Free Radical Biology and Medicine 58 (2013) 144–153

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxidative state of the liver of rats with adjuvant-induced arthritis

Jurandir Fernando Comar [a,*], Anacharis Babeto de Sá-Nakanishi [a], Andrea Luiza de Oliveira [a], Mariana Marques Nogueira Wendt [a], Ciomar Aparecida Bersani Amado [b], Emy Luiza Ishii Iwamoto [a], Rosane Marina Peralta [a], Adelar Bracht [a]

[a] Department of Biochemistry , University of Maringá, 87020900 Maringá, Brazil
[b] Department of Pharmacology and Therapeutics, University of Maringá, 87020900 Maringá, Brazil

ARTICLE INFO

Article history:
Received 25 July 2012
Received in revised form
14 November 2012
Accepted 3 December 2012
Available online 13 December 2012

Key words:
Chronic inflammation
Adjuvant-induced arthritis
Cachexia
Oxidative state
Reactive oxygen species
Free radicals

ABSTRACT

Adjuvant-induced arthritis is an experimental immunopathology in rats that is often used as a model for studying autoimmune chronic inflammation and inflammatory cachexia. In these animals oxidative stress is quite pronounced in the articular inflammation sites. The purpose of this study was to evaluate oxidative stress in the liver of arthritic rats in which morphological and metabolic alterations have been reported to occur. Oxidative injury parameters, levels and production of reactive oxygen species (ROS), and antioxidant parameters were measured in the total liver homogenate and in subcellular fractions, namely cytosol, mitochondria, and peroxisomes. Arthritic rats presented higher levels of ROS than controls in the total homogenate (46% higher) and in all subcellular fractions (51, 38, and 55% higher for mitochondria, peroxisome, and cytosol, respectively). Arthritic rats also presented higher levels of protein carbonyl groups in the total homogenate (75%) and in all subcellular fractions (189, 227, and 260%, respectively, for mitochondria, peroxisomes, and cytosol). The TBARS levels of arthritic rats were more elevated in the total homogenate (36%), mitochondria (20%), and peroxisomes (16%). Arthritic rats also presented higher levels of NO markers in the peroxisomes (112%) and in the cytosol (35%). The catalase activity of all cell compartments was strongly diminished (between 77 and 87%) by arthritis, and glutathione peroxidase activities were diminished in the mitochondria (22.7%) and cytosol (41%). Glutathione reductase activity increased (62.9%), while glucose-6-phosphate dehydrogenase, superoxide dismutase and glutathione reductase activities were not affected. The GSH content was diminished by arthritis in all cellular compartments (50 to 59% diminution). The results reveal that the liver of rats with adjuvant-induced arthritis presents a pronounced oxidative stress and that, in consequence, injury to lipids and proteins is highly significant. The higher ROS content of the liver of arthritic rats seems to be the consequence of both a stimulated pro-oxidant system and a deficient antioxidant defense with a predominance of the latter as indicated by the strongly diminished activities of catalase and glutathione peroxidase.

Introduction

Rheumatoid arthritis is an autoimmune disease characterized by chronic and systemic inflammation that affects the synovial membranes, articular cartilages, and bones. Rheumatoid arthritis occurs in 0.5–1.0% of the adult population worldwide and it is associated with an increased mortality rate, mainly due to cardiovascular complications caused by inflammatory process [1]. The pathophysiology of arthritis involves an intense hyperplasia of the articular cartilage with participation of T cells, B cells, macrophages, fibroblasts, and proinflammatory cytokines, particularly interleukin-1 (IL-1β)[1], interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) [2]. In addition to the cytokines, reactive oxygen species (ROS) also play an important role in rheumatoid arthritis. The overproduction of proinflammatory cytokines stimulates neutrophils and activated macrophages to secrete ROS in the synovial fluid, which act as mediators of tissue injury [3,4]. Superoxide ($O_2^{\bullet -}$) and its derivates, particularly the hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), the last arising from the reactions of superoxide with nitric oxide ($NO^{\bullet}$), are highly reactive and can cause tissue injury by means of oxidative damage to macromolecules, including DNA,

Abbreviations: ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; SOD, superoxide dismutase; GRd, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBARS, thiobarbituric acid-reactive substances

* Corresponding author. Fax: +55 44 30114714.
E-mail address: jurandircomar@yahoo.com.br (J.F. Comar).

0891-5849 © 2012 Elsevier Inc. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.12.003

proteins, and membrane lipids. Cytokines released into the synovium may also reach the systemic circulation and act in other tissues. In accordance, the oxidative stress biomarkers are increased in both articular inflammation sites and plasma of patients with rheumatoid arthritis [3–5].

In addition to affecting the articular cartilage, rheumatoid arthritis also evokes marked inflammatory responses and immunological alterations in other organs, such as lungs, vascular tissue, liver, and muscles [6–9]. Metabolic alterations are equally prominent, such as for example the muscle wasting condition known as rheumatoid cachexia, which is mediated by TNF-α and IL-1β and occurs in approximately two-thirds of all patients with rheumatoid arthritis [10]. With respect to the liver, several alterations caused by arthritis have been reported. Mitochondria from rats with adjuvant-induced arthritis present higher rates of oxygen uptake in the absence or presence of exogenous ADP [11]. Consistently, perfused livers from rats with adjuvant-induced arthritis also present higher rates of oxygen uptake as well as reduced gluconeogenesis from various substrates, increased glycolysis, modifications in the urea cycle, reduced metabolism of xenobiotics, and reduced albumin synthesis accompanied by elevation of the serum inflammatory globulins [11–16]. The adjuvant-induced systemic inflammation also results in higher activities of hepatic transaminases in the plasma and changes in the hepatocellular morphology, which shows irregularly shaped mitochondria and lysosomes [17]. Considering the participation of ROS in rheumatoid arthritis [3,4], as mentioned above, it is possible that the hepatic biochemical and histological alterations are associated with and influenced by changes in the oxidative state of the liver cells, particularly within the mitochondria and peroxisomes, which are the organelles directly involved in oxidative metabolism.

Taking this hypothesis into consideration, the present work was planned to investigate the oxidative status of the liver of rats with adjuvant-induced arthritis. This is an experimental immunopathology in rats that shares many features of human rheumatoid arthritis and is often used as a model for studying autoimmune chronic inflammation [18–20]. As mentioned above, several metabolic alterations caused by arthritis have been reported, but no details are known about the oxidative state of the liver cells in arthritic rats. It is also of particular interest to know how arthritis affects the oxidative state of the various cell organelles. This study aims to fill this gap by providing a detailed picture about the oxidative state of the rat liver under the influence of adjuvant-induced arthritis, which in turn, should also allow extrapolations for the liver of patients with rheumatoid arthritis.

Material and methods

Chemicals

Dinitrophenylhydrazine (DNPH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), oxidized dichlorofluorescein (DCF), 1,1′,3,3′-tetraethoxypropane, horseradish peroxidase, *o*-phthalaldehyde (OPT), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GRd), and nitrate reductase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits for albumin, AST, and ALT were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, Brazil). All other chemicals were of analytical grade.

Animals and treatments

Male Holtzman rats were fed ad libitum with a standard laboratory diet (Nuvilab, Colombo, Brazil) and maintained on a regulated light–dark cycle. For the induction of adjuvant arthritis, animals weighing 180–210 g were injected in the left hindpaw with 0.1 ml of Freund's adjuvant (heat-inactivated *Mycobacterium tuberculosis,* derived from the human strain H37Rv), suspended in mineral oil at a concentration of 0.5% (w/v). Animals showing the characteristic lesions at 21 days after adjuvant injection were selected for the experiments [18]. Rats of similar weights were injected with mineral oil and served as controls. All experiments of adjuvant arthritis induction were done in accordance with the worldwide accepted ethical guidelines for animal experimentation and previously approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol 062/08-CEEA).

Liver homogenate and subcellular fraction preparations

Rats were fasted for 18 h and then decapitated, and the livers were immediately removed, freeze-clamped, and stored in liquid nitrogen. The tissue was then homogenized in a Dounce homogenizer with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and an aliquot was separated for use as total homogenate. The remaining homogenate was centrifuged at 11,000*g* for 20 min and the supernatant separated as the soluble fraction of homogenate.

The mitochondrial and peroxisomal fractions were isolated by differential centrifugation [21]: 600*g* (10 min) and 7000*g* (10 min) for mitochondria, then the supernatant was additionally centrifuged at 15,000*g* (5 min) and 39,000*g* (10 min) for peroxisome precipitation. Disrupted mitochondria were obtained by repeated freeze-thawing procedures in liquid nitrogen. For preparing disrupted peroxisomes, the pellet was treated with Triton X-100 (final concentration of 0.04%).

