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The effect of respiratory chain impairment on β-oxidation in rat heart mitochondria

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Cardiac ischaemia leads to an inhibition of β-oxidation flux and an accumulation of acyl-CoA and acyl-carnitine esters in the myocardium. However, there remains some uncertainty as to which esters accumulate during cardiac ischaemia and therefore the site of inhibition of β-oxidation [Moore, Radloff, Hull and Sweely (1980) Am. J. Physiol. **239**, H257–H265; Latipää (1989) J. Mol. Cell. Cardiol. **21**, 765–771]. When β-oxidation of hexadecanoyl-CoA in state III rat heart mitochondria was inhibited by titration of complex III activity, flux measured as $^{14}CO_2$ release, acid-soluble radioactivity or as acetyl-carnitine was progressively decreased. Low concentrations of myxothiazol caused reduction of the ubiquinone pool whereas the NAD^+/NADH redox state was less responsive. Measurement of the CoA and carnitine esters generated under these conditions showed that there was a progressive decrease in the amounts of chain-shortened saturated acyl esters with increasing amounts of myxothiazol. The concentrations of 3-hydroxyacyl and 2-enoyl esters, however, were increased between 0 and 0.2 μM myxothiazol but were lowered at higher myxothiazol concentrations. More hexadecanoyl-CoA and hexadecanoyl-carnitine were present with increasing concentrations of myxothiazol. We conclude that 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA dehydrogenase activities are inhibited by reduction of the ubiquinone pool, and that this explains the confusion over which esters of CoA and carnitine accumulate during cardiac ischaemia. Furthermore these studies demonstrate that the site of the control exerted by the respiratory chain over β-oxidation is shifted depending on the extent of the inhibition of the respiratory chain.

INTRODUCTION

Cardiac ischaemia causes inhibition of β-oxidation owing to the obligatory oxygen dependency of this process. This causes the accumulation of CoA and carnitine esters [1,2], which are deleterious to normal cardiac function [3]. During ischaemia, terminal respiratory chain inhibition due to a lack of oxygen will lead to reduction of the ubiquinone (UQ) pool [4]. β-Oxidation is linked to the UQ pool and the respiratory chain at two stages: the 3-hydroxyacyl-CoA dehydrogenases to UQ via NAD^+/NADH and complex I, and the acyl-CoA dehydrogenases to UQ via electron transfer flavoprotein (ETF) and electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). An inhibition or deficiency of either of these proteins can lead to inhibition of β-oxidation [5,6]. ETF semiquinone (ETF_{sq}), the partly reduced form of ETF, can accumulate when the UQ pool is reduced [7] and is a potent inhibitor of acyl-CoA dehydrogenase [8]. However, ETF_{sq} disproportionates to the fully oxidized and fully reduced forms in a reaction catalysed by ETF:QO [9,10] so that the levels of the various ETF species and the importance of ETF_{sq} inhibition of the acyl-CoA dehydrogenases in intact mitochondria are unknown.

Reduction of the UQ pool will lead to inhibition of complex I activity and hence the 3-hydroxyacyl-CoA dehydrogenases, as observed with the rotenone inhibition of complex I activity and succinate-induced reversed electron transfer [5,11]. The inhibition of 3-hydroxyacyl-CoA dehydrogenase activity could also result in inhibition of the acyl-CoA dehydrogenases, as the enoyl-CoA hydratase reaction is inhibited by its 3-hydroxyacyl-CoA products [12] and the acyl-CoA dehydrogenases have a high affinity for their enoyl-CoA products, resulting in product inhibition [13].

In the ischaemic perfused heart, Rabinowitz and Hercker [14] observed, with TLC, the accumulation of small amounts of hydroxylated fatty acids generated from radiolabelled palmitate after saponification. Similarly the presence of 3-hydroxy fatty acids was demonstrated by means of GLC/MS after saponification of ischaemic rabbit hearts, although their levels were not compared with those of saturated fatty acids or other intermediates [15,16,]. These experiments were interpreted as indicating that 3-hydroxyacyl-CoA dehydrogenase is inhibited during cardiac ischaemia. When this group conducted similar experiments in rabbit heart mitochondria, 3-hydroxy fatty acids accumulated in the presence of rotenone, a complex I inhibitor, but not in the presence of cyanide, which inhibits complex IV and thus models terminal respiratory chain inhibition as seen in ischaemia [17]. Latipää [18], using enzymic assays for esters of 3-hydroxyacyl-CoA, 2-enoyl-CoA and long-chain acyl-CoA, showed that although the hypoxic perfused heart accumulated esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA, these were a small proportion of the total CoA esters accumulating. Hence there is some confusion over which of the two respiratory-chain-linked stages of β-oxidation is inhibited during ischaemia. We report here a direct analysis of intact CoA and carnitine esters accumulated during the β-oxidation of [U-^{14}C]hexadecanoyl-CoA in intact rat heart mitochondria, together with flux measurements and measurement of the redox states of the NAD^+/NADH and UQ/ubiquinol (UQH_2) pools during the titration of res-

Abbreviations used: ETF, electron transfer flavoprotein; ETF:QO, electron transfer flavoprotein:ubiquinone oxidoreductase; ETF_{sq}, electron transfer flavoprotein semiquinone; UQ, ubiquinone; UQH_2, ubiquinol.

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piratory chain activity with myxothiazol, a potent inhibitor of complex III, in order to determine which of the two possible steps is inhibited.

