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Effects of fasting on oxidative stress in rat liver mitochondria

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Abstract
While moderate caloric restriction has beneficial effects on animal health state, fasting may be harmful. The present investigation was designed to test how fasting affects oxidative stress, and to find out whether the effects are opposite to those previously found in caloric restriction studies. We have focused on one of the main determinants of aging rate: the rate of mitochondrial free radical generation. Different parameters related to lipid and protein oxidative damage were also analyzed. Liver mitochondria from rats subjected to 72 h of fasting leaked more electrons per unit of O_2 consumed at complex III, than mitochondria from *ad libitum* fed rats. This increased leak led to a higher free radical generation under state 3 respiration using succinate as substrate. Regarding lipids, fasting altered fatty acid composition of hepatic membranes, increasing the double bond and the peroxidizability indexes. In accordance with this, we observed that hepatic membranes from the fasted animals were more sensitive to lipid peroxidation. Hepatic protein oxidative damage was also increased in fasted rats. Thus, the levels of oxidative modifications, produced either indirectly by reactive carbonyl compounds ($N^{epsilon}$- malondialdehyde-lysine), or directly through amino acid oxidation (glutamic and aminoadipic semialdehydes) were elevated due to the fasting treatment in both liver tissue and liver mitochondria. The current study shows that severe food deprivation increases oxidative stress in rat liver, at least in part, by increasing mitochondrial free radical generation during state 3 respiration and by increasing the sensitivity of hepatic membranes to oxidative damage, suggesting that fasting and caloric restriction have different effects on liver mitochondrial oxidative stress.

Keywords: *Free radicals, fasting, mitochondria, lipid peroxidation, protein carbonyls*

Abbreviations: *ROS, reactive oxygen species; ETC, electron transport chain; CR, caloric restriction; GSA, glutamic semialdehyde; AASA, aminoadipic semialdehyde; MDA, malondialdehyde; MDAL, malondialdehyde lysine; DBI, double bond index; AA, antimycin A; TTFA, theonoyltrifluoroacetone; Rot, rotenone; FRL, free radical leak*

Introduction

In healthy cells, reactive oxygen species (ROS) are mainly produced at the mitochondrial electron transport chain (ETC). The mitochondrial free radical theory of aging [1,2], proposes that mitochondrially-derived ROS are causally involved in aging. Due to the high reactivity of ROS, mitochondria are not only considered as main sources of free radicals in healthy tissues, but also as principal targets for oxidative damage [2–5].

The intervention that more consistently decreases aging rate and increases mean and maximum longevity is caloric restriction [6,7]. Whereas moderate food restriction reduces aging rate and has anti-aging effects, stronger interventions involving a reduction in the caloric intake may be harmful. Thus, fasting is in essence an extreme form of caloric restriction, but

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unlike the latter, it is detrimental, and some investigations have reported pro-oxidant effects following it [8–10].

Fasting and caloric restriction (CR), however, share some similarities. Thus, some effects of fasting are reverted by refeeding [10,11], which is also the case for caloric restriction [12]. Furthermore, no clear pattern has been observed regarding levels of many types of antioxidants neither in caloric restriction nor in fasting. Different investigations have shown increases [8,10], decreases [9,13] or no changes [9] in different antioxidant levels in fasted animals, although in the case of glutathione fasting causes its depletion [14]. Similarly, although increases in antioxidant activities have been proposed to occur in CR [15], studies in skeletal muscle [16], liver [17], and brain, heart and kidney [18] have failed to describe any clear-cut overall pattern of CR-related changes in antioxidant defenses. However, most of the published data suggest that both nutritional approaches lead to different mechanisms generating either anti- aging effects or pro- oxidative effects. (i) Several studies have described that caloric restriction reduces mitochondrial oxygen free radical generation in different tissues [18–21], suggesting that the decrease in aging rate induced by CR is due, at least in part, to such decrease in mitochondrial ROS generation. On the other hand, analysis of free radical levels using electron paramagnetic resonance in rat liver have shown that 36-h fasting increases ROS levels [9]. (ii) Although some studies have reported increases in protein oxidative damage after CR [22], most investigations have shown decreases or no changes in protein oxidative damage depending on the implementation time of the dietary restriction [23–26]. None has investigated effects on protein oxidative damage in fasted animals by specific methodology. (iii) Caloric restriction investigations have also shown a decline in lipid peroxidation levels [16,17,27]. On the other hand, fasting studies have reported increases in oxidative damage to lipids [10,13].

