Aberrant mitochondrial respiration in the livers of rats infected with *Fasciola hepatica*: the role of elevated non-esterified fatty acids and altered phospholipid composition

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The non-esterified fatty acid (NEFA) content and phospholipid composition of mitochondria isolated from the livers of Wistar rats infected with *Fasciola hepatica* were examined in relation to the aberrant mitochondrial respiration previously reported [Rule, Behm, and Bygrave (1989) *Biochem. J.* 260, 517–523]. At 2 weeks post-infection, elevated NEFA levels were associated with uncoupling of mitochondrial respiration that was reversible *in vitro* by the addition of BSA. State IV respiration rates showed a strong correlation with NEFA content. At 3 weeks post-infection, NEFA content had increased further and uncoupled mitochondria no longer showed any response to BSA. ³¹P-NMR

analyses of cholate extracts of mitochondria from infected livers at 3 weeks post-infection revealed a marked loss of several major phospholipid species with a concomitant increase in catabolic products, particularly glycerophosphocholine and glycerophosphoethanolamine. Similar changes were observed in microsomal extracts. The NEFA content and phospholipid composition of mitochondria isolated from infected, athymic nude rats were not significantly different from uninfected, athymic rats. These findings suggest that uncoupling of liver mitochondria during infection with *F. hepatica* is the result of phospholipase activation mediated by the immune system of the host.

INTRODUCTION

The uncoupling of isolated mitochondria from parasitized host tissues has been documented for a variety of parasitic infections. These include *Trichinella spiralis* and *Trichinella pseudospiralis* in rat muscle [1], *Anasakis simplex* in fish muscle [2] and *Fasciola hepatica* in sheep and rat liver [3–6]. The latter study showed that not only were mitochondria that had been isolated from livers during the acute stage of the infection (2–6 weeks) uncoupled, but that there was an attenuation of respiration and a loss of oligomycin-sensitivity of the F₀F₁-ATPase at 3–4 weeks post-infection. In the case of liver fluke infection in rats, mitochondrial dysfunction is thought to be mediated by the host's immune system, as uncoupling was not evident in infected rats treated with dexamethasone, nor in infected, congenitally athymic nude rats [7].

It has been known for many years that non-esterified fatty acids (NEFA) uncouple mitochondria *in vitro* [8] though the mechanism by which this occurs remains contentious [9]. One of the characteristics of uncoupling by NEFA is that it can be reversed by the addition of defatted BSA [10]. Michejda and Boczon [11] observed such reversibility early in *T. spiralis* infection, but BSA lost effectiveness as the infection progressed.

NEFA have also been implicated in the uncoupling of mitochondria in non-pathological states such as thermogenesis in brown adipose tissue [12], in skeletal muscle during exposure to cold shock [13] and in liver and cardiac and skeletal muscle during arousal from hibernation [14,15]. However, uncoupling *in*

vivo by NEFA in tissue other than brown fat has been questioned [9].

This study presents evidence supporting a role for NEFA in the uncoupling of mitochondria isolated from rat liver during the acute phase of infection with *F. hepatica*. We have investigated the origin of these NEFA and implicated phospholipase activation as a key contributing factor in the aberrant respiratory behaviour of these mitochondria. To assess the role of the host's inflammatory response in these events, mitochondria from congenitally athymic nude rats were also examined.

MATERIALS AND METHODS

Animals

Outbred male Wistar rats and congenitally athymic (nude) rats (Lewis-derived nu/nu and nu/+) were obtained from the Animal Breeding Establishment, Australian National University. Rats were 4–6 weeks of age at the time of infection. Uninfected rats of the same age were used as controls. Control and infected rats were caged in pairs and provided with commercial rat chow and water *ad libitum*.