For obtaining the soluble cytosolic fraction, the liver was excised in 0.1 M potassium phosphate buffer (pH 7.4) and homogenized. The postmitochondrial supernatant was centrifuged at 105,000*g* for 1 h to precipitate the organelles, and the supernatant was collected as the soluble cytosolic fraction. Protein content in homogenates and subcellular fractions was measured as described by Lowry et al. [22].

Oxidative injury parameters

The liver homogenate, disrupted mitochondria, disrupted peroxisomes, and soluble cytosolic fraction were assayed for oxidative damage parameters. Lipid peroxidation was evaluated by means of the TBARS (thiobarbituric acid-reactive substances) assay [23]. The amount of lipoperoxides was calculated from the standard curve prepared with 1,1′,3,3′-etraethoxypropane and the values were expressed as nmol (mg protein)$^{-1}$. Protein carbonyl group contents were measured spectrophotometrically using DNPH ($\varepsilon_{370}=22 \times 10^3\,M^{-1}\,cm^{-1}$) and the values were expressed as nmol (mg protein)$^{-1}$ [24].

Additionally the TBARS production in intact mitochondria was measured after induction with $FeCl_3$ [25]. Briefly, mitochondria (1 mg protein) were added to 1.5 ml of a reaction mixture containing 250 mM mannitol, 6 mM α-ketoglutarate, 0.2 mM $FeCl_3$, 2 mM ADP, and 10 mM Hepes buffer (pH 7.2). The reaction mixture was incubated at 37 °C for 20 and 40 min under shaking and 0.5 ml was transferred to 2 ml of a medium containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 mM HCl, and 0.01% butylated hydroxytoluene. This suspension was incubated for 15 min at 95 °C and subsequently centrifuged for 10 min at 10,000*g*. The TBARS in the supernatant were estimated by spectrophotometry at 532 nm ($\varepsilon_{532}=1.56 \times 10^5\,M^{-1}\,cm^{-1}$) and the values expressed as nmol (mg protein)$^{-1}$.

ROS measurement

Reactive oxygen species were assessed in the homogenate supernatant, disrupted mitochondria, disrupted peroxisomes, and soluble cytosolic fraction. Total ROS content was quantified via the DCFH-DA assay as previously described [26]. Acetate groups of DCFH-DA allow it to enter the organelles. These groups are removed by esterases producing the reduced DCFH within the organelle, which can be oxidized by peroxides to the fluorescent oxidized DCF. The formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorimeter (RF-5301; Shimadzu) in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized DCF was used to express the results as nmol (mg protein)$^{-1}$.

The rate of mitochondrial ROS production (real-time ROS production), basically H_2O_2, was estimated by measuring the linear fluorescence increase (504 nm for excitation and 529 nm for emission) due to DCF formation from DCFH via oxidation by H_2O_2 in the presence of horseradish peroxidase [27]. Briefly, intact mitochondria (0.5 mg) were suspended in 2 ml of a mixture containing 250 mM mannitol, 1.36 μM DCFH-DA, 10 mM Hepes buffer (pH 7.2), and 10 mM succinate or 10 mM α-ketoglutarate as respiratory substrates. The reaction was initiated by the addition of 0.4 μM horseradish peroxidase and the fluorescence recorded for 10 min under agitation. The results were expressed as nmol min^{-1} (mg protein)$^{-1}$.

The peroxisomal H_2O_2 production was assessed by the acyl-CoA oxidase activity as described previously [28], with modifications. Briefly, intact peroxisomes (0.4 mg) were suspended in 2.0 ml of a medium containing 11 mM potassium phosphate buffer (pH 7.4), 0.04% Triton X-100, 1 mM NAD^+, 26 μM DCFH-DA, and 2 U of horseradish peroxidase. After 5 min incubation in the dark, the reaction was initiated with 30 μM palmitoyl-CoA and the fluorescence (495 nm for excitation and 520 nm for emission) was monitored for 10 min. Peroxisomal degradation of fatty acids produces H_2O_2 and other oxygen radicals, which oxidize DCFH. The results were calculated using a standard curve with oxidized DCF and the values expressed as nmol (mg protein)$^{-1}$.

Glutathione assay

GSH and GSSG were measured in the total homogenate, soluble cytosolic fraction, disrupted mitochondria, and disrupted peroxisomes. The GSH and GSSG contents were measured spectrofluorimetrically (excitation 350 nm and emission 420 nm) by means of the OPT assay as described previously [29]. The fluorescence was estimated as GSH. For the GSSG assay, the sample was previously incubated with 10 mM *N*-ethylmaleimide and subsequently with a mixture containing 1 M NaOH and 0.4 μM OPT to detect the fluorescence. The results were calculated using a standard curve prepared with GSH or GSSG and the values were expressed as μmol (g liver)$^{-1}$ or, alternatively, as nmol (mg protein)$^{-1}$.

Nitrite plus nitrate contents

The nitrite+nitrate contents were measured in the total homogenate, disrupted mitochondria, disrupted peroxisomes, and soluble cytosolic fraction. Nitrate was first converted into nitrite by adding the enzyme nitrate reductase and the total nitrite was quantified by the Griess method [30]. The results were expressed as nmol (mg protein)$^{-1}$.

Enzyme assays

Antioxidant enzymatic activities were assessed in the homogenate supernatant, cytosolic fraction, and supernatant of both disrupted mitochondria and disrupted peroxisomes. The catalase activity was estimated by measuring changes in absorbance at 240 nm using H_2O_2 as substrate and expressed as μmol min^{-1} (mg protein)$^{-1}$ [31]. The GRd activity was estimated by measuring changes in absorbance at 340 nm using NADPH and GSSG as substrates and expressed as nmol min^{-1} (mg protein)$^{-1}$ [31]. The superoxide dismutase (SOD) activity was estimated by its capacity to inhibit pyrogallol autoxidation in alkaline medium. This was measured at 420 nm [32]. One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and the results were expressed as U (mg protein)$^{-1}$. The glucose-6-phosphate dehydrogenase (G6PDH) activity was estimated by measuring the increase in absorbance at 340 nm due to $NADP^+$-dependent glucose 6-phosphate transformation and expressed as nmol min^{-1} (mg protein)$^{-1}$ [31]. The glutathione peroxidase activity was estimated by measuring changes in absorbance at 340 nm due to NADPH consumption in the presence of H_2O_2, GSH, and glutathione reductase and expressed as nmol min^{-1} (mg protein)$^{-1}$ [33]. The cytochrome *c* oxidase activity was measured in mitochondria disrupted by freeze–thawing [34]. The oxidation rate of ferrocytochrome *c* was monitored at 550 nm and quantified using the molar extinction coefficient $\varepsilon_{550\ nm}=19{,}000\ cm^{-1}\ M^{-1}$. The results were expressed as nmol min^{-1} (mg mitochondrial protein)$^{-1}$. The activity of nitric oxide synthase was measured spectrophotometrically by following the oxidation of oxyhemoglobin to methemoglobin (401 − 411 nm; $\varepsilon=38{,}600\ M^{-1}\ cm^{-1}$) and the results were expressed as nmol NO min^{-1} (mg protein)$^{-1}$ [35]. The activity of constitutive and mitochondrial nitric oxide synthase was measured in the presence of 1.0 mM $CaCl_2$ and the inducible nitric oxide synthase in the absence of $CaCl_2$ and presence of 20 mM EGTA [36]. For determination in the total homogenate, the liver was previously perfused with 0.9% NaCl to remove the excess of blood.

Plasma analytical assays

Albumin, total protein, and AST and ALT activities were assessed in the plasma. The peritoneal cavity of anesthetized rats was exposed and blood was collected from the vena cava. After centrifugation at 3000*g* for 10 min, albumin and the enzymatic activities were measured by spectrophotometry using commercial kits. Total protein was assayed as described by Lowry et al. [22].

Statistical analysis

The error parameters presented in graphs and tables are standard errors of the mean. Statistical analysis was done by means of the GraphPad Prism software (version 5.0). The Student *t* test was applied and the 5% level ($p < 0.05$) was adopted as a criterion of significance.

Results

Characterization of the experimental model

The first measurements consisted in indicators of cachexia and chronic inflammation in the rats after 21 days of adjuvant injection. The ALT and AST activities were also assessed in the plasma to evaluate the degree of liver damage. The results are shown in Table 1. The weight of the arthritic rats was 60% of that of the healthy controls at similar age and kept under the same

Table 1
Parameters of cachexia, chronic inflammation, and liver involvement in rats with adjuvant-induced arthritis.