Since food deprivation seems to have dissimilar effects depending on the intensity of the energy deficiency, it is important to discern whether or not fasting and caloric restriction involve similar underlying mechanisms. We performed the present study in the liver because during nutritional interventions it is one of the most affected tissues, it is a principal organ for supplying energy substrates to different tissues in the body and plays a main role in the adaptation of the organism to nutritional changes. Our investigation was designed to find out how fasting affects mitochondrial oxidative stress, focusing on the rate of free radical production and oxidative damage to lipids and proteins. No previous study has investigated how fasting affects mitochondrial free radical generation using a specific and highly sensitive method and allowing localization of the main sites of ROS generation within mitochondria.

Unsaturated fatty acids (UFA) are the macromolecules most sensitive to oxidative damage, with their sensitivity increasing in function of the number of double bonds per fatty acid [28]. Fasting alters fatty acid metabolism, affecting fatty acid composition and the mobilization of fatty acids in adipose tissue [11,29]. In liver, beta-oxidation increases after fasting [30,31] whereas the expression of genes encoding enzymes for lipid synthesis is down-regulated [30]. Thus, it is also possible that changes in lipid composition take place. In order to test this, we investigated mitochondrial and total tissue changes in lipid composition, as well as the sensitivity of liver membranes to lipid peroxidation in hepatic tissue.

Along with lipids, oxidative damage to proteins is especially important since they are essential for tissue structure and function. We have studied the effect of fasting on oxidative damage to mitochondrial and total tissue proteins using highly specific and sensitive techniques. We have quantified the amount of glutamic and aminoadipic semialdehydes (GSA and AASA, respectively) as well as $N^{epsilon}$-malondialdehyde lysine (MDAL). The former compounds constitute the majority of protein carbonyls generated by metal-catalyzed oxidation [32]. The second estimates covalent protein modification by a main lipid peroxidation product, MDA [33,34].

Materials and methods

Animals and treatments

Six-week-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). Animals were fed *ad libitum* with a standard rodent diet and maintained at 22 ± 2°C in a reversed 12 h (light:dark) cycle. Fasting and control animals were caged individually. Before 72 h sacrifice fasting animals were deprived of food, whereas *ad libitum*-fed animals were subjected to overnight fasting before being killed. Overnight fasting is a standard process in the particular case of liver mitochondria since food ingestion strongly affects the metabolic state of these organelles introducing variability between animals. Moreover, a main objective of this investigation was to compare the effects of fasting with previous results obtained with caloric restriction in the liver [35], and, for the same reason, the controls of these last studies had also been subjected to overnight fasting. All animals had free access to water during all stages of the study. Rats were sacrificed by decapitation and liver samples were stored at -80°C for lipid and protein analysis. For hepatic mitochondrial H_2O_2 production and oxygen consumption measurements, fresh tissue samples were processed promptly.

Isolation of mitochondria. Oxygen consumption and ROS generation

After decapitation, liver samples were immediately processed in order to obtain functional mitochondria by differential centrifugation, as previously described [35]. Mitochondrial protein concentration was measured by the Biuret method. Final mitochondrial suspensions were kept on ice, and used for oxygen consumption and H_2O_2 production measurements, performed within two hours following the isolation.

The rate of H_2O_2 production from highly-coupled liver mitochondria was assayed measuring the increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase [36]. The fluorescence was read in a LS50B Perkin Elmer fluorometer (Buckinghamshire, UK). Pyruvate/malate (2.5 mM each) or succinate (5 mM) were used as substrates. All reactions with succinate were performed in the presence of rotenone (2 μM) in order to avoid backward flow of electrons to complex I. In some assays, 2 μM antimycin A or 11 μM thenoyltrifluoroacetone (TTFA) were added to succinate+rotenone-supplemented mitochondria, and 2 μM rotenone was added to pyruvate/malate-supplemented mitochondria, to determine the maximum rates of H_2O_2 production of complexes III + II, II and I, respectively.