Infections

Metacercariae of *F. hepatica*, maintained in the snail, *Lymnaea viridis*, were obtained from Dr. J. Boray (Elizabeth Macarthur Agricultural Research Institute, Camden Park, N.S.W.,

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ER, endoplasmic reticulum; NEFA, non-esterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; SPM, sphingomyelin; GPC glycerophosphocholine; GPE glycerophosphoethanolamine; GPI, glycerophosphoinositol; DGPG, diglycerophosphoglycerol; PCho, phosphocholine; PEth, phosphoethanolamine; PLA2, phospholipase A2; TNF, tumour necrosis factor.

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Australia) and were stored at 4 °C in water. Thirty viable metacercariae in approximately 0.2 ml of water were administered via stomach tube to rats lightly anaesthetized with isoflurane. The viability of metacercariae was assessed before infection by the presence of refractile granules [16].

Subcellular fractionation of rat liver

Rats were anaesthetized with sodium pentobarbitone (approx. 50 mg/kg body weight). Livers from control and infected rats were removed, weighed, diced and washed three times in isolation medium containing 250 mM mannitol, 10 mM KCl, 5 mM Hepes/KOH and 0.5 mM EGTA (pH 7.4). The diced livers were then homogenized in a Thomas C homogenizer with three passes of a Teflon pestle rotating at 900 rev./min to give a suspension of approximately 10% (v/v) in isolation medium. The homogenate was centrifuged in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor at 750 *g* for 5 min. The resultant supernatant was centrifuged for 5 min at 5100 *g* and the supernatant kept on ice for the preparation of microsomes. The pellet from the first centrifugation was resuspended in wash medium (isolation medium lacking EGTA) and subjected to the same centrifugation procedure as the original homogenate. The two higher-speed pellets were then resuspended in wash medium, combined and centrifuged at 7700 *g* to produce the final mitochondrial pellet, which was resuspended at a concentration of 80–100 mg protein/ml. The supernatant reserved for the preparation of microsomes was then centrifuged at 20000 *g*. The resulting supernatant was then centrifuged at 100000 *g* for 1 h in a Beckman L8-M ultracentrifuge using an SW-28 rotor. The pellet was washed once and resuspended to a protein concentration of approx. 30 mg/ml. All of the above procedures were carried out at 4 °C. Protein was determined by a modification of the method of Lowry et al. [17] as previously described [7]. BSA was used as standard. Aliquots of mitochondria and microsomes destined for NEFA and phospholipid analysis were stored at –70 °C. Inner mitochondrial membranes were prepared according to the method of Wehrle et al. [18]. Preparation of inner membrane vesicles led to a 4-fold enrichment of F_1F_0 -ATPase specific activity in the control preparations [19].

The microsomal marker enzymes glucose-6-phosphatase and NADPH cytochrome *c* reductase were assayed using the methods of Bygrave and Tranter [20] and Dallner et al. [21] respectively.

Measurement of oxygen uptake

Oxygen uptake was followed polarographically with a Clark-type oxygen electrode in a medium containing 50 mM KCl, 2 mM $MgSO_4$, 15 mM Hepes/KOH, 100 mM mannitol, 0.5 mM EDTA and 2 mM KH_2PO_4 with sodium succinate (5 mM) as substrate (pH 7.4). Mitochondria were added at a final concentration of 2 mg protein/ml in a final volume of 2.5 ml at 25 °C. Fraction V BSA (Sigma) was defatted by the charcoal method.

Measurement of NEFA content

Mitochondria and microsomes were assayed for NEFA content using the method of Nixon and Chan [22]. Palmitate was used as standard.