Parameter	Controls	Arthritis
Cachexia		
Rat weight (g)	295.7 ± 5.6*	175.3 ± 2.6*
Liver fresh weight (g)	7.51 ± 0.36	7.35 ± 0.38
Chronic inflammation		
Total plasma protein (mg/ml)	59.34 ± 1.82**	67.45 ± 0.61**
Plasma albumin (mg/ml)	20.00 ± 0.85*	11.42 ± 0.51*
Plasma globulin (mg/ml)	39.36 ± 1.73*	56.03 ± 0.31*
Albumin/globulin ratio	0.51 ± 0.04*	0.20 ± 0.01*
Liver involvement		
AST (U/L)	69.6 ± 4.8**	99.8 ± 9.2**
ALT (U/L)	31.1 ± 0.5	29.8 ± 2.6

Total protein, albumin, AST, and ALT were assessed in the plasma as described under Material and methods. Plasma globulin was calculated by subtraction of albumin from the total protein concentration. The values represent the means ± standard errors of the mean of five or six observations for each experimental condition.
Pairs of values in the same line labeled with asterisks are statistically different:
* $p < 0.0001$
** $p < 0.05$

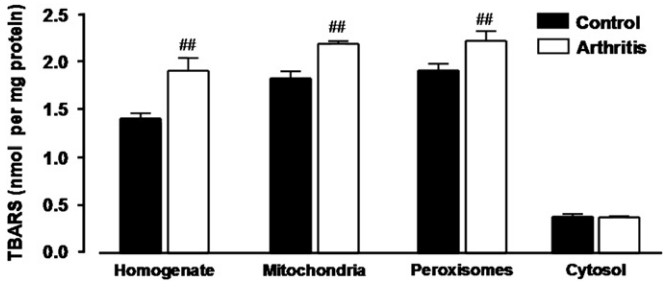

Fig. 1. Lipoperoxide levels in the homogenate and subcellular fractions of livers from control and arthritic rats. Lipid peroxidation was evaluated by means of the TBARS assay in the total homogenate, disrupted mitochondria, disrupted peroxisomes, and cytosolic soluble fraction. The amount of lipoperoxides was calculated from the standard curve prepared with 1,1′,3,3′-tetraethoxypropane and the values are expressed as nmol (mg protein)$^{-1}$. The values are the means ± standard errors of the mean of four to six animals for each experimental condition. Labeled pairs of columns represent statistically different observations: $^{\#\#}p < 0.05$.

conditions. However, no difference was noted in the fresh weight of the livers. The chronic inflammation was evidenced by the levels of the plasma proteins and the albumin/globulin ratio. Arthritic animals showed a reduction of 43% in the plasma albumin levels and an increase of 42% in the plasma globulins. It can also be observed that the plasma total proteins were 13% higher in the arthritic rats. The AST activity was only slightly increased in the plasma of arthritic rats (43%) but no changes were found in the ALT activity. The latter is usually regarded as a specific indicator for hepatic damage, whereas AST may be elevated in diseases of other organs such as the heart or muscle [37]. It should be mentioned that AST elevations under 100% are considered discrete and not indicative of serious liver damage [37].

Tissue oxidative stress

Oxidative stress in the liver was evaluated by measuring the levels of TBARS and protein carbonyl groups in the total homogenate, isolated mitochondria, isolated peroxisomes, and soluble fraction of the cytosol. The TBARS levels shown in Fig. 1 were referred to the protein content of each cell fraction. This procedure seems reasonable because the ratios of fresh liver weight per protein content in the homogenate were similar for the

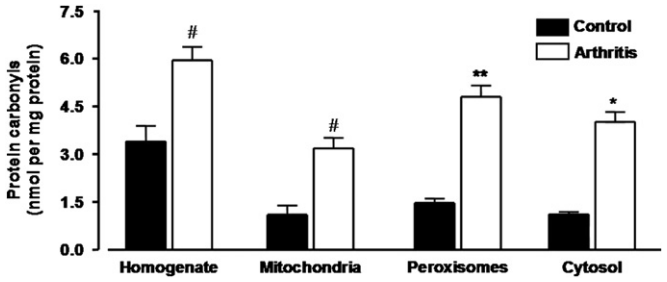

Fig. 2. Protein carbonyl contents in the homogenate and subcellular fractions of livers from control and arthritic rats. The protein carbonyl contents were measured in the total homogenate, disrupted mitochondria, disrupted peroxisomes, and soluble fractions of the cytosol. Measurements were done spectrophotometrically using DNPH ($\varepsilon_{370}=22 \times 10^3\ M^{-1}\ cm^{-1}$) and the values were expressed as nmol (mg protein)$^{-1}$ as described under Material and methods. The values are the means ± standard errors of the mean of four to six animals for each experimental condition. Labeled pairs of columns represent statistically different observations: $*p < 0.0001$, $**p < 0.001$, $^{\#}p < 0.01$.

control and arthritic conditions, i.e., 5.34 ± 0.22 and 5.91 ± 0.28 g/g ($p=0.1362$), respectively. Arthritic rats presented higher levels of TBARS in the total homogenate (36%), mitochondria (20%), and peroxisomes (16%). No difference in the cytosolic TBARS was observed. It is worth mentioning that the TBARS contents of the cytosolic fraction are the smallest of all examined fractions.

Fig. 2 shows the results of the measurements of protein carbonyl groups. Arthritic rats presented higher levels of protein carbonyl groups in the total homogenate (75%) and in all subcellular fractions (189, 227, and 260%, respectively, for mitochondria, peroxisomes, and cytosol). For this parameter the differences between arthritic and control rats were more pronounced than those found for the TBARS levels.

It is possible to induce ROS generation in isolated mitochondria by using $FeCl_3$ and, consequently, producing lipoperoxides in vitro. Because the levels of TBARS were increased in the liver mitochondria of arthritic rats, experiments were planned to quantify the TBARS generation in isolated mitochondria after the addition of $FeCl_3$. The results are shown in Fig. 3. The TBARS generation in the absence of $FeCl_3$ was minimal. In the presence of $FeCl_3$ the TBARS generation increased with time and was more pronounced in mitochondria from arthritic rats (Fig. 3A). The same experiments were done in the presence of rotenone, because inhibition of complex I of the respiratory chain tends to increase ROS generation [38]. Under these conditions the TBARS generation was increased in mitochondria from both control (32%; $p=0.011$) and arthritic rats (27%; $p=0.001$) after 40 min incubation. Also in the presence of rotenone, however, the TBARS generation was clearly higher in mitochondria from arthritic rats (Fig. 3B). It should be stressed that the adjuvant-induced arthritis does not modify the viability of the mitochondria, as can be judged from the respiratory control and ADP/O ratios [11].

ROS content and generation

Total ROS content was assessed in the total homogenate and in all subcellular fractions. The results are shown in Fig. 4. Arthritic rats presented higher levels of ROS than controls in the total homogenate (46% higher) and in all subcellular fractions (51, 38, and 55% higher for mitochondria, peroxisomes, and cytosol, respectively). Real-time ROS generation was measured in mitochondria and peroxisomes and the results are shown in Table 2. Basal ROS generation was relatively low, but most additions (substrates, aminotriazole and rotenone, as specified) to either system, mitochondria or peroxisomes, resulted in increases in