Mitochondrial oxygen consumption was measured in a Clark-type O_2 electrode (Oxygraph, Hansatech, Norfolk, UK) in the absence (State 4) and in the presence (State 3) of ADP (500 μM) under the same conditions used for H_2O_2 measurements. The mitochondrial respiratory control indexes (State 3/State 4) with succinate as substrate were 3.35 ± 0.21 in *ad libitum* fed animals and 3.41 ± 0.14 in fasted animals (mean ± SEM).

Since mitochondrial H_2O_2 production and oxygen consumption were measured in parallel under the same experimental conditions, we could calculate the fraction of electrons out of sequence, which reduce O_2 to oxygen radicals instead of reaching complex IV to reduce O_2 to water (the percent free radical leak). Two electrons are needed in order to reduce one molecule of O_2 to H_2O_2, whereas four electrons are transferred in the reduction of O_2 to water. Thus, the free radical leak (%FRL) was calculated as the rate of H_2O_2 production divided by two times the rate of oxygen consumption, and the result being multiplied by 100.

Lipid peroxidation

Lipid peroxidation was measured in liver by the thiobarbituric acid test adapted to tissues [37]. Butyl hydroxytoluene (0.07 mM) was added as an antioxidant to avoid artifact lipid peroxidation during the assay. In order to estimate the sensitivity of liver mitochondrial lipids to free radical damage, lipid peroxidation was stimulated *in vitro* by incubating mitochondria in the presence of 0.4 mM ascorbate and 0.05 mM $FeSO_4$ up to 8 h at 37°C. At different times of incubation, the lipid peroxidation assay was performed. Malondialdehyde-bis(dimethylacetal) (Merck, Germany) was used as standard. The results were expressed as nanomoles of MDA per gram of tissue.

Fatty acid analysis

Total lipids of whole homogenates and mitochondrial fraction from liver were extracted as previously described [38] for GC/MS analysis. Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett Packard 6890 Series II gas chromatograph. A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%.

The following fatty acyl indexes were calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from $n-3$ and $n-6$ series (PUFA$n-3$ and PUFA$n-6$); average chain length (ACL) = $[(\Sigma\%\text{Total}_{14} \times 14)+(\Sigma\%\text{Total}_{16} \times 16)+(\Sigma\%\text{Total}_{18} \times 18)+(\Sigma\%\text{Total}_{20} \times 20)+(\Sigma\%\text{Total}_{22} \times 22)]/100$; double bond index (DBI) = $[(1 \times \Sigma\text{mol\% monoenoic})+(2 \times \Sigma\text{mol\% dienoic})+(3 \times \Sigma\text{mol\% trienoic})+(4 \times \Sigma\text{mol\% tetraenoic})+(5 \times \Sigma\text{mol\% pentaenoic})+(6 \times \Sigma\text{mol\% hexaenoic})]$, and peroxidizability index (PI) = $[(0.025 \times \Sigma\text{mol\% monoenoic})+(1 \times \Sigma\text{mol\% dienoic})+(2 \times \Sigma\text{mol\% trienoic})+(4 \times \Sigma\text{mol\% tetraenoic})+(6 \times \Sigma\text{mol\% pentaenoic})+(8 \times \Sigma\text{mol\% hexaenoic})]$.

GSA, AASA, and MDAL measurements in proteins

GSA, AASA and MDAL concentrations in both crude homogenate and mitochondrial fractions were detected and measured by GC/MS as previously described [26]. GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector. The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally hold at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and [$^2H_8$]-lysine, *m/z* 180 and 187,

Table I. Oxygen consumption of rat liver mitochondria supplemented with pyruvate/malate or succinate in States 4 and 3.

	Pyruvate/malate		Succinate	
	Control	Fasted	Control	Fasted
STATE 4	12.58 ± 0.85	11.89 ± 0.76	36.65 ± 2.57	29.09 ± 2.17*
STATE 3	35.19 ± 2.88	29.38 ± 1.18*	120.43 ± 5.28	97.96 ± 6.06*

Values are means ± SEM from five (control) and seven (fasted) animals and are expressed as nanomoles of O_2/min mg protein. * indicates a significant difference between control and fasted groups ($p < 0.05$)

respectively; GSA and [$^2H_5$]-GSA, *m/z* 280 and 285, respectively; AASA and [$^2H_4$]-AASA, *m/z* 294 and 298, respectively; and MDAL and [$^2H_8$]-MDAL, *m/z* 474 and 482, respectively. The amounts of product were expressed as the ratio μmol GSA, AASA, or MDAL/mol lysine.

