Hepatic [2-^{13}C]acetate metabolism by jirds infected with *Echinococcus multilocularis*

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Abstract

Carbon-13 decoupled ^{1}H spin echo NMR spectroscopy, with and without population inversion, was used to study carbon flow between the host, *Meriones unguiculatus*, and the parasite, *Echinococcus multilocularis*. This was accomplished by monitoring [2-^{13}C]acetate metabolism in the liver of jirds infected with metacestodes of this parasite. Thirty minutes after injection of labelled acetate solution into the portal vein, ^{13}C enrichment was observed in hepatic acetate, β–hydroxybutyrate, succinate, alanine, lactate and glucose. For *E. multilocularis* cysts, at this time, ^{13}C enrichment was observed in the same metabolites as in livers and, in addition, citrate. At 120 min there was a significant decrease in the amount of label present in all hepatic metabolites whereas more label was found in the majority of the parasite metabolites. The results confirm that exogenous acetate, through randomization of the ^{13}C in biochemical pathways of host liver, ends up in hepatic glucose. As this biosynthetic route is not available to the parasite, the presence of ^{13}C enriched glucose in the cysts clearly indicates that the parasite is siphoning off glucose that is newly synthesized by the host. At 120 min some of this labelled glucose was stored in parasite glycogen whereas some of it had been catabolized to succinate, alanine, lactate and acetate, end products which are excreted back into the host.

Introduction

In our NMR studies on experimental alveolar echinococcosis, we have found a number of changes in the metabolic profiles of sera and organs of *Meriones unguiculatus* infected with metacestodes of *Echinococcus multilocularis*. Substantial hypoglycaemia, low glucose levels in liver, spleen and kidneys and depleted hepatic glycogen were detected in infected animals (Novak *et al.*, 1989, 1993a, 1995). These findings revealed that the parasite's extensive use of glucose exerts a systemic starvation condition in the host. Indeed, glucose is the primary source of energy for all cestodes and is either directly utilized by being broken down to lacate, alanine, succinate and acetate, or stored in the form of glycogen (Roberts, 1983). The end products of glucose metabolism were detected by ^{1}H NMR in *E. multilocularis* cysts which were grown in jirds (Novak *et al.*, 1992).

It has been postulated, although never investigated, that parasite end products excreted into the host are returned to the liver where they are utilized in metabolic pathways to produce new glucose which is desperately needed by a starving infected host. Alanine and lactate, the main gluconeogenic precursors, can enter directly into the gluconeogenic pathway via conversion to pyruvate, while succinate and acetate can be involved in this process through initial entry into the Krebs cycle, and exit at malate or oxaloacetate. In this experiment, we followed the metabolic fate of acetate in the liver of jirds infected with *E. multilocularis*. This was accomplished by introducing carbon-13 labelled acetate into the host and following the movement of labelled atoms through intermediate metabolites by analysing liver extracts with ^{13}C decoupled ^{1}H spin echo NMR spectroscopy acquired

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with, and without, ^{13}C population inversion (Novak *et al.*, 1993b).

Materials and methods

Infection

Thirty male jirds, *M. unguiculatus*, approximately 4 months old, were used in this experiment. Half of these were infected by an intraperitoneal injection of 0.5 ml of *E. multilocularis* (Alaska strain) cyst cell suspension each, whereas the other half served as uninfected controls. On days 28, 29 and 30 post infection (p.i.) the jirds were starved overnight and in the morning anaesthetized with an intramuscular injection of sodium pentobarbital (60 mg kg^{-1}; MTC Pharmaceuticals). Their abdomens were then opened and 0.1 ml of a 26% by weight solution of [2-^{13}C]acetate (99.5 atom % ^{13}C, MSD Isotopes) in 0.85% NaCl was injected into the hepatic portal vein as a bolus injection using a 1 ml syringe over a period of about 2 min. Thirty minutes later, the liver and parasite cysts were removed, frozen in liquid nitrogen (N_2), weighed and stored at –70°C until preparation of perchloric acid (PCA) extracts. The uninfected control group was treated in the same manner. To ensure that the metabolic rate of each animal was similar, all jirds were dissected between 9:00 am and 12 noon.

This experiment was repeated exactly as described above with another group of male jirds, except that in this second experiment the livers and parasite cysts were removed 120 min post injection of [2-^{13}C]acetate solution into the host.