MATERIALS AND METHODS

Materials

The sources of most materials were as described previously [20] except that BSA (fraction V, fatty acid-free) was from Advanced Protein Products (Brierley Hill, West Midlands, U.K.). Percoll, dodecyl-β-maltoside, rotenone and myxothiazol were from Sigma (Poole, Dorset, U.K.). All HPLC solvents were from Rathburn Chemicals (Walkerburn, Scotland, U.K.). HPLC-grade buffer salts were from Merck (Poole, Dorset, U.K.). Standard UQ homologues were a gift from of Eisai Chemical Company (Tokyo, Japan). Solid-phase extraction columns were from Ansys (Irvine, CA, U.S.A.). [U-^{14}C]Hexadecanoyl-CoA was synthesized as described previously [19] and CoA esters were synthesized from the acid chlorides [20] or as described previously [21].

Mitochondrial preparation

Male Wistar rats (180–250 g) were fasted for 18 h, killed by cervical dislocation, and the hearts removed and placed in ice-cold medium A [120 mM KCl/20 mM Hepes/5 mM $MgCl_2$/1 mM EGTA/5 mg/ml BSA (pH 7.4 at 25 °C)]. The hearts were chopped finely and homogenized in an Ultra-Turrax homogenizer at low speed (setting 3 for 4 s). The homogenate was centrifuged (Sorvall RC-5C, SS34 rotor, 4 °C, 4.08×10^7 rad^2·s, 830 ***g***) and the supernatant retained. The pellet was re-homogenized and re-centrifuged as above and the combined supernatants were centrifuged (1.09×10^9 rad^2·s, 16800 ***g***). The mitochondrial pellets, which were contaminated with other membrane debris, were resuspended in 20 ml of ice-cold medium B [250 mM sucrose/2 mM Hepes/0.1 mM EGTA (pH 7.4 at 25 °C)]. Percoll was added to 19 % (v/v) and the preparation was centrifuged (1.09×10^9 rad^2·s, 14400 ***g***). At this concentration of Percoll, mitochondria are found in a loose pellet whereas contaminating membrane fragments remain at the top of the tube [22]. Mitochondria were removed with a Pasteur pipette and Percoll removed by three further centrifugations in medium B (1.09×10^9 rad^2·s, 14400 ***g***). After the final centrifugation, mitochondria were resuspended in medium B at approx. 50 mg/ml protein. Mitochondrial protein was measured by the modified Lowry method of Peterson [23]. Respiratory control ratios were measured polarographically with glutamate (10 mM) plus malate (1 mM) as substrates [19].

Incubations

Incubations were performed at 30 °C in a shaking water bath, in 1 ml of medium (pH 7.4) containing 110 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 1 mM EGTA, 5 mM ADP, 1 mM carnitine, 0.2 mg/ml cytochrome *c*, and 1.5–1.8 mg of mitochondrial protein. After 3 min of preincubation, reactions were initiated with 90 μM [U-^{14}C]hexadecanoyl-CoA (specific activity 2.2 Tbq/mol, for measurement of $^{14}CO_2$ release, total acid-soluble radioactivity and CoA esters) or 90 μM unlabelled hexadecanoyl-CoA (for analysis of NAD$^+$/NADH, UQ/UQH$_2$ and carnitine esters). [U-^{14}C]Hexadecanoyl-CoA and hexadecanoyl-CoA were added as a 5:1 molar complex with fatty acid-free BSA. Myxothiazol was added as an ethanolic solution, and the concentration of stock solution determined spectrophotometrically at 313 nm ($\epsilon = 10.5$ mM·cm^{-1} [24]).

Quenching and analyses

All incubations were carried out for 4 min. Radiochemical incubations were quenched with 200 μl of acetic acid, and $^{14}CO_2$ release and total acid-soluble radioactivity were measured as described [19]. CoA esters were extracted as described previously [19,21] except that low-volume solid-phase extraction columns (SAX; Ansys) were used rather than DEAE-Sephacel, so that freeze-drying, with attendant variable losses, was no longer necessary. After drying the chloroform/methanol extract, the sample was dissolved in 3 ml of 220 mM KH_2PO_4 and applied to a 3 ml C_{18} extraction disc cartridge (Ansys), which had previously been equilibrated with acetonitrile and water, under reduced pressure (approx. 5 kPa). The sample was collected and reapplied to the column. CoA and carnitine esters were eluted with 2 ml of acetonitrile/50 mM KH_2PO_4 (1:1, v/v), pH 5.3. Water (5 ml) was added to the sample, which was then applied to a 3 ml SAX extraction disc (Ansys) that had been pre-equilibrated with water. The eluate was collected and reapplied to the disc. The disc was washed with 2 ml of 80 % (v/v) methanol and CoA esters were eluted with 1 ml of 80 % (v/v) methanol/1 M ammonium acetate/10 mM acetic acid; methanol was then removed under N_2. HPLC analysis of CoA esters was performed as described previously [25]. NAD$^+$/NADH was measured by HPLC as described [19] except that samples were quenched with phenol/chloroform/isoamyl alcohol/EDTA [26]. Ubiquinone$_9$ (UQ$_9$) and ubiquinol$_9$ (UQ$_9$H$_2$) were extracted [27] and analysed by HPLC on a Hypersil 5ODS column (250 mm × 4.6 mm; HPLC Technology, Macclesfield, Cheshire, U.K.) with a gradient of 60–86 % (v/v) ethanol (containing 50 mM $NaClO_4$) against methanol (containing 50 mM $NaClO_4$) over 20 min. Peak identity was established by reference to standard compounds and by the absorption spectra from 200 to 330 nm using a photodiode array detector. Quantification was on the basis of the absorption at 292.5 nm [28]. Carnitine esters were measured by electrospray tandem mass spectrometry as follows: to 50 μl of quenched incubation medium were added the stable isotopically labelled internal standards [*methyl*-$^2H_3$]acetyl-carnitine (1 nmol), [*methyl*-$^2H_9$]octanoyl-carnitine (100 pmol) and [*methyl*-$^2H_9$]-hexadecanoyl-carnitine (100 pmol) in 100 μl methanol. The mixture was centrifuged (11500 ***g*** for 2 min) and the supernatant evaporated to dryness under N_2 at 50 °C. The residue was butylated by treating with 50 μl of 1 M HCl in butanol at 65 °C for 15 min. The mixture was evaporated to dryness under N_2 at 60 °C and the residue dissolved in 50 μl of 60 % (v/v) acetonitrile in water. Electrospray tandem mass spectrometry was performed with a Quattro II (Fisons Instruments, Altrincham, Greater Manchester, U.K.) triple quadrupole tandem mass spectrometer equipped with a Masslynx data system and a Jasco PU980 HPLC pump. Argon was used as the collision gas at a pressure of 2 μbar (20 Pa) and the collision energy was 25 eV. Acyl-carnitines were detected with a precursor ions of m/z 85 scan function in the range m/z 250–550. The flow rate of the mobile phase (60 % acetonitrile in water) was 10 μl/min. Aliquots (10 μl) of the samples were analysed and 10 continuum scans over a 50 s sampling period were acquired and summed in a single data file. Spectra were plotted after two-point smoothing (0.74 Da) and centring. Quantification was by reference to the internal standards.