Statistical analyses

All comparisons between fasted and *ad libitum* fed animals were statistically analyzed with Students *t* test. The 0.05 level was selected as the point of minimal significance in all cases.

Results

The animals subjected to 72 h of fasting showed a lower state 4 oxygen consumption than controls with complex-II-linked substrate (succinate), but not with complex I-linked substrates (pyruvate/malate) (Table I). State 3 oxygen consumption was significantly lowered in fasted animals in the presence of both complex I- and II- linked substrates (Table I).

Regarding mitochondrial free radical generation rate, no differences were detected between fasted and control animals in state 4 (Figure 1A) with neither pyruvate/malate or succinate as substrates. During phosphorylating respiration (state 3), mitochondria from fasted animals showed a trend to higher mitochondrial free radical generation than controls with both substrates, although the difference was only statistically significant ($p = 0.014$) in the presence of succinate (Figure 1B).

Addition of rotenone to pyruvate/malate-supplemented mitochondria strongly increased mitocondrial H_2O_2 production in both control and fasted animals (compare values in Table II and Figure 1). Similarly, addition of antimycin A to succinate-supplemented mitochondria strongly increased their rate of ROS generation (compare Table II and Figure 1). However, TTFA did not increase H_2O_2 production in mitochondria supplemented with succinate. No differences in H_2O_2 generation between control and fasted animals were observed in the presence of any of the three respiratory chain inhibitors (Table II).

While fasting significantly increased the percent mitochondrial FRL in both respiratory states in succinate-supplemented mitochondria, no differences between groups were detected in pyruvate/malate supplemented mitochondria during state 4 (Figure 2A). Similarly to mitochondrial free radical production, a tendency towards a higher %FRL in state 3 of pyruvate/malate-supplemented mitochondria of fasted animals was observed, but again differences did not reach statistical significance (Figure 2B).

Fatty acid analysis was performed in liver mitochondria as well as in the whole hepatic tissue (Table III). The data obtained were very similar in mitochondrial and total tissue fractions, and only few specific fatty acids were affected differently.

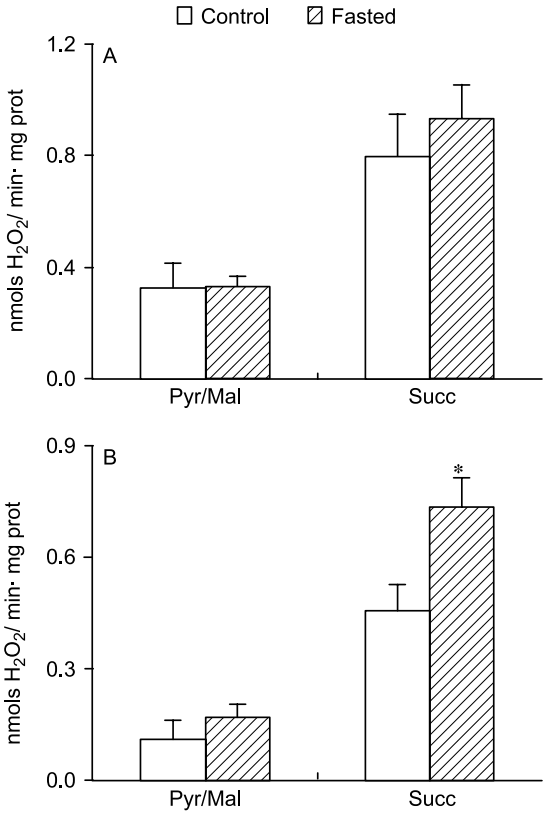

Figure 1. Effect of 72 h of fasting on the basal rates of H_2O_2 production of rat liver mitochondria during state 4 (A) or state 3 (B) respiring with pyruvate/ malate (Pyr/mal) or succinate (Suc) as substrates. Results are means ± SEM. The number of animals was seven in the fasted and five in the control group. * indicates a significant difference between groups (*$p < 0.05$)

Table II. Effect of 72 h of fasting on rates of H_2O_2 production of rat liver mitochondria stimulated by respiratory chain inhibitors.