Perchloric acid extracts

PCA extracts were prepared as previously described (Modha *et al.*, 1997). In this study, the freeze-dried residue of each sample was stirred with 1.3 ml of D_2O (99.9 atom % ^2H, CDN Isotopes) and 0.2 ml of a solution of 0.0754 g sodium [2,2,3,3-$^2H_4$]-3-trimethylsilylpropionate (TSP) in 15 ml (17.2415 g) of D_2O added as a chemical shift and intensity standard. After 2 h of stirring, the pH of each sample was adjusted to between 7.15 and 7.25, using aqueous sodium deuteroxide (NaOD). The samples were then centrifuged at 12000 rpm for 30 min at 5°C and the supernatant transferred to a 5 mm NMR tube for analysis.

NMR spectroscopy and quantification

The ^1H spin echo NMR spectra of PCA extracts were acquired at 310 K (37°C) using a Bruker AMX-500 NMR spectrometer operating at 500.13 MHz for this nucleus. The spectra were ^{13}C decoupled using the GARP composite pulse decoupling routine (Shaka *et al.*, 1985). A spectral width of 6578.95 Hz, 16 K data points and a recycle time of 15.25 s were used in data accumulation. The number of scans used for liver and cyst samples was 192. Scans acquired with, and without, ^{13}C population inversion (Bendall *et al.*, 1981) were acquired alternatively in blocks of 16 scans and stored in separate computer memory locations. Peak assignments were based on published data (Evanochko *et al.*, 1984; Gilroy *et al.*, 1988; Desmoulin *et al.*, 1990; Sze & Jardetzky, 1990; Rafter *et al.*, 1991; Yacoe *et al.*, 1991) and the spectra of authentic compounds. Concentrations of metabolites present (in $\mu mol\ g^{-1}$ of tissue) were calculated from integration data of metabolite peaks, relative to that of TSP, in the [$^{12}C + {}^{13}C$] spectra. The percent carbon-13 in metabolites was calculated using the [$^{12}C + {}^{13}C$] spectrum and the sum of the [$^{12}C + {}^{13}C$] and [$^{12}C - {}^{13}C$] spectra. Data obtained were analysed statistically using an analysis of variance (ANOVA) with $\alpha = 0.05$.

Table 1. Concentrations of metabolites from livers of uninfected and infected *Meriones unguiculatus*, and from *Echinococcus multilocularis* cysts, 30 min after introduction of [2–^{13}C]acetate into the host.

Metabolite	$\mu mol\ g^{-1}$ wet wt (mean ± S.D.)		
	Uninfected (n = 15)	Infected (n = 15)	Cysts (n = 14)
Glycogen	1.12 ± 0.76[a]	0.32 ± 0.09[b]	1.14 ± 0.43[*]
Glucose	4.66 ± 1.11[a]	3.20 ± 1.02[b]	0.77 ± 0.43[*]
Citrate	–	–	1.08 ± 0.29
Succinate	0.40 ± 0.06[a]	0.50 ± 0.21[a]	1.06 ± 0.50[*]
Acetate	0.65 ± 0.35[a]	0.91 ± 0.31[b]	2.10 ± 0.25[*]
Alanine	0.10 ± 0.06[a]	0.26 ± 0.43[a]	1.91 ± 0.56[*]
Lactate	0.58 ± 0.33[a]	0.76 ± 0.74[a]	5.82 ± 0.86[*]
β-HB	0.57 ± 0.23[a]	0.88 ± 0.46[b]	0.58 ± 0.25[*]

[a,b]Different letters denote a significant difference between concentrations of metabolites in uninfected and infected groups ($\alpha \leq 0.05$). [*]Indicates a significant difference between concentrations of metabolites in infected and cysts groups.

Results

Total concentrations of metabolites

The total concentrations (labelled plus unlabelled material) of metabolites which contained ^{13}C enrichment, from livers of uninfected and infected jirds and *Echinococcus* cysts removed 30 min post injection of [2-^{13}C]acetate, are presented in table 1. When compared to uninfected controls, the livers from infected animals had less glycogen and glucose, but more acetate and β-hydroxybutyrate (β-HB). The parasite had higher concentrations of glycogen, succinate, acetate, alanine and lactate, but lower concentrations of glucose and β-HB than the liver of its host. Two hours post injection of [2–^{13}C]acetate (table 2), the livers from infected jirds contained less glycogen and glucose than those of uninfected animals. *Echinococcus* cysts, at this time, had higher concentrations of succinate, acetate, alanine and lactate but lower concentrations of glucose and β-HB than the livers of infected hosts.