Complex III assay

Complex III was assayed as UQ$_2$H$_2$:cytochrome *c* reductase by the method described in [29]. Dodecyl-β-maltoside (0.58 mM) was included, and the assay performed on a Cobas Bio fast

centrifugal analyser. Mitochondria were freeze–thawed three times in liquid nitrogen before assay, and the maximum reducible cytochrome *c* was obtained by the addition of a few grains of ascorbate. The increase in absorbance rapidly becomes nonlinear, and the activity was expressed as an apparent first-order rate constant. The degree of inhibition of complex III by myxothiazol was determined by a comparison of the first-order rate constants when myxothiazol was added to the mitochondria before freeze–thawing.

RESULTS

Mitochondrial preparation

Mitochondria isolated by the above method were coupled (the respiratory control ratio with 10 mM glutamate plus 1 mM malate was 3.5–5) and seemed to be free from contaminating membrane fragments. The use of proteolytic agents was avoided as this has been reported to cause loss of some enzyme activities in the outer mitochondrial membrane [30]; a second homogenization of the nuclear pellet increased the yield of mitochondria (results not shown).

Products of β-oxidation during the inhibition of β-oxidation by myxothiazol

Measurement of β-oxidation flux as acid-soluble radioactivity with different concentrations of [U-^{14}C]hexadecanoyl-CoA gave maximal flux at 90 μM and this was used as the substrate concentration in all subsequent incubations. β-Oxidation flux, whether measured as total acid-soluble radioactivity, as $^{14}CO_2$ release, as acetyl-carnitine accumulation or as total ^{14}C-labelled products (i.e. total acid-soluble radioactivity plus $^{14}CO_2$ release), was progressively inhibited by myxothiazol (Figure 1). Small amounts of myxothiazol (0.2 μM) inhibited both $^{14}CO_2$ release and acetyl-carnitine production by 32% whereas total acid soluble radioactivity accumulation was unchanged with this amount of myxothiazol.

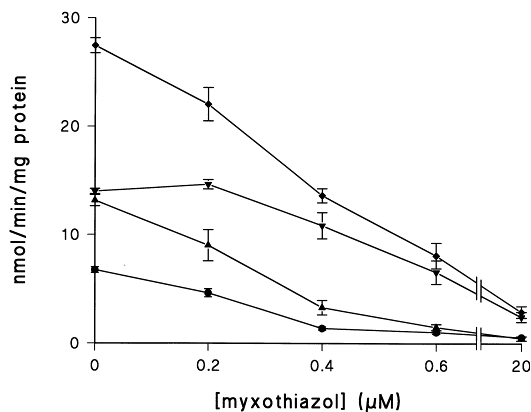

Figure 1 Products of β-oxidation during inhibition by myxothiazol

Rat heart mitochondria were incubated with 90 μM [U-^{14}C]hexadecanoyl-CoA and various amounts of myxothiazol for 4 min. Reactions were quenched and products analysed as described in the Materials and methods section. The results are calculated on the basis of acetyl units. Results means ± S.E.M. for three to five incubations. Symbols: ◆, total ^{14}C-labelled products, ▼, total acid-soluble radioactivity, ▲, $^{14}CO_2$ release, ●, acetyl-carnitine.

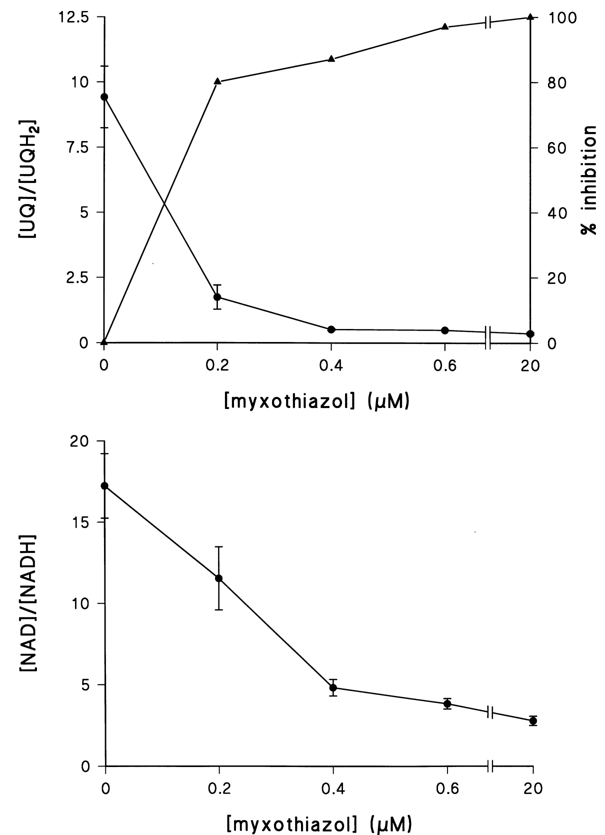

Figure 2 UQ and NAD redox states during the inhibition of β-oxidation by myxothiazol

Rat heart mitochondria were incubated with 90 μM hexadecanoyl-CoA and various amounts of myxothiazol for 4 min. Reactions were quenched and UQ, UQH_2, NAD^+ and NADH analysed as described in the Materials and methods section. Results are means ± S.E.M. for three incubations. Upper panel: ●, UQ/UQH_2; ▲, corresponding inhibition of complex III. Lower panel: NAD^+-to-NADH ratio.