^{31}P -NMR spectroscopy

Mitochondria and microsomes (10–25 mg of protein in 300 μ l of wash medium) were dissolved in 1 ml of 5% (w/v) cholate solution containing 50 mM EDTA and 100 mM Hepes/KOH,

pH 8.0. The solution was incubated for 5 min at 100 °C, cooled on ice and centrifuged at 8000 *g* for 10 min in a benchtop centrifuge. A 540 μ l volume of the supernatant was removed and mixed with 60 μ l of 2H_2O . Methylene diphosphonate (400 μ M) was added as internal standard and the sample transferred to a 5 mm NMR tube. ^{31}P -NMR spectroscopy was carried out at the Australian National University NMR Centre in a Varian VXR300S spectrometer operating at 121.42 MHz with broadband WALTZ-16 proton decoupling. Spectra were acquired over 16 h at 25 °C with a sweep-width of 20000 Hz, acquisition time 0.82 s, interpulse delay 5 s, number of data points 32768 and pulse angle 45°. Peaks were assigned from spectra of standards acquired under the same conditions. Peak heights were used for quantification.

Chemicals

Oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cholate, 1,3-diphenylcarbazide, palmitate, methylene diphosphonate, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), sphingomyelin (SPM), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), phosphocholine (PCho) and phosphoethanolamine (PEth) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. ADP was from Boehringer Mannheim, Germany. 2H_2O was from Australian Nuclear Science and Technology Organisation, Lucas Heights, NSW, Australia. All other reagents were of analytical grade.

Statistics

Results are expressed as mean $\pm$ S.E.M. for $n \geq 3$ experiments. The statistical significance *P* was determined by unpaired, two-tailed Student's *t*-test.

RESULTS

Mitochondrial respiration

It has been shown previously that mitochondria isolated from infected livers at 2 weeks post-infection are uncoupled [6]. We have now found that coupling can be restored at this stage of infection by adding defatted BSA to the incubation mixture (Figure 1a). State IV respiration rates and acceptor control ratios which were significantly different from control values in the absence of BSA, approached control values in its presence (Table 1). However, BSA had no observable effect on the respiration rate of uncoupled mitochondria isolated at 3 weeks post-infection (Figure 1b). This suggests that uncoupling at this stage of infection may be due not only to the presence of a proton-translocating agent such as NEFA, but that the inner membrane has sustained structural damage leading to an increase in membrane permeability. The presence of BSA (0.5 g/l) or the phospholipase inhibitor dibucaine (400 μ M) in all isolation media also did not affect uncoupling at this stage.

At 3 weeks post-infection, mitochondria from infected livers may display uncoupled respiration characterized by the oxygen electrode trace depicted in Figure 1(b). That is, there is an increase in State IV respiration rate accompanied by a failure to respond to ADP and the chemical uncoupler CCCP. There is also no response to the F_0F_1 -ATPase inhibitor oligomycin (results not shown). Alternatively, at three weeks post-infection this response may be attenuated (Figure 1c, upper trace) [7]. Mitochondria displaying attenuated respiration also show no

Figure 1 The effect of BSA on oxygen uptake by mitochondria isolated from infected livers

Mitochondria were prepared and respiration measured as described in the Materials and methods section. Traces are representative of responses to ADP (0.1 mM), oligomycin (4 μ g/ml) and CCCP (2 μ M). Mitochondria were always added to medium supplemented with 5 mM succinate. BSA was added at a concentration of 0.8 mg/ml except in (c) (lower trace) where the concentration was 2.4 mg/ml. Mitochondria were from livers at: (a) 2 weeks post-infection; (b) 3 weeks post-infection; (c) 3 weeks post-infection (attenuated respiration). The traces in (c) represent the respiratory behaviour of the same mitochondria in the absence of BSA (upper trace) and in its presence (lower trace). (d) Shows mitochondria from uninfected livers uncoupled by the addition of mitochondria from 3 week-infected livers (0.3 mg of protein).

Table 1 Effect of bovine serum albumin on State IV respiration rates and acceptor control ratios in isolated liver mitochondria at 2 weeks post-infection

Mitochondria were isolated and oxygen uptake measured as described in the Materials and methods section. When present, the concentration of BSA was 0.8 mg/ml. Respiration is expressed as nmoles oxygen $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ protein. Values are mean $\pm$ S.E.M. for five separate experiments; * indicates value is significantly different from the corresponding control value ($P \leq 0.01$).