Percent carbon-13 in metabolites

The following is a list of the metabolites found to contain label in both the liver and the parasite, with the carbon atom(s) used to monitor that labelling: glycogen, C-1; glucose, C-1; succinate, C-2/C-3; acetate, C-2; alanine, C-3; lactate, C-3; and β-HB, C-4 (table 3). In addition, *E. multilocularis* had label in citrate, which was monitored by the equivalent carbon atoms, 2 and 4. In all

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Table 2. Concentrations of metabolites from livers of uninfected and infected *Meriones unguiculatus*, and from *Echinococcus multilocularis* cysts, 120 min after introduction of [2–^{13}]acetate into the host.

Metabolite	μmol g^{-1} wet wt (mean ± S.D.)		
	Uninfected (n = 14)	Infected (n = 15)	Cysts (n = 15)
Glycogen	2.86 ± 1.95^{a}	0.67 ± 0.74^{b}	1.32 ± 0.43
Glucose	5.25 ± 0.98^{a}	4.18 ± 1.38^{b}	1.02 ± 0.45*
Citrate	–	–	1.94 ± 0.60
Succinate	0.38 ± 0.08^{a}	0.46 ± 0.10^{a}	1.04 ± 0.33*
Acetate	0.32 ± 0.09^{a}	0.22 ± 0.16^{a}	1.41 ± 0.48*
Alanine	0.17 ± 0.25^{a}	0.19 ± 0.15^{a}	2.98 ± 0.87*
Lactate	0.90 ± 0.96^{a}	0.74 ± 0.39^{a}	8.58 ± 2.85*
β-HB	0.72 ± 0.38^{a}	0.82 ± 0.36^{a}	0.48 ± 0.22*

^{a,b}Different letters denote a significant difference between concentrations of metabolites in uninfected and infected groups ($\alpha \leq 0.05$). *Indicates a significant difference between concentrations of metabolites in infected and cyst groups.

cases, the atoms chosen for monitoring had directly attached protons whose resonances were minimally encumbered, at most, by other resonances, thereby permitting accurate integration, and were indicative of the maximum degree of labelling. After 30 min, livers from infected jirds had a higher percentage label in glycogen than those in the control group. However, for hepatic alanine and lacate, the percent carbon-13 was less in the infected group than in uninfected controls. In the parasite cysts, all metabolites, except for glucose and acetate, had less carbon-13 than the corresponding hepatic metabolites of their infected hosts. The percent carbon-13 in metabolites from livers of uninfected and infected jirds in the 120 min group did not differ statistically. Most of the label remaining in the liver was in acetate. In contrast, after 120 min, *E. multilocularis* cysts contained more label in glycogen, glucose, succinate, acetate, alanine and lactate than corresponding hepatic metabolites.

When the flow of label from [2-^{13}C]acetate to hepatic and cyst metabolites was considered by comparing data at 30 min with that at 120 min, it was found that, in general, the percent carbon-13 in liver metabolites decreased whereas that in cyst metabolites increased with time. In uninfected animals, there was a lower percentage carbon-13 after 120 min than after 30 min in all hepatic metabolites except glycogen, which contained a similar percentage to that from the 30 min group. In livers from infected jirds, all metabolites including glycogen, contained less label 2 h post injection of labelled acetate than those in the 30 min group. In contrast, there was a greater percentage carbon-13 in parasite cyst glycogen, glucose, succinate, alanine and lactate after 120 min than in the corresponding metabolites of cysts which were harvested at 30 min. However, as in the liver, the percentage carbon-13 in cyst acetate and β-HB decreased with time. The percent carbon-13 in citrate remained unchanged.

Discussion

Detection of carbon-13 beyond natural abundance in hepatic glucoses provides solid evidence that exogenous [2–^{13}C]acetate was utilized in host gluconeogenic pathways. Labelled glucose synthesized by the liver of infected host had three possible fates. Some of it was stored in glycogen and the presence of label in lactate and alanine is consistent with its use in catabolic processes. Also, some of the labelled glucose was found in the parasite. This indicated that *E. multilocularis*, located in the peritoneal cavity, siphoned off labelled glucose produced and released by the host's liver, as carbon-13 could only end up in the glucose molecule through entry of [2-^{13}C]acetate, as acetyl CoA (labelled at C-2), into the Krebs cycle followed by subsequent participation of labelled metabolites in gluconeogenesis. As cestodes do not have a complete Krebs cycle (Smyth & McManus, 1989), it is unlikely that the parasite could incorporate labelled atoms from acetate into glucose. Further, there is no convincing evidence of measurable gluconeogenesis in these organisms (Bryant & Behm, 1989; Tielens &

Table 3. Percent carbon-13 in metabolites from livers of uninfected and infected *Meriones unguiculatus*, and from *Echinococcus multilocularis* cysts, after introduction of [2-^{13}C]acetate into the host.