UQ/UQH_2 redox state during the inhibition of β-oxidation by myxothiazol

The maximum succinate-reducible UQ pool for each mitochondrial preparation was determined by the incubation of 1.5–1.8 mg of mitochondria with 1.5 mM succinate, 60 μM myxothiazol and 1 mM cyanide, and the values for UQ/UQH_2 were corrected for this value. The proportion of the total UQ pool not available for reduction was approx. 10–15%, and probably represents UQ in membrane fragments or storage forms; it is not due to reoxidation of UQH_2 during the extraction, because dithionite-reduced mitochondria gave 100% UQH_2, and exogenous $UQ_{10}H_2$ added to mitochondrial suspensions and extracted was not oxidized during the extraction. The finding of a non-succinate reducible UQ pool is similar to that of Kröger and Klingenberg [31] in beef heart submitochondrial particles and of van den Bergen et al. [27] using plant mitochondria and the same extraction method as ourselves. The UQ-to-UQH_2 ratio during the inhibition of β-oxidation by myxothiazol is shown in Figure 2 (top panel). The UQ pool is largely oxidized during the β-oxidation of hexadecanoyl-CoA but becomes progressively reduced by titration of complex III with myxothiazol. Concentrations of 0.2, 0.4, 0.6 and 20 μM myxothiazol corresponded to 80, 87, 97 and 100% inhibition of complex III respectively. The largest change in the UQ-to-UQH_2 ratio was

Table 1 Accumulation of acyl-CoA esters during the inhibition of β-oxidation by myxothiazol

Results are means ± S.E.M. for three separate mitochondrial preparations. CoA esters are indicated as following: C_2, acetyl; $C_{12:1}$, dodec-2-enoyl; C_{12}, dodecanoyl-; C_{140H}, 3-hydroxytetradecanoyl; $C_{14:1}$, tetradec-2-enoyl; C_{14}, tetradecanoyl; C_{160H}, 3-hydroxyhexadecanoyl-; $C_{16:1(3,4)}$, hexadec-3-enoyl; $C_{16:1}$, hexadec-2-enoyl; C_{16}, hexadecanoyl. Abbreviation: n.d., not detected.

		Acyl-CoA ester concentration (nmol/mg of protein)				
CoA ester	Myxothiazol concentration (μM)...	0	0.2	0.4	0.6	20
C_2		0.398 ± 0.012	0.965 ± 0.073	n.d.	n.d.	n.d.
$C_{12:1}$		0.061 ± 0.012	0.026 ± 0.005	n.d.	n.d.	n.d.
C_{12}		0.142 ± 0.019	0.105 ± 0.013	0.035 ± 0.020	n.d.	n.d.
C_{140H}		0.090 ± 0.007	0.036 ± 0.007	n.d.	n.d.	n.d.
$C_{14:1}$		0.026 ± 0.003	0.063 ± 0.012	0.021 ± 0.005	0.010 ± 0.006	0.008 ± 0.005
C_{14}		1.051 ± 0.090	0.439 ± 0.031	0.286 ± 0.047	0.155 ± 0.016	0.085 ± 0.018
C_{160H}		0.125 ± 0.015	0.196 ± 0.040	0.103 ± 0.014	0.107 ± 0.013	0.068 ± 0.015
$C_{16:1(3,4)}$		0.030 ± 0.030	0.120 ± 0.0 21	n.d.	n.d.	n.d.
$C_{16:1}$		0.436 ± 0.031	0.541 ± 0.100	0.434 ± 0.049	0.322 ± 0.027	0.072 ± 0.020
C_{16}		24.644 ± 2.841	24.133 ± 1.620	25.466 ± 1.483	29.481 ± 2.984	36.087 ± 3.880