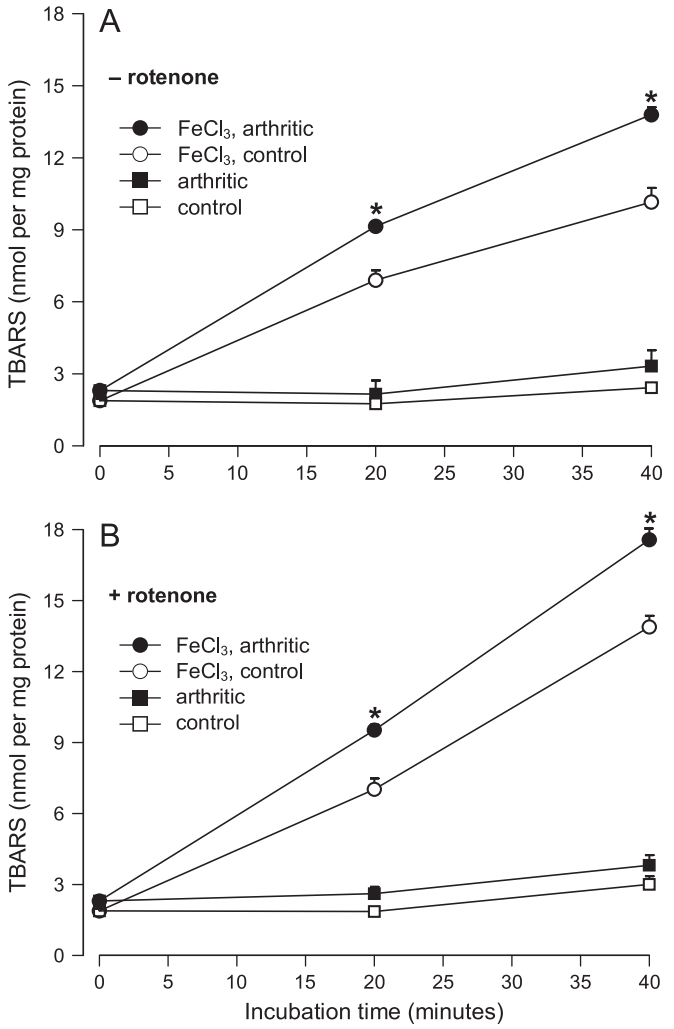

Fig. 3. Production of TBARS induced by $FeCl_3$ in isolated liver mitochondria from control and arthritic rats. The mitochondrial TBARS generation was determined at 20 and 40 min of incubation in (A) the absence and (B) the presence of rotenone as described under Material and methods. The incubation conditions were 10 mM Hepes buffer (pH 7.2), 250 mM mannitol, 6 mM α-ketoglutarate, 0.2 mM $FeCl_3$, 2 mM ADP, 10 μM rotenone, and mitochondria (0.67 mg protein/ml). The values are the means ± standard errors of the mean of four animals for each experimental condition. Asterisks indicate statistical significance of the differences between control and arthritis ($*p < 0.01$; Student's t test).

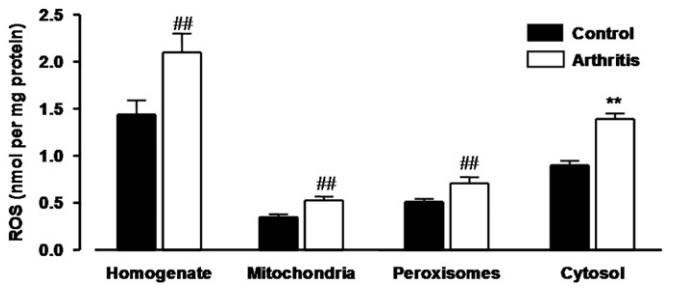

Fig. 4. Reactive oxygen species content of the homogenate and subcellular fractions of livers from control and arthritic rats. The ROS content was measured in the total homogenate, disrupted mitochondria, disrupted peroxisomes, and cytosolic soluble fraction. The ROS contents were quantified via the DCFH-DA assay. The formation of DCF was measured fluorimetrically (excitation and emission wavelengths were set at 504 and 529 nm). A standard curve with oxidized DCF was used to express the results as nmol (mg protein)$^{-1}$. The values are the means ± standard errors of the mean of four to six animals for each experimental condition. Labeled pairs of columns represent statistically different observations: $**p < 0.001$, $^{\#\#}p < 0.05$ for difference between control and arthritic rats.

Table 2
ROS generation in isolated liver mitochondria and peroxisomes from control and arthritic rats.

Substrate	Control	Arthritis
Mitochondrial ROS generation (mmol/min per mg protein)		
No additions	0.097 ± 0.021	0.085 ± 0.011
Succinate	0.205 ± 0.017	0.157 ± 0.024
Succinate + rotenone	0.336 ± 0.014	0.352 ± 0.058
Succinate + aminotriazole	0.364 ± 0.045	0.404 ± 0.018
α-Ketoglutarate	0.188 ± 0.024	0.176 ± 0.045
α-Ketoglutarate + rotenone	0.161 ± 0.030	0.173 ± 0.014
α-Ketoglutarate + aminotriazole	0.295 ± 0.036	0.330 ± 0.034
Peroxisomal ROS generation (mmol/min per mg protein)		
No additions	0.447 ± 0.054	0.373 ± 0.031
Palmitoyl-CoA	0.669 ± 0.066	0.558 ± 0.040
Palmitoyl-CoA + aminotriazole	1.125 ± 0.099	1.194 ± 0.080

The generation of reactive oxygen species, predominantly H_2O_2, was measured in isolated mitochondria and peroxisomes as described under Material and methods. Rotenone (10 μM) and aminotriazole (a catalase inhibitor; 40 mM) were added to enhance the ROS production. The values represent the means ± standard errors of the mean of four or five animals for each experimental condition.

ROS generation. The exception was the addition of rotenone in the presence of α-ketoglutarate, which did not produce an increase in ROS generation. In no case, however, was there any significant difference between the control and the arthritic condition.

Enzyme activities

The activities of the enzymes catalase, glutathione peroxidase, glutathione reductase, SOD, G6PDH, cytochrome *c* oxidase, and NO synthase were measured in the total homogenate and subcellular fractions. Table 3 shows the enzymatic activities measured in the total homogenate. The results are presented per gram of fresh tissue and per milligram of protein. The latter corresponds to the specific activity. No differences were found for the SOD and glutathione reductase activities, but the catalase and glutathione peroxidase activities were lower in the arthritic condition. The difference was more pronounced for the catalase activity, the arthritic condition presenting only one-third of the activity found for the control condition. On the other hand, the G6PDH activity was clearly higher in the arthritic condition. The activity of the constitutive NO synthase in the total homogenate was not different between control and arthritic rats, but the inducible form was 64% higher in the arthritic condition.

The results obtained with the subcellular fractions are shown in Table 4. Here again no significant differences between arthritic and control rats were found for the SOD activity. The glutathione reductase activity was clearly lower in the cytosolic fraction of arthritic rats. The catalase activity was considerably lower for the arthritic condition in all subcellular fractions: 86, 87, and 77% less, respectively, in the mitochondria, peroxisomes, and cytosol. The glutathione peroxidase activity of arthritic rats was also lower in the mitochondria and cytosol from arthritic rats, but in the peroxisomes it was approximately equal to the corresponding activity found for the control rats. The cytosolic glucose-6-phosphate dehydrogenase activity was higher in the cytosolic fraction from arthritic rats, an observation that is consistent with the measurements in the whole liver homogenate (Table 3) if one considers the notion that this enzyme is mainly cytosolic. The cytochrome *c* oxidase activity, which is restricted to the mitochondria, was revealed to be considerably increased in the arthritic condition (+62%). The peroxisomes were revealed to possess the highest activities of both constitutive and inducible NO synthases [36]. In the arthritic condition these activities were clearly higher, 119.3% in the case of the constitutive enzyme.

Table 3
Activities of antioxidant enzymes, respiratory enzymes, and NO synthases (constitutive and inducible) in the liver homogenate of control and arthritic rats.

Parameter	Referred to wet tissue weight		Referred to protein content	
	Control	Arthritis	Control	Arthritis
Catalase	88.61 ± 3.20* mmol/min per g	29.43 ± 1.10* mmol/min per g	868.0 ± 65.7** mmol/min per g	327.9 ± 33.1** mmol/min per g
Glutathione reductase	4.78 ± 0.18# mmol/min per g	3.99 ± 0.13# mmol/min per g	48.4 ± 2.2 mmol/min per g	38.7 ± 3.6 mmol/min per g
SOD	226.5 ± 12.9 U/g	184.9 ± 17. U/g	2.32 ± 0.13 U/mg	2.15 ± 0.14 U/mg
Glutathione peroxidase	25.83 ± 1.67** mmol/min per g	14.52 ± 1.20** mmol/min per g	258.8 ± 24.5## mmol/min per g	172.9 ± 22.7## mmol/min per g
G6PDH	2.39 ± 0.28## mmol/min per g	3.56 ± 0.30## mmol/min per g	22.8 ± 3.2** mmol/min per g	40.2 ± 1.9** mmol/min per g
Constitutive NOS	0.328 ± 0.03 mmol/min per g	0.359 ± 0.01 mmol/min per g	1.96 ± 0.05 mmol/min per g	1.58 ± 0.15 mmol/min per g
Inducible NOS	0.54 ± 0.03* mmol/min per g	0.88 ± 0.02** mmol/min per g	0.173 ± 0.01** mmol/min per g	0.298 ± 0.02* mmol/min per g

NOS, nitric oxide synthase. The enzymatic activities were measured in the liver as described under Material and methods and expressed per gram of wet tissue and per milligram of protein in the homogenate supernatant (specific activity). The values represent the means ± standard error of the mean of five to seven animals for each experimental condition. Pairs of values in the same line labeled with superscripts are statistically different according to Student's *t* test:

*$p < 0.0001$.

** $p < 0.001$.

# $p < 0.01$.

$p < 0.05$.

Table 4
Activities of antioxidant enzymes, respiratory enzymes, and NO synthases (constitutive and inducible) in the subcellular fractions of livers from control and arthritic rats.