Metabolite	Percent ^{13}C (mean ± S.D.)					
	30 min			120 min		
	Uninfected (n = 15)	Infected (n = 15)	Cysts (n = 14)	Uninfected (n = 14)	Infected (n = 15)	Cysts (n = 15)
Glycogen	1.67±1.00^{a}	3.40±2.77^{b}	1.70±0.80*	1.60±0.51^{a}	1.55±0.31^{a+}	4.72±1.60^{*+}
Glucose	3.62±1.19^{a}	2.70±1.09^{a}	2.31±1.24	1.98±0.85^{a+}	1.57±0.26^{a+}	5.98±2.72^{*+}
Citrate	–	–	2.93±0.97	–	–	2.75±0.76
Succinate	5.31±2.16^{a}	5.91±1.67^{a}	2.69±0.94*	1.98±0.63^{a+}	1.87±0.87^{a+}	3.75±1.26^{*+}
Acetate	24.75±14.81^{a}	30.62±14.14^{a}	38.35±8.26*	4.83±4.41^{a+}	3.22±5.53^{a+}	14.52±5.32^{*+}
Alanine	4.87±3.55^{a}	3.07±1.82^{b}	1.63±0.80*	2.37±1.02^{a+}	1.57±0.41^{a+}	4.98±1.73^{*+}
Lactate	3.90±1.78^{a}	2.79±0.82^{b}	1.64±0.31*	1.97±0.42^{a+}	1.62±0.38^{a+}	3.54±0.81^{*+}
β-HB	16.64±3.78^{a}	15.22±4.53^{a}	11.30±3.53*	1.83±0.76^{a+}	1.49±0.33^{a+}	3.01±1.03^{+}

^{a,b}Different letters denote a significant difference ($\alpha \leq 0.05$) between percent ^{13}C in metabolites in uninfected and infected groups. *Denotes a significant difference ($\alpha \leq 0.05$) in percent ^{13}C in metabolites between corresponding infected and cyst groups.

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van den Bergh, 1993). Thus the presence of labelled glucose in *Echinococcus* cysts and the decrease in the amount of this carbohydrate in livers of infected jirds demonstrates competition between the host and parasite for this compound. Although, 30 min into the experiment, some of the labelled glucose was used in host glycogenesis, the total amount of hepatic glycogen was significantly lower in infected jirds than in controls. This again illustrates that *E. multilocularis* induces a persistent starvation condition in its host.

Carbon-13 enrichment of hepatic metabolites decreased with time. At the end of two hours, most of the labelled metabolites were processed in host biochemical pathways and/or distributed to the extrahepatic tissues. Also, some other carbon-13 labelled compounds in addition to glucose, namely acetate and β-HB, were taken up by *Echinococcus* cysts. In the parasite, the catabolism of glucose labelled at C-1 and C-6 would result predominantly in the end products [3-^{13}C]lactate, [3-^{13}C]alanine, [2-^{13}C]succinate and [2-^{13}C]acetate (Blackburn *et al.*, 1986; Mathews & van Holde, 1990; Voet & Voet, 1995). These and the isotopomers resulting from other glucose isotopomers were then excreted into the host.

Recent experiments from our laboratory (Corbin *et al.*, 1998) revealed the presence of significant amounts of citrate in culture media in which tetrathyridia of *Mesocestoides vogae* had been incubated. This finding indicates formation of citrate in the mitochondrion by the citrate synthase catalysed reaction of oxaloacetate and acetyl CoA, and suggests that in *Mesocestoides* further steps in the forward direction of the Krebs cycle are either very slow or not operating. Similarly, detection of citrate, labelled at C-2, in *E. multilocularis* indicates pronounced citrate synthase activity. Indeed, citrate synthase and other Krebs cycle enzymes have been found in *E. multilocularis* cysts (McManus & Bryant, 1995). However, as intermediates resulting from the catabolism of citrate were not detected, the possibility exists that some of the produced citrate was also excreted.

Acetate produced by the parasite from [1-^{13}C]- and [6-^{13}C]-glucose becomes labelled at C-2. However, it seems unlikely that the high level of carbon-13 enrichment of acetate observed in the cysts could have been produced solely by catabolism of labelled glucose. It was reported previously (Novak *et al.*, 1992) that in unstarved jirds infected with *E. multilocularis* the concentration of acetate in intraperitoneal cysts is lower than that of lactate, alanine and succinate, whereas in this study it is only consistently less than lactate. Further, the percent carbon-13 in acetate decreased with time in spite of the increase in the percent carbon-13 in glucose. Therefore, it seems that the majority of labelled acetate found in cysts in this experiment was the result of direct uptake by the parasite. As acetate can be used in host metabolic pathways, it can be assumed that the acetate of parasite origin, together with exogenous acetate, is eventually disposed of in biochemical pathways of the host.