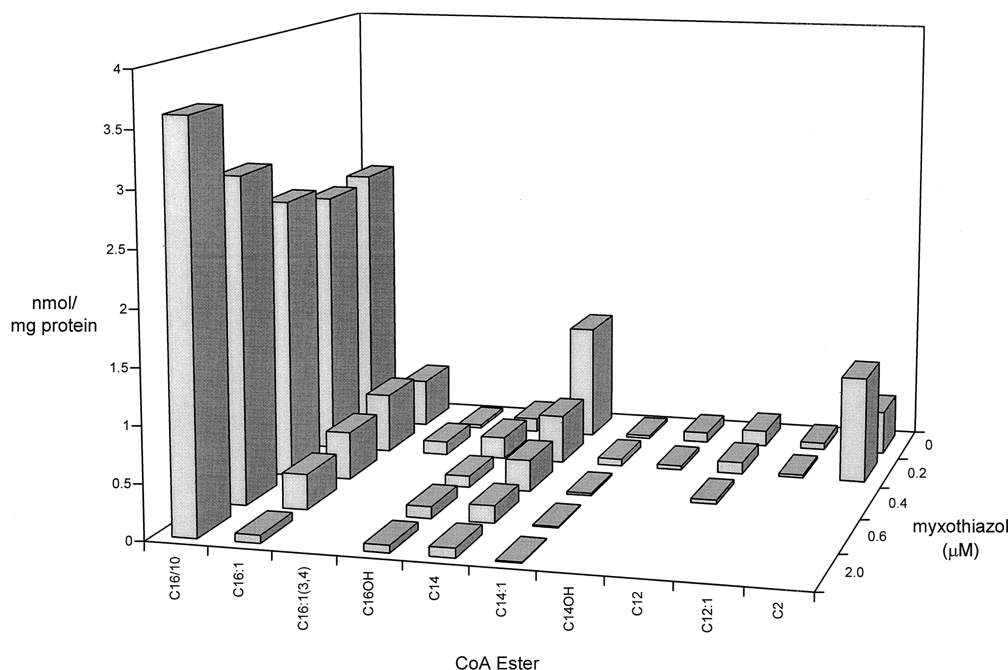

Figure 3 CoA esters generated during the inhibition of β-oxidation by myxothiazol

Rat heart mitochondria were incubated with 90 μM [U-^{14}C]hexadecanoyl-CoA and various amounts of myxothiazol for 4 min. Reactions were quenched and CoA esters analysed as described in the Materials and methods section. Results are means for three incubations. CoA esters are indicated as in Table 1 and the concentration of hexadecanoyl-CoA has been divided by 10 for clarity.

observed between 0 and 80% inhibition of complex III. Some UQ was in the oxidized form even at 20 μM myxothiazol (Figure 2); the reason for some oxidation of the UQ pool when isolated complex III was completely inhibited is not known, although the complex III inhibition could be less than complete in intact mitochondria or some UQH_2 might be re-oxidized by other factors.

NAD^+/NADH redox state during the inhibition of β-oxidation by myxothiazol

The NAD^+-to-NADH ratio measured directly by HPLC was lowered progressively by myxothiazol, as shown in Figure 2 (lower panel). In the absence of myxothiazol, NAD^+/NADH was largely oxidized (NAD^+ to NADH = 17), indicating that β-oxidation of hexadecanoyl-CoA was not limited by the availability of intramitochondrial NAD^+. This finding is similar to that demonstrated in skeletal muscle mitochondria oxidizing hexadecanoate [19]. The NAD^+/NADH redox state was less responsive to myxothiazol than the UQ redox state, in keeping with the function of the UQ pool so that complex I activity is only impaired when complex III has much of the control strength over electron flux to oxygen. Thus at 0.2 μM myxothiazol the reduction level of NAD^+/NADH increases from 6% to 8%

Table 2 Accumulation of acyl-carnitine esters during the inhibition of β-oxidation by myxothiazol

Results are means ± S.E.M. for three separate mitochondrial preparations. Abbreviation: n.d., not detected. Carnitine esters are indicated with the same abbreviations as for the CoA esters in Table 1, with the addition of C_4, butyryl; C_6, hexanoyl; C_8, octanoyl; C_{10}, decanoyl; C_{12OH}, 3-hydroxydodecanoyl.

		Acyl-carnitine ester concentration (nmol/mg of protein)				
Carnitine ester	Myxothiazol concentration (μM)...	0	0.2	0.4	0.6	20
C_2		27.011 ± 1.031	18.486 ± 1.482	5.518 ± 0.619	4.133 ± 0.619	2.206 ± 0.115
C_4		0.376 ± 0.041	0.180 ± 0.014	0.049 ± 0.018	0.036 ± 0.010	0.022 ± 0.006
C_6		0.678 ± 0.096	0.375 ± 0.010	0.127 ± 0.035	0.077 ± 0.017	0.032 ± 0.016
C_8		0.379 ± 0.064	0.244 ± 0.014	0.077 ± 0.033	0.052 ± 0.014	0.017 ± 0.009
C_{10}		0.645 ± 0.106	0.481 ± 0.028	0.155 ± 0.080	0.098 ± 0.013	0.046 ± 0.005
C_{12OH}		0.023 ± 0.015	0.013 ± 0.007	0.011 ± 0.006	0.015 ± 0.009	0.000 ± 0.000
$C_{12:1}$		0.013 ± 0.007	0.025 ± 0.013	0.015 ± 0.008	0.007 ± 0.004	0.004 ± 0.004
C_{12}		0.390 ± 0.098	0.330 ± 0.067	0.233 ± 0.044	0.208 ± 0.053	0.224 ± 0.014
C_{14OH}		0.029 ± 0.008	0.025 ± 0.006	0.009 ± 0.006	0.003 ± 0.003	0.005 ± 0.005
$C_{14:1}$		0.015 ± 0.009	0.008 ± 0.006	0.006 ± 0.006	0.017 ± 0.002	0.017 ± 0.008
C_{14}		0.315 ± 0.094	0.295 ± 0.085	0.125 ± 0.017	0.097 ± 0.021	0.098 ± 0.016
C_{16OH}		0.029 ± 0.009	0.043 ± 0.004	0.037 ± 0.004	0.039 ± 0.009	0.023 ± 0.004
$C_{16:1}$		0.019 ± 0.011	0.062 ± 0.028	0.047 ± 0.018	0.046 ± 0.011	0.023 ± 0.002
C_{16}		0.306 ± 0.083	0.506 ± 0.190	0.856 ± 0.268	0.837 ± 0.231	0.986 ± 0.307