	Control		Infected	
	— BSA	+ BSA	— BSA	+ BSA
State IV respiration	13.8 $\pm$ 1.0	12.9 $\pm$ 1.2	27.8 $\pm$ 4.5*	19.5 $\pm$ 3.7
Acceptor control ratio	3.3 $\pm$ 0.3	3.5 $\pm$ 0.3	1.7 $\pm$ 0.4*	2.6 $\pm$ 0.5

respiratory response to ADP, CCCP and oligomycin. When BSA was added to attenuated mitochondria, the rate of respiration increased, but there was still no response to ADP, CCCP or oligomycin (Figure 1c, lower trace). This effect was observed only when BSA was present at 3–4 times the concentration necessary to reverse uncoupling at 2 weeks post-infection. As well as these effects of BSA on mitochondria isolated from infected livers, it was found that control mitochondria became uncoupled in the presence of a small amount (approx. 0.3 mg of

Table 2 NEFA content of isolated mitochondria

Mitochondria were isolated from livers at 2 and 3 weeks post-infection and NEFA content was determined as described in the Materials and methods section. Results are presented as mean $\pm$ S.E.M. ($n = 5$). Significant difference from the corresponding control value is shown as * $P < 0.05$ and ** $P < 0.005$.

Weeks post-infection	Control	Infected
2	42.5 $\pm$ 4.6	87.8 $\pm$ 16.0*
3	64.2 $\pm$ 7.8	211.9 $\pm$ 31.6**

Figure 2 The relationship between State IV respiration rate and NEFA content of isolated mitochondria

Liver mitochondria were isolated from uninfected rats (+) and rats infected for 2 (■) and 3 weeks (□). Respiration rates and NEFA content were determined as described in the Materials and methods section. Each data point represents one animal.

protein) of 'infected' mitochondria. BSA reversed this (Figure 1d), but dibucaine had no effect (results not shown). Oligomycin was included in the incubation medium to prevent mitochondrial fatty acid activation contributing to the increase in respiration rate observed on addition of 'infected' mitochondria [22]. There was no observable oxygen consumption when an equivalent amount of mitochondria from infected liver was added to the incubation mixture alone.

NEFA content of isolated mitochondria

As NEFA are known to uncouple mitochondria and this effect can be reversed by defatted BSA [10], the NEFA content of isolated mitochondria was assessed. Table 2 shows that the NEFA content of mitochondria isolated from livers of rats infected for 2 weeks was double that of control mitochondria, increasing by up to 5 times control values at 3 weeks post-infection. At 2 weeks post-infection, State IV respiration rates showed a strong correlation with NEFA content (Figure 2, $r = 0.96$). Of the group of five animals assayed at 3 weeks post-infection, four showed attenuated respiration. Figure 2 shows that these animals form a distinct group, with levels exceeding 200 nmoles/mg protein, well in excess of levels from uncoupled mitochondria.

When mitochondria exhibiting attenuated respiration were added to control mitochondria, State IV respiration rates

Figure 3 ^{31}P -NMR spectra of isolated mitochondria

Representative ^{31}P -NMR spectra of cholate extracts of mitochondria isolated from control (a) and 3 week-infected (b) livers. Spectra are shown on the same vertical scale at absolute intensity, Fourier-transformed with 1.0 Hz line-broadening. The PC peak (1) has been truncated in (a). Samples were prepared and spectra acquired as described in the Materials and methods section. Peak assignments: 1, PC; 2, PI; 3, PS; 4, LPC; 5, PE; 6, SPM; 7, GPC; 8, GPI; 9, CL; 10, LPE; 11, GPE; 12, DGPG; P_i , orthophosphate; 13, Pcho.

increased in proportion to the amount added up to a point which corresponds to approximately 30 nmoles of NEFA added per mg control mitochondrial protein (results not shown). At higher concentrations, the respiration rate declined. A similar effect was reported by Rottenberg and Hashimoto using oleate [23]. However, it was not possible to inhibit respiration by this means to the extent seen in the attenuated state.