	Control	Fasted
Pyr/ Mal + Rot	1.02 ± 0.10	1.05 ± 0.12
Succ + TTFA	0.86 ± 0.16	1.04 ± 0.10
Succ + AA	5.54 ± 0.23	4.75 ± 0.49

Values are means ± SEM from five (control) and seven (fasted) animals and are expressed as nanomoles of H_2O_2/ min mg protein. Pyr/ Mal + Rot = pyruvate/ malate + rotenone; Suc + TTFA = succinate + thenoyltrifluoroacetone; Succ + AA = succinate + antimycin A. No significant differences between control and fasted animals were observed in any case.

The overall results (Table III) were identical in both fractions, showing that fasting significantly increased ($p < 0.0001$) double bond (DBI) and peroxidizability indexes (PI). This increase in double bonds was mainly caused by decreases in monounsaturated ($16{:}1n-7$ and $18{:}1n-9$) and increases in polyunsaturated ($18{:}2n-6$, $20{:}4n-6$ and $22{:}6n-3$) fatty acids in fasted animals. Accordingly, the study of the sensitivity of hepatic membranes to oxidative damage showed that *in vitro* lipid peroxidation was significantly higher ($p < 0.009$) in fasted than in control animals (Figure 3).

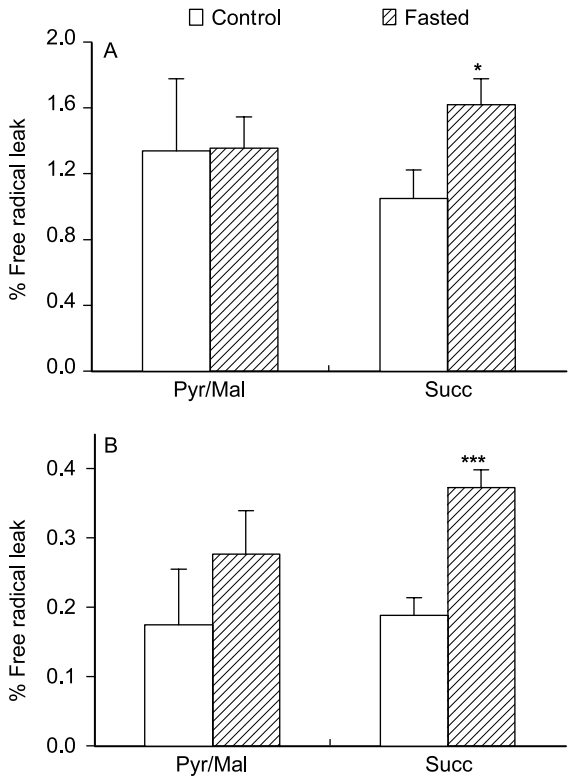

Figure 2. Effect of 72 h of fasting on the percent free radical leak of rat liver mitochondria during state 4 (A) or state 3 (B) respiring with pyruvate/malate (Pyr/Mal) or succinate (Succ) as substrates. Results are means ± SEM. The number of animals was seven in the fasted and five in the control group. * indicates a significant difference between groups (* $p < 0.05$; *** $p < 0.001$)

Regarding oxidative and lipoxidative protein damage, AASA, MDAL and GSA were significantly higher in fasted rats than in *ad libitum* fed ones both in the total and in the mitochondrial fractions (Figure 4). Thus, the steady-state level of protein oxidative stress is generally increased by fasting both through direct oxidation and by lipoxidation-derived protein modification.

Discussion

The current study shows that 72 h of fasting increases oxidative stress in rat liver, at least in part, by enhancing mitochondrial free radical production rate during state 3 respiration. The results suggest that such an increase takes at complex III level. Fasting also modifies the fatty acid composition of liver membranes, making them more prone to oxidative damage. The increased mitochondrial oxidative stress was associated with an augmentation of total and mitochondrial protein oxidative damage in animals subjected to 72 h of fasting when compared to *ad libitum* fed animals.