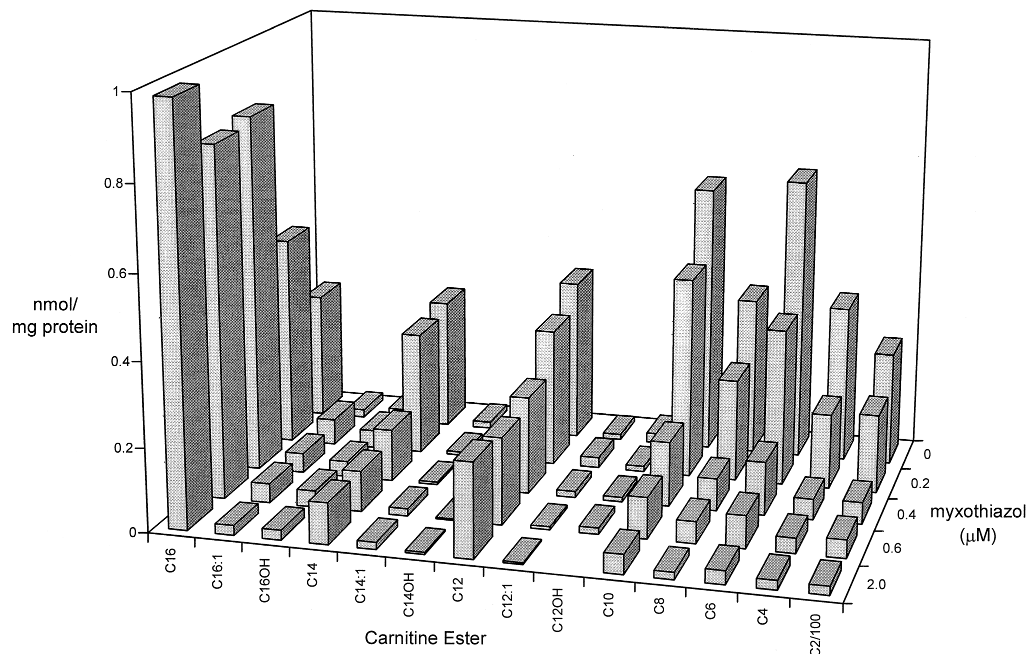

Figure 4 Carnitine esters generated during the inhibition of β-oxidation by myxothiazol

Rat heart mitochondria were incubated with 90 μM hexadecanoyl-CoA and various amounts of myxothiazol for 4 min. Reactions were quenched and carnitine esters analysed as described in the Materials and methods section. Results are means for three incubations. Carnitine esters are indicated as in Table 2, and the concentration of acetyl-carnitine has been divided by 100 for clarity.

whereas that of the UQ pool increases from 10 % to 33 %; this is when complex III has been inhibited by 80 %. The apparent K_m of beef heart mitochondrial NADH-ubiquinone reductase (or oxidase) for UQ is close to the mitochondrial content of UQ [32] and at 0.2 μM myxothiazol the oxidized UQ level is still within the error limits of the K_m value determined by Estornell et al. [32], so complex I activity will be essentially in the forward direction unless the UQ pool is highly reduced and there is an input of energy.

CoA esters generated during the inhibition of β-oxidation by myxothiazol

The generation of CoA esters is shown in Table 1 and graphically in Figure 3. The amount of the substrate, hexadecanoyl-CoA, remaining at the end of the incubation increased with decreased β-oxidation flux. The amounts of hexadec-2-enoyl-CoA and 3-hydroxyhexadecanoyl-CoA were increased at 0.2 μM myxothiazol compared with incubations conducted in the absence of

myxothiazol but were progressively decreased in the presence of greater amounts of myxothiazol. Tetradecanoyl-CoA was significantly decreased (from 1.05 to 0.44 nmol/mg of protein; $P < 0.005$) in the presence of 0.2 μM myxothiazol and decreased further in the presence of increasing amounts of myxothiazol. Tetradec-2-enoyl-CoA and dodecanoyl-CoA followed trends similar to those of hexadec-2-enoyl-CoA and tetradecanoyl-CoA respectively, whereas 3-hydroxytetradecanoyl-CoA decreased in amount at 0.2 μM myxothiazol and was absent at higher concentrations. Acetyl-CoA was only found in the absence of myxothiazol or the presence of 0.2 μM myxothiazol, as was hexadec-3-enoyl-CoA. The ratio of (hexadec-2-enoyl-CoA + hexadec-3-enoyl-CoA + 3-hydroxyhexadecanoyl-CoA) to tetradecanoyl-CoA, which we have previously used as a protein-independent measure of the inhibition of 3-hydroxyacyl-CoA dehydrogenase activity relative to that of the acyl-CoA dehydrogenase [33], was significantly raised from 0.56 in the absence of myxothiazol to 1.95 in the the presence of 0.2 μM myxothiazol ($P < 0.005$). This ratio was 1.88, 2.76 and 1.65 in the presence of 0.4, 0.6 and 20.0 μM myxothiazol respectively. The ratio of hexadecenoyl-CoA to 3-hydroxyhexadecanoyl-CoA was 3.73, 3.37, 4.21, 3.10 and 1.06 in the presence of 0, 0.2, 0.4, 0.6 and 20.0 μM myxothiazol respectively.

Carnitine esters generated during the inhibition of β-oxidation by myxothiazol

The carnitine esters generated during the oxidation of hexadecanoyl-CoA with increasing amounts of myxothiazol are shown in Table 2 and graphically in Figure 4. The most striking difference between the CoA and carnitine ester intermediates was the presence of large amounts of medium- and short-chain carnitine esters whereas the corresponding CoA esters were not detected. This does not seem to be due to the measurement of carnitine esters by tandem mass spectrometry rather than radio-HPLC, because a similar phenomenon has been noted in fibroblasts in which both CoA and carnitine esters were measured by radio-HPLC [34]. Otherwise the accumulation of carnitine esters followed a similar pattern to the CoA esters. There was a progressive rise in hexadecanoyl-carnitine with increasing myxothiazol concentration, although high levels were not reached, even when β-oxidation was almost completely inhibited. There was a rise in both hexadecenoyl-carnitine and 3-hydroxyhexadecanoyl-carnitine esters between 0 and 0.2 μM myxothiazol, with a subsequent decline as the amount of myxothiazol was increased, although levels were still higher than in the absence of myxothiazol. The ratio of (hexadecenoyl-carnitine + 3-hydroxyhexadecanoyl-carnitine) to tetradecanoyl-carnitine was lower than in the corresponding CoA esters (0.15, 0.36, 0.67, 0.88, 0.47 in the presence of 0, 0.2, 0.4, 0.6 and 20.0 μM myxothiazol respectively), probably owing to the lower specificity of CPT for 3-hydroxyacyl-CoA and 2-enoyl-CoA than for saturated CoA esters [19,25,35,36].