Parameter	Mitochondria		Peroxisomes		Cytosol	
	Control	Arthritis	Control	Arthritis	Control	Arthritis
Catalase (mmol/min per g)	4786.2 ± 512.9*	676.7 ± 96.8*	958.0 ± 138.2*	127.1 ± 17.8*	1265.2 ± 57.8*	291.8 ± 49.3*
Glutathione reductase (mmol/min per g)	13.24 ± 1.76	11.00 ± 0.60	25.2 ± 1.6	18.3 ± 2.7	110.0 ± 4.5#	80.5 ± 4.6#
SOD (U/mg)	2.18 ± 0.10	2.30 ± 0.14	1.05 ± 0.13	0.89 ± 0.15	4.07 ± 0.25	3.48 ± 0.10
Glutathione peroxidase (mmol/min per g)	206.6 ± 16.7##	136.8 ± 4.9##	75.20 ± 8.47	69.23 ± 7.19	501.9 ± 19.6*	296.1 ± 13.2*
G6PDH (mmol/min per g)	—	—	—	—	30.79 ± 1.50##	48.60 ± 4.95##
Cytochrome *c* oxidase (mmol/min per g)	290.9 ± 46.5##	471.6 ± 45.7##	—	—	—	—
Constitutive NOS (mmol/min per g)	0.10 ± 0.01##	0.40 ± 0.12##	1.41 ± 0.69	3.11 ± 0.88	Not detected	Not detected
Inducible NOS (mmol/min per g)	Not detected	Not detected	1.24 ± 0.29##	2.72 ± 0.41##	0.063 ± 0.02**	0.30 ± 0.03**

NOS, nitric oxide synthase. The enzyme activities in the various subcellular fractions were measured as described under Material and methods and referred to the protein content. The values represent the means ± standard errors of the mean of four to six animals for each experimental condition.
Pairs of values in the same line labeled with superscripts are statistically different according to Student's *t* test:

*$p < 0.0001$

** $p < 0.001$.

# $p < 0.01$.

$p < 0.05$.

The mitochondrial NO synthase, which is constitutive, revealed only a discrete activity that was three times higher in the arthritic condition, whereas the inducible enzyme was not detected in this organelle. The opposite was found in the cytosol: no constitutive NO synthase activity was detected, but a relatively low inducible NO synthase activity was detected, which was four times higher in the arthritic condition.

Nitrite plus nitrate levels

The indirect markers of NO, its decomposition products nitrate and nitrite, were measured in the total homogenate and in all subcellular fractions. The results are shown in Fig. 5. The nitrite+nitrate contents were 112 and 35% higher, respectively, in the peroxisomes and in the cytosol of arthritic rats compared to the controls. No difference was found in the mitochondria. Surprisingly, in the total homogenate of arthritic rats the nitrite+nitrate levels were 32% lower.

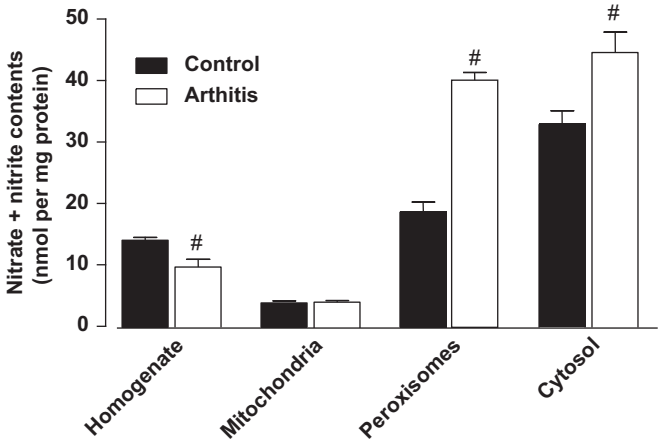

Fig. 5. Nitrite+nitrate concentrations in the homogenate and subcellular fractions of control and arthritic rat livers. The nitrite+nitrate contents were measured in the total homogenate, disrupted mitochondria, disrupted peroxisomes, and cytosolic soluble fraction as described under Material and methods. The values are the means ± standard error of the mean of three to four animals in each experimental condition. #$p < 0.05$ for the difference between control and arthritic rats.

Glutathione levels

Table 5 shows the levels of oxidized and reduced glutathione that were found in the liver homogenate. Here again the results were referred to the liver fresh weight and, alternatively, to the protein content. Irrespective of the way in which the contents were calculated it turns out that the GSH/GSSG ratio was considerably diminished in the arthritic condition (arthritic around 40% of the control). This was the consequence of both a diminution of the GSH content and an increase in the GSSG

Table 5
Reduced (GSH) and oxidized (GSSG) glutathione contents in the liver homogenate of control and arthritic rats.

Parameter	Referred to wet tissue weight		Referred to protein content	
	Control	Arthritis	Control	Arthritis
GSH	$3.39 \pm 0.22^{**}$ μmol/g	$1.84 \pm 0.17^{**}$ μmol/g	$15.84 \pm 1.10^{\#\#}$ nmol/mg	$10.93 \pm 1.59^{\#\#}$ nmol/mg
GSSG	$0.365 \pm 0.025^{\#\#}$ μmol/g	$0.515 \pm 0.053^{\#\#}$ μmol/g	$1.78 \pm 0.24^{\#\#}$ nmol/mg	$3.00 \pm 0.34^{\#\#}$ nmol/mg
GSH+(2 × GSSG)	$4.12 \pm 0.23^{\#\#}$ μmol GSH units/g	$2.87 \pm 0.15^{\#\#}$ μmol GSH units/g	19.40 ± 1.50 nmol GSH units/mg	17.93 ± 1.86 nmol GSH units/mg
GSH/GSSG	$9.28 \pm 0.92^{**}$	$3.57 \pm 0.60^{**}$	8.89 ± 0.90	$3.64 \pm 0.57^{**}$

Measurements are described under Material and methods. The results are expressed per gram of wet tissue and per milligram of protein in the homogenate. The values represent the means ± standard error of the mean of five animals in each experimental condition.
Pairs of values in the same line labeled with superscripts are statistically different:

** $p < 0.001$.
$^{\#\#}$ $p < 0.05$.

Table 6
Reduced and oxidized glutathione content in the subcellular fractions of livers from control and arthritic rats.

Parameter	Mitochondria		Peroxisomes		Cytosol	
	Control	Arthritis	Control	Arthritis	Control	Arthritis
GSH (nmol/mg)	$5.07 \pm 0.36^{**}$	$2.51 \pm 0.36^{**}$	$1.27 \pm 0.23^{\#\#}$	$0.52 \pm 0.07^{\#\#}$	$20.37 \pm 2.80^{\#\#}$	$11.06 \pm 1.00^{\#\#}$
GSSG (nmol/mg)	$1.12 \pm 0.10^{*}$	$2.02 \pm 0.08^{*}$	0.38 ± 0.05	0.47 ± 0.05	$9.19 \pm 0.47^{\#\#}$	$11.67 \pm 0.84^{\#\#}$
GSH+(2 × GSSG) (nmol GSH units/mg)	7.31 ± 0.52	6.53 ± 0.45	2.03 ± 0.55	1.46 ± 0.38	38.75 ± 2.08	34.40 ± 1.96
GSH/GSSG	$4.52 \pm 0.38^{*}$	$1.24 \pm 0.18^{*}$	3.34 ± 1.03	1.11 ± 0.25	$2.21 \pm 0.41^{\#\#}$	$0.95 \pm 0.19^{\#\#}$

Measurements are described under Material and methods. The results are expressed per milligram of protein. The values represent the means ± standard error of the mean of five or six animals in each experimental condition.
Pairs of values in the same line labeled with superscripts are statistically different:

* $p < 0.0001$.
** $p < 0.001$.
$^{\#\#}$ $p < 0.05$.

content. The total content of both forms, i.e., GSH+GSSG, may have slightly decreased in the arthritic condition, but a statistically significant difference was found only when the contents were expressed in terms of the fresh liver weight. Similar decreases in the GSH/GSSG ratio for the arthritic condition were found in mitochondria, peroxisomes, and cytosol, as revealed by Table 6. In peroxisomes this was the consequence of a diminished GSH content. In the mitochondria and in the cytosol it was the result of both diminished GSH content and increased GSSG content. The total content, i.e., the sum GSH+GSSG, presented no significant differences in the cellular fractions analyzed.