Phospholipid composition of the mitochondrial membranes

As uncoupling could not be reversed by BSA at 3 weeks post-infection, ^{31}P -NMR was used to investigate changes that may have occurred in the membrane phospholipid composition, thereby leading to an increase in membrane permeability. Representative ^{31}P -NMR spectra of cholate extracts of isolated mitochondria are shown in Figure 3. The most striking feature of these spectra is the substantial loss of the two major phospholipid species PC (peak 1) and PE (peak 5) in the material from infected animals, with the concomitant appearance of peaks representing their respective catabolic products GPC (peak 7) and GPE (peak

Table 3 Major phosphorus-containing species observed by ^{31}P -NMR in cholate extracts of mitochondria isolated from livers of rats at 3 weeks post-infection

Conditions were as described in the Materials and methods section. Units are nmoles/mg mitochondrial protein. Results are expressed as mean $\pm$ S.E.M. ($n = 3$). Significant difference from the corresponding control value is shown as ** $P < 0.005$ and * $P < 0.05$. Shifts were determined using authentic compounds except in the case of DGPG and GPI, where only putative (p) identification was made on the basis of shifts relative to formally identified species. Methylene diphosphonate was used as internal standard (16.4 p.p.m.) and for quantification.

Species	Shift (p.p.m.)	Control	Infected
Phosphatidylcholine	-0.581	91.5 $\pm$ 5.7	30.8 $\pm$ 3.1**
Phosphatidylethanolamine	-0.008	58.7 $\pm$ 4.3	38.3 $\pm$ 2.2*
Phosphatidylinositol	-0.461	21.7 $\pm$ 2.2	6.6 $\pm$ 0.4**
Phosphatidylserine	-0.219	7.7 $\pm$ 0.9	4.5 $\pm$ 0.3
Cardiolipin	-0.404	25.8 $\pm$ 2.2	26.5 $\pm$ 3.0
Lysophosphatidylcholine	-0.109	2.5 $\pm$ 0.4	6.5 $\pm$ 1.3
Lysophosphatidylethanolamine	0.434	0	10.1 $\pm$ 0.6**
Glycerophosphocholine	0.072	0	41.7 $\pm$ 4.7**
Glycerophosphoethanolamine	0.625	2.6 $\pm$ 0.6	38.4 $\pm$ 1.7**
Phosphocholine	3.470	8.6 $\pm$ 0.2	12.7 $\pm$ 0.5**
Phosphoethanolamine	3.983	0	0
Sphingomyelin	0.032	10.4 $\pm$ 2.30	11.8 $\pm$ 0.8
Diglycerophosphoglycerol (p)	0.987	0	10.4 $\pm$ 6.2**
Glycerophosphoinositol (p)	0.132	0	8.7 $\pm$ 1.2**
PC:PE	-	1.6 $\pm$ 0.1	0.8 $\pm$ 0.1**

11). Quantification of these results is shown in Table 3. The phospholipid composition of control mitochondria shown is in agreement with literature values [24]. Not all phospholipids which decreased in concentration did so to the same degree. PC and PI levels were approximately 60 % lower in extracts from infected livers, whereas PE decreased by only 30 %. No significant change was observed in PS (peak 3), CL (peak 9) or SPM (peak 6). The PC:PE ratio of 1.6 ± 0.1 in control preparations was similar to that obtained by Pollesello et al. [25] using ^1H -NMR, with the decrease to 0.8 ± 0.1 in the infected state emphasizing the differential loss of these two major phospholipids. Overall, these changes represent a loss of approximately 40 % of total phospholipid.