DISCUSSION

In our previous experiments in which the CoA and carnitine ester intermediates of β-oxidation in liver and skeletal muscle mitochondria were measured, we used [U-^{14}C]hexadecanoate as the substrate [19,25]. In the experiments described here it was necessary to use [U-^{14}C]hexadecanoyl-CoA owing to the high ATP-ase activity present in heart mitochondria. Thus even when 5 mM ATP was included, the formation of [U-^{14}C]hexadecanoyl-CoA from [U-^{14}C]hexadecanoate for β-oxidation was limited by the availability of ATP (results not shown). We therefore chose to use [U-^{14}C]hexadecanoyl-CoA as the substrate, with state 3 conditions obtained by the addition of 5 mM ADP. As extra-mitochondrial CoA was not included, all the CoA esters observed with the exception of [U-^{14}C]hexadecanoyl-CoA were necessarily intramitochondrial.

The finding of a progressive inhibition of β-oxidation flux with increasing amount of myxothiazol was as expected; at 20 μM myxothiazol the β-oxidation flux measured as acetyl-carnitine, as $^{14}CO_2$ release or as acid soluble radioactivity was diminished to 10 % or less of control values. Acetyl-carnitine accumulation and $^{14}CO_2$ release were lowered in the presence of 0.2 μM myxothiazol whereas total acid-soluble radioactivity was unchanged, presumably because the tricarboxylic acid cycle (and therefore CO_2 release) was inhibited markedly by low levels of myxothiazol, causing an accumulation of tricarboxylic acid cycle intermediates and hence an increased proportion of total acid-soluble radioactivity.

Measurement of the redox state of the NAD^+/NADH and UQ pools during the inhibition of β-oxidation by titration of complex III with myxothiazol showed a progressive reduction of these pools with increasing myxothiazol concentrations. In the absence of myxothiazol, the UQ pool was largely oxidized, suggesting that complex III is unlikely to have a high control strength over β-oxidation of hexadecanoyl-CoA under aerobic conditions. The UQ pool was greatly reduced by 80 % inhibition of complex III activity and the pool became further reduced with increasing myxothiazol concentrations. During the inhibition of complex III with antimycin in submitochondrial particles, the UQ pool became reduced with a titration curve that was strongly dependent on the substrate used [31,37]. The redox state of NAD^+/NADH was less sensitive to inhibition of complex III, but again was largely oxidized in the absence of myxothiazol, showing that β-oxidation of hexadecanoyl-CoA is unlikely to be inhibited by the intramitochondrial NAD^+/NADH redox state in heart mitochondria. This is entirely consistent with UQ pool function.

The UQ pool and ETF equilibrate quickly in submitochondrial particles [7,38] and in intact liver mitochondria [39], so that the redox state of ETF would be expected to be responsive to that of the UQ pool. However, we were unable to measure the ETF redox state directly in heart mitochondria by either the methods of Halestrap and Dunlop [40] or Kunz [39,42]. Ramsay et al. [10], using a reconstituted system, found the K_m of ETF:QO for UQ_1 to be 57.1 μM. However, it is impossible to compare this value with the apparent K_m for UQ_{10} of NADH-cytochrome *c* reductase of 1.7 nmol/mg protein [32]. Moreover the disproportionation of ETF_{sq}, which inhibits the acyl-CoA dehydrogenases [8], catalysed by ETF:QO [10] means that the redox state of ETF cannot be estimated from that of the UQ pool. Furthermore although the concentration of ETF is thought to exceed that of the acyl-CoA dehydrogenases [7,38] it is not known whether it behaves as a single pool.

In the absence of myxothiazol, the CoA ester intermediates of β-oxidation of [U-^{14}C]hexadecanoyl-CoA were similar to those reported previously for β-oxidation of [U-^{14}C]hexadecanoate or [U-^{14}C]hexadecanoyl-carnitine in rat skeletal muscle mitochondria [19], i.e. significant amounts of esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA were formed in the absence of significant changes in the gross intramitochondrial redox state of NAD^+/NADH. As the concentration of myxothiazol increased, the amount of tetradecanoyl-CoA, the first chain-shortened intermediate, decreased. The amounts of 3-hydroxy-hexadecanoyl-CoA and hexadec-2-enoyl-CoA increased so that the ratio of (hexadec-2-enoyl-CoA + hexadec-3-enoyl-CoA + 3-hydroxyhexadecanoyl-CoA) to tetradecanoyl-CoA was

