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5-Hydroxydecanoate is metabolised in mitochondria and creates a rate-limiting bottleneck for β-oxidation of fatty acids

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5-Hydroxydecanoate (5-HD) blocks pharmacological and ischaemic preconditioning, and has been postulated to be a specific inhibitor of mitochondrial ATP-sensitive K^+ (K_{ATP}) channels. However, recent work has shown that 5-HD is activated to 5-hydroxydecanoyl-CoA (5-HD-CoA), which is a substrate for the first step of β-oxidation. We have now analysed the complete β-oxidation of 5-HD-CoA using specially synthesised (and purified) substrates and enzymes, as well as isolated rat liver and heart mitochondria, and compared it with the metabolism of the physiological substrate decanoyl-CoA. At the second step of β-oxidation, catalysed by enoyl-CoA hydratase, enzyme kinetics were similar using either decenoyl-CoA or 5-hydroxydecenoyl-CoA as substrate. The last two steps were investigated using l-3-hydroxyacyl-CoA dehydrogenase (HAD) coupled to 3-ketoacyl-CoA thiolase. V_{max} for the metabolite of 5-HD (3,5-dihydroxydecanoyl-CoA) was fivefold slower than for the corresponding metabolite of decanoate (L-3-hydroxydecanoyl-CoA). The slower kinetics were not due to accumulation of D-3-hydroxyoctanoyl-CoA since this enantiomer did not inhibit HAD. Molecular modelling of HAD complexed with 3,5-dihydroxydecanoyl-CoA suggested that the 5-hydroxyl group could decrease HAD turnover rate by interacting with critical side chains. Consistent with the kinetic data, 5-hydroxydecanoyl-CoA alone acted as a weak substrate in isolated mitochondria, whereas addition of 100 μM 5-HD-CoA inhibited the metabolism of decanoyl-CoA or lauryl-carnitine. In conclusion, 5-HD is activated, transported into mitochondria and metabolised via β-oxidation, albeit with rate-limiting kinetics at the penultimate step. This creates a bottleneck for β-oxidation of fatty acids. The complex metabolic effects of 5-HD invalidate the use of 5-HD as a blocker of mitochondrial K_{ATP} channels in studies of preconditioning.

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The phenomenon of preconditioning, whereby short ischaemic or pharmacological interventions transiently protect the heart from infarction following a subsequent prolonged period of ischaemia, is of considerable clinical interest (Yellon & Downey, 2003). Despite enormous research efforts in the past one to two decades, the molecular and cellular mechanisms underlying preconditioning have not been conclusively identified. Recently, mitochondrial ATP-sensitive K^+ (K_{ATP}) channels have been implicated as important mediators of preconditioning (Garlid *et al.* 2003; Gross & Peart, 2003; Yellon & Downey, 2003; O'Rourke, 2004). However, the evidence for their role in preconditioning is primarily based on the effects of pharmacological agents, in particular, diazoxide and 5-hydroxydecanoate (5-HD). Diazoxide was reported to be a specific opener of mitochondrial K_{ATP} channels (Garlid *et al.* 1996), but recent studies have suggested that its mechanism of cardioprotection may be due to other actions, such as inhibition of succinate dehydrogenase (Hanley *et al.*

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2002*a*; Lim *et al.* 2002), activation of sarcolemmal K_{ATP} channels (Suzuki *et al.* 2003) or transient opening of the mitochondrial permeability transition pore (Hausenloy *et al.* 2004).

Elucidating the action of 5-HD may provide a key to understanding the mechanisms involved in cardioprotection, since it blocks nearly all forms of preconditioning. Several years ago, 5-HD was deduced to be a specific blocker of mitochondrial K_{ATP} channels (Jaburek *et al.* 1998). More recent work has shown that the situation is much more complex since we now know that 5-HD, like other fatty acid substrates, is activated via acyl-CoA synthetase, yielding 5-hydroxydecanoyl-CoA (5-HD-CoA) (Hanley *et al.* 2002*a*; Lim *et al.* 2002). Following extramitochondrial activation, acyl-CoA esters are taken up into mitochondria via carnitine palmitoyltransferases (CPT-I and CPT-II) and carnitine acylcarnitine translocase (Bartlett & Eaton, 2004), but, at this stage, it is not clear whether 5-HD-CoA can be transported via this system. Using isolated heart and liver mitochondria, Lim *et al.* (2002) reported that 5-HD acted either as a weak substrate or inhibited metabolism, and they suggested that 5-HD-CoA could not access the matrix.