Mitochondrial free radical generation is closely associated with aging rate both in comparative and in CR models [3]. Unlike in the case of CR, where a reduction in the percentage of mitochondrial free radical leak and ROS generation rate in liver mitochondria has been reported [35,39,40], we observed an increase in the percentage of free radical leak in succinate-supplemented mitochondria after fasting. Since differences between fasted and control animals were detected using succinate as substrate (in the presence of rotenone, avoiding backwards flow of electrons to complex I), it can be deduced that the increment in free radical leak takes place in mitochondrial complex II and/or III. With pyruvate/ malate electrons flow through both complex I and III. However, no significant differences were detected with these last substrates possibly due to the lower sensitivity obtained with pyruvate/malate, since fasted animals also showed a non-significant trend to increase ROS production in state 3 and not in state 4. Data obtained after addition of the specific inhibitors antimycin A and TTFA to succinate-supplemented mitochondria reveal that complex III is an important generator of free radicals while complex II is not, because antimycin A strongly increased ROS production whereas the addition of TTFA did not change it. Thus, the results suggest that fasting makes complex III leakier, increasing the risk of free radical generation. In fact, we observed a higher mitochondrial ROS generation rate when succinate was used as substrate during phosphorylating respiration.

Which mechanisms could lead to an enhancement in free radical leak after 72 h of fasting? The difference in mitochondrial ROS generation between control and

Table III. Fatty acid composition (mol%) of total phospholipids in total and mitochondrial fractions of rat liver after 72 h of fasting.

	Hepatic tissue			Liver mitochondria		
	Control	*Fasted*	*p*	*Control*	*Fasted*	*p*
14:0	0.33 ± 0.01	0.23 ± 0.01	0.001	0.20 ± 0.01	0.10 ± 0.008	0.0001
16:0	22.74 ± 0.11	22.09 ± 0.32	0.071	18.48 ± 0.35	17.20 ± 0.20	0.011
16:1$n-7$	2.73 ± 0.12	0.24 ± 0.01	0.0001	2.49 ± 0.19	1.25 ± 0.05	0.0001
18:0	18.21 ± 0.11	19.91 ± 0.19	0.0001	16.01 ± 0.29	19.25 ± 0.23	0.0001
18:1$n-9$	10.05 ± 0.23	8.00 ± 0.31	0.001	10.26 ± 0.39	6.00 ± 0.05	0.0001
18:2$n-6$	14.36 ± 0.19	15.49 ± 0.32	0.011	18.79 ± 0.22	20.12 ± 0.30	0.006
18:3$n-3$	0.19 ± 0.01	0.11 ± 0.006	0.001	0.11 ± 0.007	0.08 ± 0.009	0.043
20:2$n-6$	0.74 ± 0.03	0.64 ± 0.01	0.049	0.64 ± 0.06	0.50 ± 0.02	0.054
20:3$n-6$	0.16 ± 0.01	0.11 ± 0.004	0.004	0.33 ± 0.04	0.08 ± 0.008	0.0001
20:4$n-6$	24.12 ± 0.23	26.10 ± 0.25	0.0001	24.59 ± 0.36	26.73 ± 0.28	0.001
20:5$n-3$	0.29 ± 0.02	0.06 ± 0.006	0.0001	0.19 ± 0.02	0.06 ± 0.006	0.0001
22:4$n-6$	0.39 ± 0.02	0.37 ± 0.02	0.516	0.25 ± 0.01	0.15 ± 0.004	0.0001
22:5$n-6$	0.36 ± 0.04	0.27 ± 0.01	0.109	0.39 ± 0.03	0.21 ± 0.01	0.001
22:5$n-3$	0.40 ± 0.02	0.20 ± 0.02	0.001	0.52 ± 0.02	0.29 ± 0.05	0.005
22:6$n-3$	4.85 ± 0.16	6.11 ± 0.16	0.001	6.69 ± 0.12	7.92 ± 0.16	0.0001
ACL	18.22 ± 0.01	18.36 ± 0.006	0.0001	18.40 ± 0.008	18.51 ± 0.006	0.0001
SFA	41.30 ± 0.14	42.23 ± 0.35	0.027	34.70 ± 0.53	36.56 ± 0.19	0.009
UFA	58.69 ± 0.14	57.76 ± 0.35	0.027	65.29 ± 0.53	63.43 ± 0.19	0.009
MUFA	12.79 ± 0.25	8.25 ± 0.31	0.0001	12.75 ± 0.55	7.25 ± 0.08	0.0001
PUFA	45.90 ± 0.19	49.50 ± 0.31	0.0001	52.54 ± 0.36	56.17 ± 0.14	0.0001
PUFA$n-3$	5.74 ± 0.19	6.49 ± 0.18	0.022	7.52 ± 0.11	8.37 ± 0.20	0.004
PUFA$n-6$	40.16 ± 0.14	43.01 ± 0.45	0.0001	45.01 ± 0.44	47.80 ± 0.24	0.0001
DBI	176.60 ± 0.97	186.55 ± 0.82	0.0001	198.07 ± 1.02	206.98 ± 0.75	0.0001
PI	159.43 ± 1.48	174.90 ± 1.18	0.0001	180.24 ± 0.78	195.53 ± 1.24	0.0001