Discussion

Oxidative damage and ROS contents

The results of this investigation reveal that the oxidative stress is increased in the liver of rats with adjuvant-induced arthritis compared with normal healthy animals. This can be concluded from the higher levels of TBARS and protein carbonyl groups found in the total liver homogenate as well as in the mitochondria, peroxisomes, and cytosol. This conclusion is also allowed by the clearly higher levels of ROS in the whole liver and of the NO markers nitrite+nitrate in the cytosol and peroxisomes. These observations are highlighted in Fig. 6, which attempts to represent in a schematic way the events that could have led arthritic rats to (1) increased hepatic production of reactive oxygen and nitrogen species coupled to a (2) diminished capacity of detoxification via the ROS scavenger system. These two aspects are discussed separately in the following paragraphs. Concerning more specifically the observations about oxidative damage, higher TBARS levels in the total liver homogenate had already been reported [39]. In the present study we have extended the experimental approach by measuring also protein carbonyl groups, which allows a more complete and reliable evaluation of oxidative stress. The protein carbonyl groups should be preferred when oxidative stress is evaluated in isolated organelles because the protein content is almost unaffected during the isolation procedures, whereas TBARS are protein-independent and can get lost. An increase in protein carbonyl groups has been also demonstrated in the articular fluids of patients with rheumatoid arthritis [40].

Although ROS have rather short half-lives, the technique used in this work for their detection, the DCFH-DA probe, has been employed successfully in several studies [41]. Even under appropriate conditions, H_2O_2 and other ROS are normally decomposed or lost during the organelle isolation, mainly because of membrane diffusion or inactivation. Thus, the ROS contents found in the mitochondria, peroxisomes, and cytosolic fractions do not represent the situation just after extraction. However, the higher levels that were found in hepatic cellular fractions from arthritic rats may be reflecting the fact that the fractional loss and inactivation of ROS are independent of their concentrations so that higher levels at a given time also represent higher initial levels. On the other hand, it is important to highlight that the DCFH probe is not specific for H_2O_2 and ROS, because it also reacts with other reactive species, including lipid hydroperoxides [42]. Moreover, DCFH can also be oxidized in a ROS-independent manner by cytochrome *c* and Fe^{+2} [42]. For this reason the ROS contents as measured by the DCFH probe must always be examined in the context of the other oxidative stress indicators.

The enhanced ROS generation

The scheme in Fig. 6 proposes that the increased oxidative metabolism in the liver of arthritic rats is at least partly responsible for an increased generation of reactive oxygen species. The rats used in this study revealed many signs of cachexia and chronic inflammation (see Table 1 and [10]), including diminished

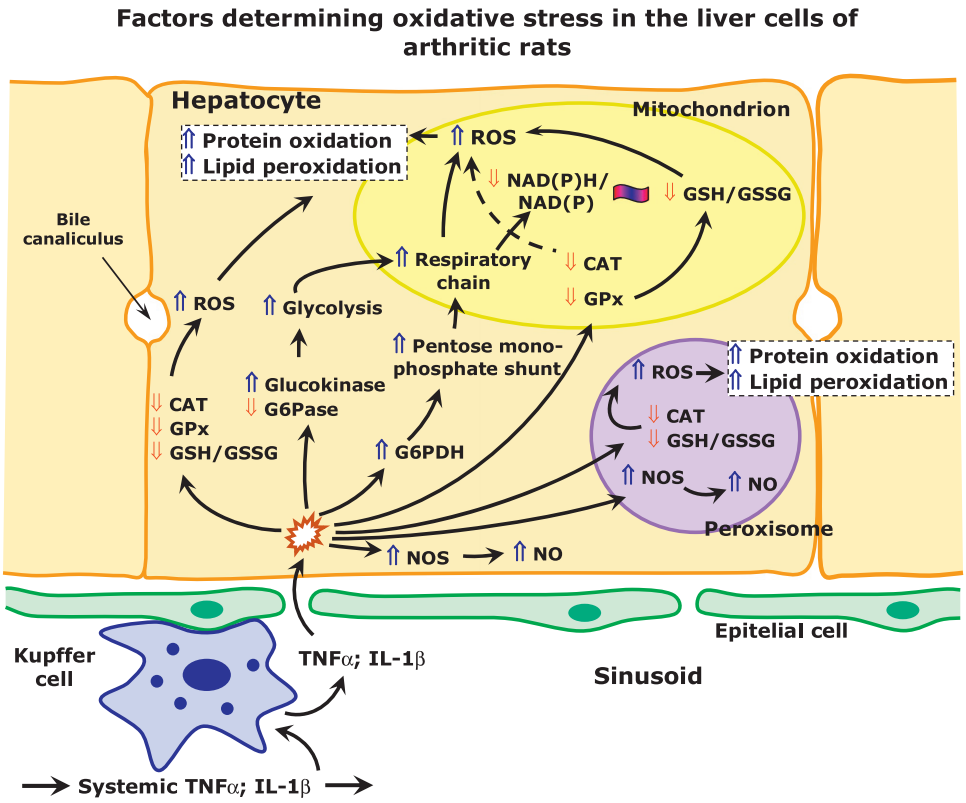

Fig. 6. Schematic representation of the events leading to increased oxidative stress in the liver cells of rats with adjuvant arthritis. The scheme is discussed in the text and is based on the results of the current work and on previously published data. The symbol ↑ means upregulation and ↓ means downregulation. Abbreviations: TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; GSH, reduced glutathione; GSSG, oxidized glutathione; CAT, catalase; GPx, glutathione peroxidase; G6PDH, glucose-6-phosphate dehydrogenase; G6Pase, glucose 6-phosphatase; NOS, nitric oxide synthase.

plasma albumin and increased plasma globulins and elevated levels of plasma TNF-α, IL-1β, and IL-6, which are well-known metabolic regulators [13]. High catabolic activity is characteristic of cachexia and work published over the past years has confirmed that the oxidative metabolism is considerably enhanced in the liver of adjuvant-induced arthritic rats [11,14,15,43]. Fig. 6 illustrates the most important observations. Livers from arthritic rats present higher activity of glucokinase and a diminished activity of glucose 6-phosphatase, a combination that leads to higher levels of glucose 6-phosphate and also to higher rates of glycolysis [15,43]. The pentose–monophosphate shunt should be also increased, as can be expected from the higher levels of glucose 6-phosphate [43] combined with the increased activity of glucose-6-phosphate dehydrogenase observed in this work. It should be mentioned that the higher activity of glucose-6-phosphate dehydrogenase in arthritic rats is in accordance with the induction of both activity and expression of this enzyme by agents that promote oxidative stress in the rat liver, such as hydrogen peroxide and *t*-butylhydroperoxide [44]. Furthermore, the glucose-6-phosphate dehydrogenase activity is increased in the liver of rats with cachexia induced by TNF-α [45]. Perfused livers from arthritic rats are generally characterized by higher rates of oxygen uptake under several conditions [11]. Although this can be brought about by an increased flux of reducing equivalents from the cytosol in the case of increased glycolysis, there is equally evidence for an increased activity of the mitochondrial respiratory chain. In fact, isolated mitochondria from arthritic rats respire more intensely in both the presence and the absence of ADP [11], an observation that is consistent with the increased cytochrome *c* activities found in this work. Taken as a whole all these observations are consistent with a more intense oxidative metabolism in the liver of arthritic rats, a phenomenon that should also generate a more oxidizing environment and a more intense production of ROS.

Another factor that can also be responsible for the increased generation of both reactive oxygen species and reactive nitrogen species in arthritis is the enhanced NO-synthase activity (Fig. 6). The same agents that also increase oxidative metabolism, TNF-α, IL-1β, IL-6, and others [13], are equally responsible for increasing the activity of the inducible NO synthase in several tissues. Increased tissue levels of NO result in the formation of reactive nitrogen species such as peroxynitrite, equally able to produce oxidative tissue injury by itself or to generate reactive oxygen species [46]. It should be remarked that our observations with respect to the NO levels (as indicated by the nitrate+nitrite contents) and NO-synthase activities are compatible with the enhanced nitrate excretion and the increased expression of NO synthase mRNA found in the liver and joints of adjuvant- and collagen-induced arthritic rats [47]. It should be recalled, however, that we found considerably higher nitrate+nitrite levels in the peroxisomes and cytosol of arthritic rats, but a smaller content of these markers in the total homogenate, which includes also nuclei and extra- and intracellular membranes. This observation can also be indicating a drastic change in the compartmentation of NO in the liver of arthritic rats, a matter certainly deserving clarification by future work.

The fact that no increased ROS production was found in isolated hepatic mitochondria and peroxisomes from arthritic rats does not disprove that the phenomenon occurs in vivo because incubations with isolated organelles do not reproduce the in vivo conditions, as inflammatory agents, for example, are absent. In fact, isolated mouse hepatocytes presented higher

mitochondrial ROS production in response to proinflammatory cytokines [48] and it has also been demonstrated in rats that inflammatory liver injuries correlate with increases in ROS production and cytokine release [49].