Once in the matrix, acyl-CoA esters are sequentially oxidised and shortened by two carbon atoms via the β-oxidation pathway, such that decanoyl-CoA would be shortened to octanoyl-CoA after one round, and 5-HD-CoA would be shortened to 3-hydroxyoctanoyl-CoA (that is, the 5-hydroxyl group would become a 3-hydroxyl group). It should be noted that metabolism of racemic 5-HD-CoA will give rise to two different enantiomers of the products, and the extent to which the different enantiomers are metabolised by the downstream enzymes is not known. Thus, L-3-hydroxyoctanoyl-CoA, formed after one round of 5-HD-CoA β-oxidation, could be metabolised normally, whereas the other enantiomer (D-3-hydroxyoctanoyl-CoA) could exert an inhibitory effect on β-oxidation. In a recent paper (Hanley *et al.* 2003), we have shown that activated 5-HD, similar to decanoyl-CoA, is a good substrate for purified human liver (as well as pig kidney) medium-chain acyl-CoA dehydrogenase, the first step of β-oxidation. In the present study, we provide a comprehensive analysis of the metabolism of 5-HD via the subsequent three steps of β-oxidation. Some of the results have been published in preliminary form (Hanley *et al.* 2004).

Methods

Enzymes of the β-oxidation pathway

Activity of enoyl-CoA hydratase (EC 4.2.1.17), which catalyses the hydration of enoyl-CoA esters, was assayed spectrophotometrically. In each assay, 0.5 μg bovine liver enoyl-CoA hydratase (Fluka) was added to a 1 ml cuvette and absorbance changes were measured at 263 nm. Recombinant human heart L-3-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35) was expressed and purified as previously described (Barycki *et al.* 2000). HAD catalyses the oxidation of the L-3-hydroxyl group to a keto group concomitant with the reduction of NAD^+. Purified pig-heart 3-ketoacyl-CoA thiolase (3-ketothiolase), the ultimate enzyme of β-oxidation, was kindly provided by Professor Horst Schulz (City University of New York, NY, USA). Unless stated otherwise, HAD was assayed indirectly by coupling it to the 3-ketothiolase reaction, which circumvented the problem of product inhibition (Noyes & Bradshaw, 1973; He *et al.* 1989). The coupled assay mixture contained 2.78 μg HAD, 0.5 mM NAD^+, 0.25 mM CoASH and 0.4 μg 3-ketothiolase. Absorbance changes were measured at 340 nm. All experiments using the aforementioned enzymes were performed at 25°C in standard solution containing 50 mM potassium phosphate, 0.3 mM EDTA and 10% glycerol (pH 7.6). Titrations were performed in duplicate. In separate experiments, free coenzyme A (CoASH) concentration was determined at various time intervals using DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) (Hanley *et al.* 2003).

Synthesis and purification of β-oxidation intermediates

5-HD-CoA was synthesised essentially as previously described (Hanley *et al.* 2003). In brief, acyl-CoA synthetase (EC 6.2.1.3; Sigma) was used to synthesise 5-HD-CoA from 5-HD and CoASH in tris(hydroxymethyl)aminomethane (Tris) buffer solution containing (mM): 1.2 Na_2ATP, 100 Tris, 9.1 EDTA and 1.8 EDTA (pH 7.5 with HCl). The enoyl-CoA esters decenoyl-CoA and 5-hydroxydecenoyl-CoA (5-HD-enoyl-CoA) were prepared enzymatically using human liver medium-chain acyl-CoA dehydrogenase and ferricenium hexafluorophosphate (Kumar & Srivastava, 1994). Finally, L-3-hydroxydecanoyl-CoA and 3,5-dihydroxydecanoyl-CoA were synthesised from their respective enoyl-CoA esters using bovine liver enoyl-CoA hydratase. The various decanoyl-CoA and 5-HD-CoA derivatives were purified by preparative reversed-phase HPLC.