A similar reduction in carbon-13 label with time was observed for β-HB in livers of jirds and *E. multilocularis* cysts. As cestodes do not posses a pathway for β-oxidation of fatty acid chains (Smyth & McManus, 1989), which is the usual source of acetyl CoA for β-HB synthesis in the mammalian liver, it is likely that this ketone body was taken up from the host rather than synthesized *de novo*. Even if *E. multilocularis* could synthesize β-HB, due to an incomplete Krebs cycle and non-functional gluconeogenic pathway, this ketone body cannot be utilized in these organisms.

Although lactate, alanine and succinate were excreted by *Echincococcus* into the host, there was no difference in the total concentration of these metabolites between livers of uninfected and infected jirds. This could mean, that in order for the concentrations to be the same, lactate, alanine and succinate are metabolized in some way at a greater rate in infected animals. This kind of metabolic adjustment has indeed been observed in various malignancies where the host adapts to the enhanced anaerobic glycolysis of the tumour with a concomitant increase in hepatic gluconeogenesis so that lactate, disposed of by the tumour, is rapidly utilized (Douglas & Shaw, 1990; Dills, 1993). Therefore, if in the present experiments, more lactate and alanine were utilized in the gluconeogenic pathways of infected animals, this could account for the unchanged concentration and lower percent enrichment of these metabolites 30 min post injection. Similarly, the unchanged concentration of hepatic succinate in infected jirds could also be the result of increased hepatic utilization of this intermediate by these hosts. However, unlike lactate and alanine, the percent carbon-13 in succinate in uninfected and infected jirds did not differ. Since [2–^{13}C]acetate must be metabolized via the Krebs cycle, all intermediates in the cycle, including succinate, should therefore have a constant influx of carbon-13 label. Thus, in the infected host, carbon-13 enrichment of succinate seems to reflect a balance between metabolism of this and other Krebs cycle intermediates with the influx of carbon-13 label from [2-^{13}C]acetate.

After 30 min, the total concentration of hepatic acetate was higher in infected hosts. It is known that acetate enters into mammalian biochemical pathways by conversion to acetyl CoA via acetyl CoA synthase (Crabtree *et al.*, 1990). It seems likely that in infected animals there will be a higher concentration of acetyl CoA due to greater β-oxidation of fatty acids and the acetate of parasite origin added to the exogenous acetate. A high concentration of generated acetyl CoA would, in turn, inhibit the conversion of acetate to acetyl CoA in this readily reversible reaction. This was not the case two hours into the experiment, as at this time the acetate overload was fully utilized by the infected host. Regardless of time, the majority of the carbon-13 label in the liver was present in acetate. There was no difference in the percent carbon-13 of hepatic acetate between uninfected and infected hosts. This label was most likely due to residual exogenous [2-^{13}C]acetate and, in the infected host, also due to a contribution of labelled acetate produced and excreted by the parasite. In additon, some of the [2-^{13}C]acetate was carried by blood to *E. multilocularis* cysts and, since the parasite cannot metabolize this compound, it eventually had to be returned to and recycled by the host.

Our results clearly indicate that the metabolism of acetate in the host was not carried out solely by direct oxidation in the Krebs cycle. The relatively high carbon-13 enrichment of β-HB in both uninfected and infected hosts

after 30 min suggests that a large amount of exogenous acetate was initially diverted to ketone body synthesis. Indeed, more β–HB was produced in infected animals than in the controls. This is in contrast with an earlier observation from our laboratory (Novak *et al.*, 1995), where a decrease in hepatic β-HB in *E. multilocularis* infected jirds was found when exogenous glucose was administered in a bolus injection. Under those experimental conditions, β-oxidation of fatty acids would have been the primary source of acetate and acetyl CoA for ketogenesis (Guyton, 1991), and the host was probably using β-HB faster than acetate could be liberated from fatty acids. In the current experiment, labelled acetate was probably diverted to ketogenesis as an alternate means of metabolizing excess acetyl CoA. As discussed in Orten & Neuhaus (1982), when the amount of acetyl CoA exceeds that of oxaloacetate, the accumulating acetyl CoA is directed toward ketone body synthesis. However, after 120 min, as the excess acetate was metabolized, nearly all labelled β-HB left the liver and also there was no difference in the concentration of hepatic β-HB between uninfected and infected jirds.

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