As mentioned above, the most abundant catabolic products identified were GPC and GPE, which were present in similar amounts. LPE (peak 10), which had not been detected in control preparations, increased significantly, but was present at only about 25 % of the concentration of GPE. The apparent increase in LPC (peak 4) proved to be statistically insignificant. Lyso-phospholipids therefore comprised approximately 12 % of total phospholipid in mitochondrial membranes from infected livers compared with about 1 % of control membranes. PEth was not found in any extract, in contrast to Pcho (peak 13) which showed a small but significant increase in the infected state. By extrapolation from the downstream shift changes observed for the hydrolytic products of PC and PE, we have identified the peak at 0.987 p.p.m. (peak 12) as putative diglycerophosphoglycerol (DGPG), which results from the complete deacylation of cardiolipin. This assumption is supported by the absence of this peak, along with that corresponding to CL in the ^{31}P -NMR spectra of microsomal extracts (Table 4). Therefore, despite the lack of any net change in CL, there is evidence that this important phospholipid is not exempt from degradation in the infected state. Putative glycerophosphoinositol (GPI) (0.132 p.p.m., peak 8) is present in preparations from infected livers, which is consistent with the substantial loss of PI noted above.

Table 4 NEFA content and major phosphorus-containing species observed by ^{31}P -NMR in cholate extracts of microsomes isolated from livers of rats at 3 weeks post-infection

Conditions were as described in the Materials and methods section and in the legend to Table 3. Units are nmoles/mg microsomal protein. Results are expressed as mean $\pm$ S.E.M. ($n = 3$). Significant difference from the corresponding control value is shown as ** $P < 0.005$ and * $P < 0.05$.

Species	Control	Infected
Phosphatidylcholine	399.6 $\pm$ 39.0	122.1 $\pm$ 22.3**
Phosphatidylethanolamine	143.8 $\pm$ 32.1	54.4 $\pm$ 6.4*
Phosphatidylinositol	114.2 $\pm$ 17.2	35.9 $\pm$ 5.0*
Phosphatidylserine	33.6 $\pm$ 4.5	20.6 $\pm$ 5.0
Cardiolipin	0	0
Lysophosphatidylcholine	11.5 $\pm$ 1.4	3.8 $\pm$ 2.2*
Lysophosphatidylethanolamine	0	0
Glycerophosphocholine	0	56.1 $\pm$ 17.1*
Glycerophosphoethanolamine	0	16.1 $\pm$ 2.3**
Phosphocholine	3.7 $\pm$ 3.7	11.3 $\pm$ 5.1
Phosphoethanolamine	0	0
Sphingomyelin	36.0 $\pm$ 6.8	50.9 $\pm$ 16.3
Diglycerophosphoglycerol (p)	0	0
Glycerophosphoinositol (p)	0	14.9 $\pm$ 6.4**
PC:PE	3.0 $\pm$ 0.5	2.2 $\pm$ 0.2
NEFA content	45 $\pm$ 7	667 $\pm$ 46**

In an attempt to identify changes in the phospholipid composition of the inner mitochondrial membrane, inner membrane vesicles were prepared and analysed (results not shown). In contrast to whole mitochondria, there was a 50% loss of CL at 3 weeks post-infection, suggesting that the inner mitochondrial membranes from infected livers have an increased susceptibility to preparative damage. Given that CL is intimately associated with various inner membrane proteins including the F_1F_0 -ATPase [26], this is consistent with previous findings demonstrating the leakage of mitochondrial ATPase activity from the membranes during preparation of inner membrane vesicles from infected livers [19]. In contrast to control preparations, ATPase specific activity of the latter failed to become enriched.

Microsomes

Analyses of the microsomal fraction showed a pattern of changes similar to that found in mitochondria. That is, levels of PC, PE and PI decreased, while GPC, GPE and putative GPI all increased; there was no significant change in PS or SPM (Table 4). In contrast to mitochondria, microsomal LPC actually decreased in the infected livers and LPE was not detected. NEFA content increased from 45 ± 7 nmoles/mg protein to 667 ± 46 nmoles/mg protein. Overall, the damage to the microsomes was more profound than to the mitochondria, with a loss of total phospholipid approaching 60%. However, the PC:PE ratio did not change significantly.