Impaired ROS scavenging and low GSH/GSSG ratios due to arthritis

The results of this work strongly indicate that the ROS-scavenging system is at least partly impaired in the liver of arthritic rats. The superoxide dismutase activity is practically normal in the liver of arthritic rats. If one takes into account the likely possibility discussed above of an accelerated oxidative metabolism in the liver of arthritic rats this could equally mean higher rates of hydrogen peroxide production (↑respiratory activity→$O_2^{\bullet -}$→H_2O_2). Decomposition of H_2O_2, in turn, can be expected to be impaired in the arthritic condition by virtue of the marked deficiency in catalase, which is reduced by at least 70% in the arthritic condition. In the case of the GSH peroxidase reaction, decomposition of H_2O_2 is possibly further impaired by the fact that the GSH concentration and the GSH/GSSG ratio were revealed to be considerably lower in the arthritic condition. The catalase found in mitochondrial preparations deserves a few comments. The presence of this enzyme in mitochondria is controversial and it is not clear if catalase is an intrinsic component of the mitochondrial antioxidant system [50] or if its presence in mitochondrial preparations results from a contamination by peroxisomes [51]. We tend to believe that the catalase activity found in the mitochondrial preparations represents at least partly the mitochondrial enzyme rather than a peroxisomal contamination because higher activities were found in the mitochondria.

The levels of GSH and GSSG found in this work in the total liver homogenate are very close to those reported previously for freeze-clamped livers from fasted rats [52]. These levels reflect, thus, most probably the true physiologic conditions. Losses of GSH occur during tissue processing [52] so that the absolute levels found in the various cell fractions do not reflect the physiologic conditions. Both the GSH contents and the GSH/GSSG ratios in the arthritic condition were, however, consistently lower in all cell fractions that were examined so that, in comparative terms, they are possibly still reflecting the difference between the healthy and the arthritic condition.

The lower GSH/GSSG ratio in the liver cells of arthritic rats is caused mainly by the oxidation of GSH to GSSG without reduction back to GSH, as can be deduced from the observation that the sum (GSH+(2 × GSSG)) was much less affected by arthritis than the individual GSH or GSSG contents. In principle the low GSH/GSSG ratios may have several causes. High rates of transformation of GSH into GSSG by the GSH peroxidase is one of these causes. However, the catalytic activity of the hepatic GSH peroxidase in arthritic rats is lower. This fact would require considerably higher concentrations of the second substrate, namely H_2O_2, to produce higher reaction rates. The kinetics of GSH peroxidase is complex, but the general belief is that the enzyme is predominantly in the reduced form in the presence of physiological concentrations of the substrates [53,54]. Under such conditions the rate of H_2O_2 decomposition is given by the product $k_{+1}[E_0][H_2O_2]$, where k_{+1} is a second-order rate constant and $[E_0]$ is the total GSH peroxidase concentration [53]. Recent studies have shown that this relation holds unless the H_2O_2 concentration increases to considerably high levels so as to exceed $[E_0]$ [55], an improbable situation in this study. Arthritis reduces $[E_0]$ to 56.2% of its normal levels so that equal rates of H_2O_2 decomposition in the normal and arthritic conditions would require correspondingly higher H_2O_2 concentrations. The required $[H_2O_2]_{arthritic}/[H_2O_2]_{normal}$ ratio for equal rates of H_2O_2 decomposition in normal and arthritic rats can be calculated from the GSH peroxidase activities given in Table 3as

$$\frac{[H_2O_2]_{arthritic}}{[H_2O_2]_{normal}} = \frac{[E_0]_{normal}}{[E_0]_{arthritic}} = \frac{25.83}{14.52} = 1.77.$$

A $[H_2O_2]_{arthritic}/[H_2O_2]_{normal}$ ratio higher than 1.77 would thus be required to produce in the liver of arthritic rats a higher net flux through the GSH peroxidase. This is not very likely if one takes into account the ROS contents of livers from arthritic rats, which were revealed to be approximately 1.44 higher than those in healthy rats (see Fig. 3). It is thus probable that the actual flux through the glutathione peroxidase is lower in the liver of arthritic rats so that it is also unlikely that its reaction is an important cause of the low GSH/GSSG ratios. More important is possibly the GSSG reduction catalyzed by the GSH reductase. The hepatic activity of this enzyme is almost normal in arthritic rats, but the more oxidized environment, suggested by a number of observations discussed above, could be producing lower NADPH/NAD^+ ratios, thus reducing the substrate availability for the GSH reductase. This enzyme works far from equilibrium in vivo and the velocity of GSSG reduction is known to be dependent only on the availability of the substrates GSSG and NADPH [52,56]. It is thus possible that the low GSH/GSSG ratios reflect the general more oxidized state of the arthritic liver, although other causes cannot be excluded.

Concluding remarks

In conclusion it can be said that the results of this study reveal that the liver of rats with adjuvant-induced arthritis present a pronounced oxidative stress and that, in consequence, injury to lipids and proteins is highly significant. The higher ROS content of the liver of arthritic rats seems to be the consequence of both a stimulated pro-oxidant system and a deficient antioxidant defense with a predominance of the latter as indicated by the strongly diminished activities of catalase and glutathione peroxidase. This is an unbalanced situation that certainly contributes to the malignancy and morbidity of the arthritis disease.

Acknowledgments

The authors are grateful for (a) the technical assistance of Célia Akemi Gasparetto, (b) the financial support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico, and (c) the financial support of the Fundação Araucária.

References

[1] Gabriel, S. E.; Michaud, K. Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases. *Arthritis Res. Ther.* **11:**229–245; 2009.
[2] Choy, E. H. S.; Panayi, G. S. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* **344:**907–916; 2001.
[3] Kamanli, A.; Naziroglu, M.; Aydilek, N.; Hacievliyagil, C. Plasma lipid peroxidation and antioxidant levels in patients with rheumatoid arthritis. *Cell Biochem. Funct.* **22:**53–57; 2004.
[4] Seven, A.; Guzel, S.; Aslan, M.; Hamuryudan, V. Lipid, protein, DNA oxidation and oxidant status in rheumatoid arthritis. *Clin. Biochem.* **41:**538–543; 2008.
[5] Sarban, S.; Kocyigit, A.; Yazar, M.; Isikan, U. E. Plasma total antioxidant capacity, lipid peroxidation and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. *Clin. Biochem.* **38:**981–986; 2005.
[6] Sangha, O. Epidemiology of rheumatic diseases. *Rheumatology* **39(2):**3–12; 2000.
[7] Sattar, N.; Mccarey, D. W.; Capell, H.; Mcinnes, I. B. Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation* **108:**2957–2963; 2003.
[8] Haruna, Y.; Morita, Y.; Komai, N.; Yada, T.; Sakuta, T.; Tomita, N.; Fox, D. A.; Kashihara, N. Endothelial dysfunction in rat adjuvant-induced arthritis:

