed by cholestasis or exposure to bile salts, due to mitochondrial permeability transition pore (MPTP) opening (Gores et al. 1998; Rolo and Palmeira 2000; Rolo et al. 2001b). The MPTP is a proteinaceous channel, formed when mitochondria are overloaded with calcium or in oxidative stress conditions. It is characterized by mitochondrial membrane depolarization, mitochondrial calcium release, and an increase in non-specific permeability of the inner membrane to low-molecular-weight solutes, leading to mitochondrial swelling and inhibition of oxidative phosphorylation.

Nevertheless, our results provide evidence that kidney mitochondria of ANIT-treated rats presented an improved oxidative phosphorylation system over that of the control group. Therefore, respiratory complexes and ATP-synthase, as well as membrane integrity, seem not to be affected by the increased oxidative stress in kidney mitochondria from cholestatic rats. Additional to these results, we observed that kidney mitochondria from ANIT-treated rats presented a decreased susceptibility to MPT induction with Ca^{2+}/P_i system, since a smaller amplitude of swelling after the addition of calcium was observed (compared with control rats). Cholestasis-induced rats also presented an increased capacity to accumulate calcium, as demonstrated by membrane potential fluctuations in the presence of calcium pulses, and measurements of extramitochondrial calcium fluxes using the fluorescent calcium dye Calcium Green-5N.

In summary, our results indicate that despite being submitted to increased oxidative stress (Holt et al. 1999; Ljubuncic et al. 2000), kidney from cholestasis-induced rats present some defense mechanisms to circumvent this process. Moreover, despite some previous reports indicating that bile acids may exert their cytotoxic effect by causing mitochondrial dysfunction, our results indicate that kidney mitochondria from ANIT-treated rats show improved phosphorylative capacity and, moreover, show a decreased susceptibility to MPTP induction, probably due to adaptive mechanism of calcium transport (Gores et al. 1998; Rolo and Palmeira 2000; Rolo et al. 2001b).

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