Values are mean ± SE from six different animals. ACL = average chain length; SFA = saturated fatty acids; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; DBI = double bond index; PI = peroxidizability index. For calculations of fatty acid indexes, see "Materials and methods" section.

fasted animals disappeared after addition of respiratory chain inhibitors. In the absence of inhibitors, respiratory complexes are only partly reduced, whereas in their presence, they are fully reduced. Thus, the degree of reduction of complex III seems to be involved in the mechanism leading to a higher free radical generation in fasting. In agreement with a previous report [11], we observed a reduction in mitochondrial oxygen consumption in fasted animals. Since degree of reduction of the electron carriers increases when the rate of electron flux in the ETC decreases, it is likely that the reduction in mitochondrial oxygen consumption is involved in the increased mitochondrial free radical production during fasting. A similar mechanism is operative during caloric restriction in yeast mitochondria, where a higher rate of oxygen consumption leads to a lower electron leak and a reduction in free radical generation [41].

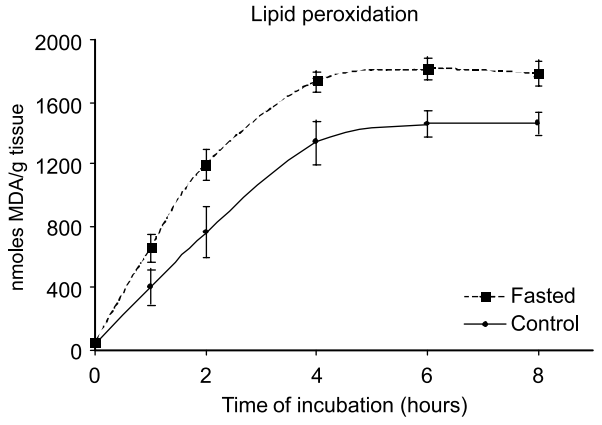

Figure 3. Sensitivity to *in vitro* lipid peroxidation of total liver lipids. The tissue lipids were incubated in a free radical generation system and lipid peroxidation was measured at different times of incubation. Results are means ± SEM from six different animals. Stimulated lipid peroxidation was significantly higher in fasted than in control animals ($p < 0.001$).

Another factor that could affect free radical leak in liver mitochondria during fasting is the alteration in mitochondrial fatty acid composition. Such changes could affect the stability, structure and activity of membrane proteins [42], including complexes of the ETC. It has been described that particular phospholipids, like cardiolipin, are important in the stabilization of mitochondrial respiratory chain supercomplexes [43,44]. However, previous investigations indicate that the degree of fatty acid unsaturation does not modify basal (substrate alone without inhibitors) mitochondrial free radical generation, or mitochondrial oxygen consumption [45]. On the other hand, the investigation showed that increasing the degree of fatty acid unsaturation of heart mitochondria could increase the rates of mitochondrial oxygen radical generation in situations in which the degree