vascular superoxide production by NADP(H) oxidase and uncoupled endothelial nitric oxide synthase. *Arthritis Rheum.* **54:**1847–1855; 2006.
[9] Haruna, Y.; Morita, Y.; Yada, T.; Satoh, M.; Fox, D. A.; Kashihara, N. Fluvastatin reverses endothelial dysfunction and increased vascular oxidative stress in rat adjuvant-induced arthritis. *Arthritis Rheum.* **56:**1827–1835; 2007.
[10] Roubenoff, R.; Roubenoff, R. A.; Cannon, J. G.; Kehayias, J. J.; Zhuang, H.; Dowson-Hughes, B.; Dinarello, C. A.; Rosenberg, I. H. Rheumatoid cachexia: cytokine-driven hypermetabolism and loss of lean body mass in chronic inflammation. *J. Clin. Invest.* **93:**2379–2386; 1994.
[11] Caparroz-Assef, S. M.; Bersani-Amado, C. A.; Nascimento, E. A.; Kelmer-Bracht, A. M.; Ishii-Iwamoto, E. L. Effects of the nonsteroidal anti-inflammatory drug nimesulide on energy metabolism in livers from adjuvant-induced arthritic rats. *Res. Commun. Mol. Pathol. Pharmacol* **99:**93–116; 1998.
[12] Morton, D. M.; Chatfield, D. H. The effects of adjuvant-induced arthritis on the liver metabolism of drugs in rats. *Biochem. Pharmacol.* **19:**473–481; 1970.
[13] Billingham, M. E. J.; Gordon, A. H. The role of the acute phase reaction in inflammation. *Agents Actions* **6:**195–200; 1976.
[14] Fedatto Jr Z.; Ishiii-Iwamoto, E. L.; Amado, C. B.; Vicentini, G.; D'urso-Panerari, A.; Bracht, A.; Kelmer-Bracht, A. M. Gluconeogenesis in the liver of arthritic rats. *Cell. Biochem. Funct.* **17:**271–278; 1999.
[15] Fedatto Jr Z.; Ishii-Iwamoto, E. L.; Bersani-Amado, C.; Maciel, E. R. M.; Bracht, A.; Kelmer-Bracht, A. M. Glucose phosphorylation capacity and glycolysis in the liver of arthritic rats. *Inflammation Res* **49:**128–132; 2000.
[16] Yassuda-Filho, P.; Bracht, A.; Ishii-Iwamoto, E. L.; Lousano, S. H.; Bracht, L.; Kelmer-Bracht, A. M. The urea cycle in liver of arthritic rats. *Mol. Cell. Biochem.* **243:**97–106; 2003.
[17] Hung, D. Y.; Siebert, G. A.; Chang, P.; Whitehose, M. W.; Fletcher, L.; Crawford, D. H.; Roberts, M. S. Hepatic pharmacokinetics of propranolol in rats with adjuvant induced systemic inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290:**G343–G351; 2005.
[18] Pearson, C. M.; Wood, F. D. Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. *Am. J. Pathol. Philadelphia* **42:**93–95; 1963.
[19] Bendele, A. M.; McComb, J.; Gould, T.; McAbee, T.; Sennelle, G.; Chlipala, E.; Guy, M. Animal models of arthritis: relevance to human disease. *Toxicol. Pathol.* **27:**134–142; 1999.
[20] Szekanecz, Z.; Halloran, M. M.; Volin, M. V.; Woods, J. M.; Strieter, R. M.; Haines, G. K.; Kunkel, S. L.; Burdick, M. D.; Koch, A. E. Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. *Arthritis Rheum.* **43:**1266–1277; 2000.
[21] Natarajan, S. K.; Eapen, C. E.; Pullimood, A. B. Balasubramanian. Oxidative stress in experimental liver microvesicular steatosis: role of mitochondria and peroxisomes. *J. Gastrointest. Hepatol* **21:**1240–1249; 2006.
[22] Lowry, O. H.; Rosebrough, N. J.; Lewis Farr, A.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275; 1951.
[23] Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **52:**302–310; 1978.
[24] Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **186:**464–478; 1990.
[25] Takayanagi, R.; Takeshige, K.; Minakami, S. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles: dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol. *Biochem. J* **192:**853–860; 1980.
[26] Siqueira, I. R.; Fochesatto, C.; Torres, I. L. S.; Dalmaz, C.; Netto, C. A. Aging affects oxidative state in hippocampus, hypothalamus and adrenal glands of Wistar rats. *Life Sci.* **78:**271–278; 2005.
[27] Zaccagnino, P.; Saltarella, M.; D'oria, S.; Corcelli, A.; Saponetti, M. S.; Lorusso, M. N-arachidonylglycine causes ROS production and cytochrome c release in liver mitochondria. *Free Radic. Biol. Med.* **47:**585–592; 2009.
[28] Small, G. M.; Burdett, K.; Connock, M. J. A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. *Biochem. J.* **227:**205–210; 1985.
[29] Hissin, P. J.; Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74:**214–226; 1976.
[30] Bryan, N. S.; Grisham, M. B. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med.* **43:**645–657; 2007.
[31] Bergmeyer, H. U., editor. *Methods of Enzymatic Analysis.* Weinheim/London: Verlag Chemie-Academic Press; 1974.
[32] Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem* **47:**469–474; 1974.
[33] Tappel, A. L. Glutathione peroxidase and hydroperoxides. *Methods Enzymol.* **52:**506–513; 1978.
[34] Mason, T. L.; Poyton, R. O.; Wharton, D. C.; Schatz, G. Cytochrome c oxidase from Bakers' yeast. *J. Biol. Chem.* **248:**1346–1354; 1973.
[35] Knowles, R. G.; Merrett, M.; Salter, M.; Moncada, S. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.* **270:**883–896; 1990.
[36] Liaudet, L.; Soriano, F. G.; Szabo, C. Biology of nitric oxide signaling. *Crit. Care Med.* **28:**N37–N52; 2000.
[37] Pratt, D. S.; Kaplan, M. M. Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N. Engl. J. Med.* **342:**1266–1271; 2000.
[38] Li, N.; Ragheb, K.; Lawler, G.; Sturgis, J.; Rajwa, B.; Melendez, J. A.; Robinson, J. P. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J. Biol. Chem.* **278:** 8516–8525; 2003.
[39] Vijayalakshmi, T.; Muthulakshmi, V.; Sachdanandam, P. Salubrious effect of Semecarpus anacardium against lipid peroxidative changes in adjuvant arthritis studied in rats. *Mol. Cell. Biochem.* **175:**65–69; 1997.
[40] Mantle, D.; Falkous, G.; Walker, D. Quantification of protease activities in synovial fluid from rheumatoid and osteoarthritis cases: comparison with antioxidant and free radical damage markers. *Clin. Chim. Acta* **284:**45–58; 1999.
[41] Wardman, P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med* **43:**995–1022; 2007.
[42] Tarpey, M. M.; Wink, D. A.; Grisham, M. B. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **286:**R431–444; 2004.
[43] Kelmer-Bracht, A. M; Santos, C. P. B.; Ishii-Iwamoto, E. L; Broetto-Biazon, A. C.; Bracht, A. Kinetic properties of the glucose 6-phosphatase of the liver from arthritic rats. *Biochim. Biophys. Acta* **1638:**50–56; 2003.
[44] Cascales, M.; Martín-Sanz, P.; Craciunescu, D. G.; Mayo, I.; Aguiliar, A.; Robles-Chillide, E.; Cascales, C. Alterations in the hepatic peroxidation mechanisms in thioacetamide-induced tumors in rats. *Carcinogenesis* **12:**233–240; 1991.
[45] Yasmineh, W. G.; Parkin, J. L.; Caspers, J. I.; Theologides, A. Tumor necrosis factor/cachectin decreases catalase activity of rat liver. *Cancer Res.* **51:**3990–3995; 1991.
[46] Grisham, M. B.; Jourd'heuil, D.; Wink, D. A. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am. J. Physiol.* **276:**G315–G321; 1999.
[47] Cannon, G. W.; Openshaw, S. J.; Hibbis Jr J. B.; Hoidal, J. R.; Hueckstead, T. P.; Griffiths, M. M. Nitric oxide production during adjuvant-induced and collagen-induced arthritis. *Arthritis Rheum.* **39:**1677–1684; 1996.
[48] Adamson, G. M.; Billings, R. E. Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes. *Arch. Biochem. Biophys.* **294:**223–229; 1992.
[49] Jaeschke, H. Reactive oxygen and mechanisms of inflammatory liver injury: present concepts. *J. Gastroenterol. Hepatol.* **26(1):**173–179; 2011.
[50] Salvi, M.; Battaglia, V.; Brunati, A. M.; La Rocca, N.; Tibaldi, E.; Pietrangeli, P.; Marcocci, L.; Mondovi, B.; Rossi, C. A.; Toninello, A. Catalase takes part in rat liver mitochondria oxidative stress defense. *J. Biol. Chem.* **282:**24407–24415; 2007.
[51] Halliwell, B., Gutteridge, J. M. C., editors. *Free Radicals in Biology and Medicine*. 4th edition. London: Oxford Univ. Press; 2007.
[52] Viña, J.; Hems, R.; Krebs, H. A. Maintenance of glutathione content in isolated hepatocytes. *Biochem. J.* **170:**627–630; 1978.
[53] Flohé, L.; Loschen, G.; Günzler, W. A.; Eichele, E. Glutathione peroxidase. V. The kinetic mechanism. *Hoppe-Seylers Z. Physiol. Chem* **353:**987–999; 1972.
[54] Splittgerber, A. G.; Tappel, A. L. Steady-state and pre-steady state kinetic properties of rat liver selenium–glutathione peroxidase. *J. Biol. Chem.* **254:**9807–9813; 1979.
[55] Ng, C. F.; Schafer, F. Q.; Buettner, G. R.; Rodgers, V. G. J. The rate of cellular hydrogen peroxide removal shows dependency on GSH: mathematical insight into in vivo H_2O_2 and GPx concentrations. *Free Radic. Res.* **41:**1201–1211; 2007.
[56] Ziegler, D. M. Role of reversible oxidation–reduction of enzyme thiols–disulfides in metabolic regulation. *Annu. Rev. Biochem* **54:**305–329; 1985.