significntly raised from 0.56 in the absence to 1.95 in the presence of 0.2 μM myxothiazol. This implies an inhibition of the NAD^+-linked 3-hydroxyacyl-CoA dehydrogenase step of β-oxidation between 0 and 0.2 μM myxothiazol. When the myxothiazol concentration was increased, the tetradecanoyl-CoA concentration decreased further, as did the concentrations of 3-hydroxyhexadecanoyl-CoA and hexadec-2-enoyl-CoA. This implies that β-oxidation is inhibited at a point proximal to hexadec-2-enoyl-CoA, i.e. the acyl-CoA dehydrogenase. This is confirmed by the presence of increased amounts of hexadecanoyl-CoA and hexadecanoyl-carnitine, although the intra-mitochondrial amounts of these two esters are not known. We interpret these results as indicating that as the respiratory chain is inhibited, first the 3-hydroxyacyl-CoA dehydrogenase step is inhibited (elevated 3-hydroxyhexadecanoyl-CoA and hexadec-2-enoyl-CoA between 0 and 0.2 μM myxothiazol); then as the respiratory chain is further inhibited and the UQ/UQH_2 ratio decreases, the acyl-CoA dehydrogenase is inhibited, either by ETF_{sq} or by the lack of the fully oxidized form of ETF. This is manifested at the level of hexadecanoyl-CoA rather than tetradecanoyl-CoA because of diminished flux. The fact that tetradecanoyl-CoA is not increased could reflect the preferential oxidation of chain-shortened intermediates rather than hexadecanoyl-CoA, which has not yet entered the β-oxidation spiral. Despite the decreased overall flux, and the decreased flux through the acyl-CoA dehydrogenase, the continued presence of esters of 3-hydroxyhexadecanoyl-CoA and hexadec-2-enoyl-CoA implies that 3-hydroxyacyl-CoA dehydrogenase is further inhibited. If all the inhibition of β-oxidation in the presence of 0.4 μM myxothiazol or greater were at the acyl-CoA dehydrogenase, then only hexadecanoyl-CoA would be observed, as in ETF and ETF:QO deficiency [6,34]. Another possible interpretation of these results is that esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA are acting as feedback inhibitors of the acyl-CoA dehydrogenases [12,13,41] so that β-oxidation flux is determined by the activity of 3-hydroxyacyl-CoA dehydrogenase, with a subsequent feedback inhibition causing accumulation of esters of hexadecanoyl-CoA and hexadecanoyl-carnitine. However, this explanation is unlikely because (i) the levels of esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA were diminished rather than constant in the presence of 0.4–2.0 μM compared with 0.2 μM myxothiazol and (ii) higher levels of esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA have been observed in succinate-induced reverse electron flow [11], 3-hydroxyacyl-CoA dehydrogenase deficiency [43], and alcoholic liver disease [33], so presumably a much greater accumulation of esters of 3-hydroxy-CoA and 2-enoyl-CoA is necessary before inhibition of the acyl-CoA dehydrogenases occurs. The pattern of carnitine esters observed supports this interpretation of the results, although the ratios are less pronounced. We assume that this is because of the lower activity of the CPT system towards esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA [35,36] and is similar to what we have observed in both muscle [19] and liver [25] mitochondria. The presence of large amounts of short-chain acyl-carnitines in the absence of their corresponding CoA esters probably reflects the respective volumes available to the CoA and carnitine esters; CoA esters are restricted to the intramitochondrial volume whereas carnitine esters are able to accumulate throughout the incubation volume. Hence there is a sideways 'pull' to carnitine esters.

The inhibition of 3-hydroxyacyl-CoA dehydrogenase activity by small changes in the NAD^+-to-NADH ratio (in the presence of small amounts of myxothiazol) is probably due to a sub-pool of intramitochondrial NAD^+/NADH between the trifunctional protein of β-oxidation and complex I. We have suggested such a subcompartmentation to be operative in skeletal muscle mitochondria to account for the presence of esters of 3-hydroxyacyl-CoA and 2-enoyl-CoA in the absence of gross changes in NAD^+/NADH [19]. The possibility that myxothiazol inhibited the trifunctional protein directly was excluded; the activity of the isolated trifunctional protein with hexadec-2-enoyl-CoA as substrate was not inhibited by myxothiazol concentrations of at least 20 μM (results not shown). Other factors might be responsible for the inhibition of 3-hydroxyacyl-CoA dehydrogenase activity by myxothiazol, for example medium pH changes due to greater respiratory rates in the absence of myxothiazol. However, we consider this to be unlikely owing to the high activity of ATPase, and consider the direct effect of myxothiazol exerted via the UQ and NAD^+/NADH pools to be more likely.

Although tandem mass spectrometry of carnitine esters accumulated in fibroblasts and leukocytes is increasingly being used for diagnosis of inborn errors of β-oxidation [44,45] and studies of the control of β-oxidation [46], the observation that great changes in β-oxidation flux can take place without great changes in the CoA and carnitine ester intermediates observed means that such results should always be interpreted together with a reliable flux measurement.

These observations explain the previous somewhat confusing observations of the accumulation of 3-hydroxyacyl esters in the ischaemic heart. Rabinowitz and Hercker [14] and Moore et al. [15,16] demonstrated the accumulation of 3-hydroxy fatty acid in the ischaemic heart, although the levels that they observed were not compared with those of saturated fatty acids or other intermediates, and implied 3-hydroxyacyl-CoA dehydrogenase inhibition during cardiac ischaemia. In another study, although esters of 2-enoyl-CoA and 3-hydroxyacyl-CoA accumulated in the ischaemic heart, they formed only a small percentage of the total accumulation of CoA esters [18]. In rabbit heart mitochondria, 3-hydroxy fatty acids accumulated only in the presence of rotenone, a complex I inhibitor, although there was a small accumulation in the presence of small amounts of cyanide but not in the presence of greater amounts [17]. The effect of cardiac ischaemia is gradual in that cytochrome oxidase becomes progressively inhibited as the concentration of oxygen falls below the K_m of cytochrome oxidase [47]. Hence 3-hydroxyacyl esters accumulate before the inhibition of β-oxidation at the acyl-CoA dehydrogenase level becomes more important, and hexadecanoyl-CoA and hexadecanoyl-carnitine accumulate; hence the observation that 2-enoyl-CoA and 3-hydroxyacyl-CoA esters form only a small proportion of the accumulating CoA esters in cardiac ischaemia [18]. However, in the intact heart, the inhibition of the respiratory chain by ischaemia will be exacerbated by the chronic depression of complex I and III activities [48].

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