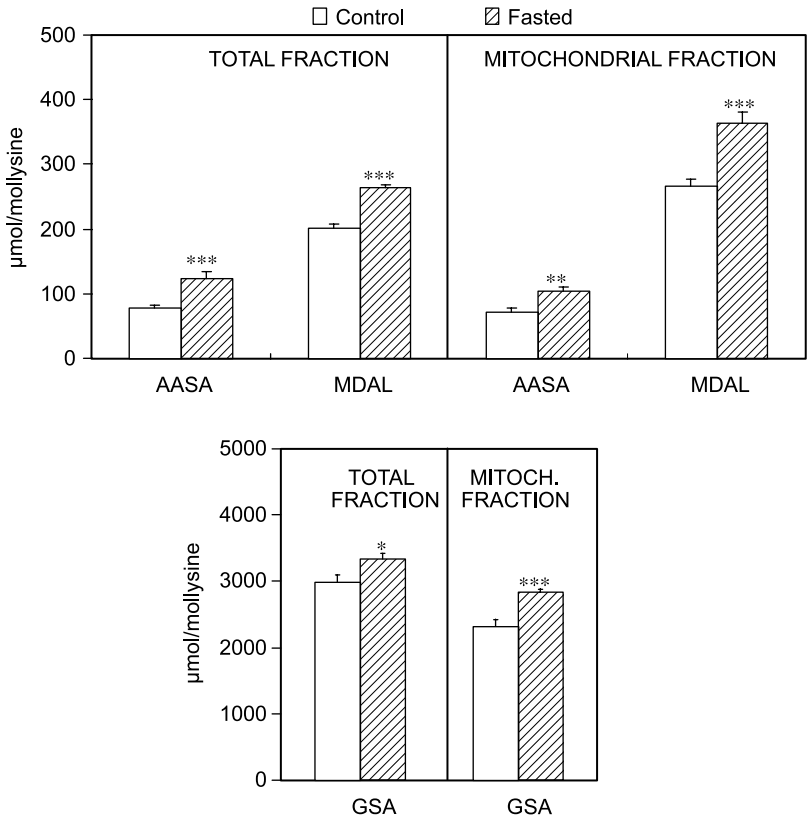

Figure 4. Effect of 72 h of fasting on the levels of $N^{epsilon}$-malondialdehyde lysine (MDAL), aminoadipic semialdehyde (AASA) and glutamic semialdehyde (GSA) adducts in total and mitochondrial proteins of rat liver. Values are means ± SEM from six different animals. * indicates significant differences between control and fasted animals (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

of reduction of complex III is higher than normal. Although we have performed the present investigation in the liver, the situation described in that study can be similar to that found in mitochondria from fasted animals, since complex III is probably more reduced in fasted animals than in *ad libitum* fed ones due to a lower mitochondrial oxygen consumption. Moreover, it has been recently described that severe modification of mitochondrial membrane fatty acid composition in rat liver can modify mitochondrial H_2O_2 generation [46].

The high fatty acid unsaturation in the hepatic membranes of fasted animals also enhanced their sensitivity to oxidative damage, as we detected by *in vitro* lipid peroxidation measurements. This means that the hepatic lipids from fasted animals are more prone to oxidative damage due to their higher content of UFA rich in double bonds. This leads to a higher generation of lipid peroxides in fasted than in *ad libitum* fed animals. Different results have been previously reported on *in vivo* MDA levels, with increases [10,13] or no changes [9,47], as well as decreases in old rats [47]. While classic methods of detection of lipid peroxidation suffer from significant interference when applied to *in vivo* measurements, after strong stimulation of lipid peroxidation *in vitro* (more than 40-fold in our study) these interferences are minimized and become irrelevant. Thus, our results globally indicate two reasons why the propensity to lipid peroxidation would be higher in fasted animals, because their tissue lipids are more sensitive to oxidative damage and because their mitochondria increase their production of free radicals.

Regarding proteins, investigations studying effects of fasting on hepatic proteins have mainly focused on protein metabolism, reporting changes in gene expression for catabolic pathways [30] and in amino acid levels [48]. We have focused on protein oxidative damage. Lipid peroxidation products react with different macromolecules. Thus, MDA can be attached to lysine residues in proteins. The observed increase in the levels of oxidatively modified proteins containing MDAL in liver of fasted animals indicates that the higher sensitivity to oxidative damage of hepatic lipids leads to an increase in lipid peroxidation dependent protein modification. Since we also observed higher levels of GSA and AASA, we conclude that in fasted rodents, liver proteins are not only oxidatively modified indirectly by reactive carbonyl compounds, but also directly through amino acid oxidation, in agreement with the higher mitochondrial free radical generation rate observed in fasted animals. Furthermore, the results of the current study show that glutamic semialdehyde is the

predominant form of protein carbonyls in rat liver mitochondria (higher than AASA), in agreement with previous reports in liver [23] and heart mitochondria [26].

In summary, our investigation suggests that fasting and caloric restriction have opposite effects with respect to mitochondrial oxidative stress in liver. Caloric restriction decreases mitochondrial oxidative stress by reducing mitochondrial free radical production. Contrary to this, fasting increases mitochondrial oxidative stress, at least in part, by increasing mitochondrial free radical leak and ROS generation at complex III level, increases protein oxidative damage, and changes fatty acid composition, leading to a higher degree of fatty acid unsaturation, lipid peroxidation and lipoxidation-derived protein modification.

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