As similar changes were observed in both mitochondria and microsomes, it was important to assess the extent of contamination by microsomes in the mitochondrial preparations. NADPH cytochrome *c* reductase specific activity was distributed similarly between microsomal and mitochondrial fractions in control and infected preparations, the specific activity found in the mitochondria being approximately 11% of that found in the microsomes. Hence the changes in mitochondrial phospholipids largely cannot be attributed to microsomal contamination. Glucose-6-phosphatase was found to be an unsuitable marker enzyme in this instance as little or no activity could be detected

in microsomes or mitochondria isolated from 3 week-infected livers.

Athymic, nude rats

An equivalent amount of NEFA was found in mitochondria isolated from the athymic infected and uninfected groups (38.9 ± 2.1 and 30.5 ± 4.4 nmoles/mg protein respectively). These levels were comparable with those found in uninfected Wistar rats (Figure 2). The NEFA content of the infected heterozygote group (200.0 ± 54.1 nmoles/mg protein) was elevated to a level similar to infected Wistar rats at the same stage of infection. In confirmation of previous work [7], mitochondria isolated from both the athymic groups displayed coupled respiration, whereas those from the infected heterozygote group did not.

^{31}P -NMR analysis of cholate extracts revealed no statistically significant differences in the phospholipid composition of athymic infected and uninfected rat liver mitochondria (results not shown). The phospholipid composition of membranes from both of these sources was comparable to that found above for uninfected Wistar rats. Differences in phospholipid composition between athymic infected and infected heterozygote groups were similar to those already noted between control and infected Wistar rats. As with the athymic controls, the athymic infected animals showed little or no evidence of the various products of phospholipid breakdown. In these groups, levels of GPC, GPE, putative DGPG and GPI remained at or near zero, whereas GPC and GPE were major components of the extracts from infected heterozygotes, with putative DGPG and GPI also increasing significantly.

DISCUSSION

Evidence has been presented in this paper that links NEFA with aberrant liver mitochondrial bioenergetic metabolism during the acute stage of infection with *F. hepatica*. At 2 weeks post-infection, when NEFA content was shown to be elevated, uncoupling could be restored with BSA and there was a strong positive correlation between State IV respiration rates and NEFA content. In addition, the concentration-dependent and BSA-reversible uncoupling of control mitochondria by mitochondria isolated from infected livers is consistent with the action of NEFA in causing uncoupling at this stage of the infection.

By 3 weeks post-infection, when NEFA content was elevated further, a change from BSA-reversible to -irreversible uncoupling was observed. Attenuation of mitochondrial respiration at this stage was associated with very high levels of NEFA. As the attenuation could be alleviated by BSA, it is possible that high concentrations of NEFA may interact with the membrane in such a way as to disrupt electron transport. Alternatively, albumin may have a non-specific stabilizing effect on the mitochondria similar to its effect on many enzymes [27] and the association of NEFA with attenuation might not be a causative one.

Progression from BSA-reversible to -irreversible uncoupling has also been reported for mitochondria isolated from rat skeletal muscle infected with *T. spiralis* [11] and from S180 sarcoma in mice following administration of endotoxin *in vivo* [28]. The latter authors suggested that the damage involved mitochondrial phospholipase A2 (PLA2) activity producing NEFA and lysophospholipids. This hypothesis is supported by early work *in vitro* examining the effect of heated snake venom on mitochondria, which produced a similar result [29]. That is, an initial increase in oxygen consumption, reversible in its early stages by the addition of BSA, was followed by a cessation of respiration. Later, Burstein et al. [30] also found BSA to be protective to

mitochondria incubated with low concentrations of phospholipase, but it offered increasingly less protection as the enzyme concentration was raised.

Consistent with these observations, we have observed changes indicative of the sequential action of a phospholipase A and lysophospholipase in ^{31}P -NMR spectra of mitochondrial membrane extracts at 3 weeks post-infection. The most compelling evidence for this was the marked increase in the phosphodiester GPC and GPE. These metabolites can arise only from phospholipid hydrolysis, and in liver they are considered to be the major catabolic products of PC and PE respectively [31]. The lack of change in SPM content is also consistent with PLA2 activation as this phospholipid is not considered to be a substrate for this enzyme [32]. The low level of lysophospholipids relative to phosphodiester indicates the presence in the liver of a lysophospholipase of high activity. The rapid removal of lysophospholipids would be protective to the cells, as lysophospholipids, like NEFA, are able to uncouple mitochondrial respiration and at high concentrations also have detergent effects on membranes [33,34]. Since BSA binds lysophospholipids as well as NEFA, it appears likely that lysophospholipids also contribute to the respiratory lesions observed.

We observed a net loss of approximately 40 % of mitochondrial phospholipids at 3 weeks post-infection. This, along with the 60 % loss of phospholipid from the microsomal fraction, is in accord with the > 50 % loss of total phospholipid from whole liver preparations reported elsewhere [35]. Such a depletion of membrane phospholipid could account for the BSA-irreversible uncoupling of oxidative phosphorylation seen at this stage. It is possible that these changes in phospholipid content were due not only to activation of phospholipid catabolism, but also to differential changes in biosynthetic rates, as not all phospholipid species in the mitochondria were equally affected. For example, the PC:PE ratio declined, and DGPG increased while CL remained unchanged. Further experiments are needed to explore this.

It is possible that the NEFA found in the mitochondrial fraction could have originated elsewhere, becoming associated with the mitochondria during the isolation procedure. However, we have presented evidence that the mitochondrial membranes have undergone hydrolytic attack, a consequence of which is the release of NEFA. Mitochondria from infected livers contained over 200 nmoles NEFA/mg protein in excess of control preparations, which is compatible with the observed loss of around 100 nmoles/mg protein of mitochondrial phospholipid. Therefore, it seems likely that the NEFA have arisen from the mitochondrial membranes themselves. Further work is under way to assess any contribution from other possible sources. Preliminary results indicate that the NEFA did not arise from an increase in *de novo* hepatic synthesis (L. M. Lenton, C. A. Behm and F. L. Bygrave, unpublished work). Our results therefore support the hypothesis that uncoupling of liver mitochondria during fasciolosis in rats is causally related to phospholipase activation, with the ensuing accumulation of toxic products, such as NEFA and lysophosphatides, as well as the removal of phospholipids contributing to the loss of membrane integrity.

In the athymic, nude rat model we reported the absence of uncoupling of mitochondria during infection [7] and now we have demonstrated no changes in the products of phospholipid catabolism in these animals. The involvement of T-lymphocytes in the pathology of infection and the products of their interactions with Kupffer cells can lead to a wide range of outcomes, including production of reactive oxygen species and phospholipase activation [7]. Maffei Facino et al. [36] have suggested that the initial event in the development of liver pathology is the

respiratory burst of the phagocytic cells, followed by peroxidation of the hepatic tissue. This could indirectly lead to phospholipase activation [37]. On the other hand, the macrophage products tumour necrosis factor- α (TNF- α) and IL-1 have also been reported to induce PLA2 synthesis and secretion directly [38]. Moreover, in some systems, TNF-induced cytotoxicity is thought to be mediated via phospholipase activation and in others via the induction of reactive oxygen formation in the mitochondria [39], either of which could be relevant to the lesions in *F. hepatica*-infected livers.

In conclusion, this report describes major degenerative changes in the phospholipid composition of mitochondria and microsomes at a time when mitochondrial bioenergetic metabolism is impaired. Work to elucidate in more detail the sequence of events leading to host liver pathology during infection with *F. hepatica* is under way.

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