Synthesis of pure D- and L-3-hydroxyoctanoyl-CoA esters

Racemic 3-hydroxyoctanoyl-CoA was prepared by reacting CoASH with a 1.2-fold excess of 3-hydroxyoctanoyl-imidazole in 4 ml of tetrahydro-furan/125 mM $NaHCO_3$ (1:1, v/v), in accord with the method of acyl-CoA ester synthesis described by

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Kawaguchi *et al.* (1981). The imidazole derivative was obtained by adding 3-hydroxyoctanoic acid (Larodan Fine Chemicals AB, Malmö, Sweden) to a 1.3-fold excess of 1,1-carbonyldiimidazole in tetrahydrofuran. After evaporation of the solvent, the residue was dissolved in ethylacetate and washed with 3 N H_3PO_4, 0.75 N H_3PO_4 and finally with water, before being dried. Analysis by thin layer chromatography (TLC) revealed a single spot (retention factor (R_f) 0.78). The CoA-ester reaction was allowed to proceed for 6 h, then dried down, dissolved in 8 ml 1 M NaCl and distributed over four C18-SPE (solid-phase extraction) cartridges (1 g), activated with methanol and equilibrated with 1 M NaCl. Cartridges were washed with 1 M NaCl, 0.5 M NaCl and water, and eluted with increasing concentrations of acetonitrile in water.

The enantiomers of 3-hydroxyoctanoyl-CoA were prepared from octanoyl-CoA by the combined action of acyl-CoA oxidase with either peroxisomal l-specific multifunctional protein (MFP1) or peroxisomal D-specific multifunctional protein (MFP2). The multifunctional proteins were isolated as previously described (Novikov *et al.* 1994). Octanoyl-CoA (5 μmol; P-L Biochemicals, Milwaukee, WI, USA) was desaturated at 30°C with 0.5 U acyl-CoA oxidase (*Arthrobacter* species; Sigma) in 10 ml reaction mixture, which contained 100 mM potassium phosphate buffer (pH 7.5), 5 μM FAD and 5 U ml^{-1} catalase. Upon completion (~40 min; monitored at 280 nm; $\Delta\varepsilon = 3.6$), multifunctional proteins were added (either 12.6 μg MFP1 or 5.4 μg MFP2) and disappearance of the 2-enoyl-CoA was monitored at 280 nm. After reaching equilibrium (~60 min), reaction mixtures were loaded on activated C18-SPE cartridges (500 mg; Waters). Cartridges were washed with 2 M ammonium acetate and the CoA-esters were eluted with methanol/water (80:20) containing 10 mM ammonium acetate. After evaporation of the solvent under reduced pressure, the samples were purified by preparative reversed-phase HPLC. Major peaks were collected, and those corresponding to 3-hydroxyoctanoyl-CoA (and remaining 2-octenoyl-CoA) were evaporated and lyophilised (the yield for 3-hydroxyoctanoyl-CoA esters was ~55%).

Analytical reversed-phase HPLC revealed a single peak for the isolated 3-hydroxyoctanoyl-CoA isomers, whereas a small contaminant (4.9%) was present in the racemic CoA-ester. Further tests showed that the isolated 3-L-hydroxyoctanoyl-CoA was dehydrogenated in the presence of NAD$^+$ to 3-oxooctanoyl-CoA by purified MFP1, but not by MFP2. The D-isomer was a substrate for MFP2 but not for MFP1, confirming that the pure isomers had been assigned the correct configurations. The racemic compound was converted by both enzymes.

Molecular modelling

A structure of HAD in complex with NAD$^+$ and 3-keto-octanoyl-CoA (1.8 Å, $R = 20.1\%$, $R_{\text{free}} = 23.1\%$; J. J. Barycki and L. J. Banaszak, unpublished data) was used as a template for the modelling studies. To generate a model of 3,5-dihydroxydecanoyl-CoA bound to the active site, a hydroxyl group at position C5 of the substrate was modelled in both the D and L configurations, and two additional carbon atoms were added to the acyl chain using the program O (Jones *et al.* 1991). Minor arrangements of adjacent amino acid side chains were required to accommodate 3,5-dihydroxydecanoyl-CoA in the active site of the enzyme, but typically, displacements were less than 0.2 Å.

Isolated liver and heart mitochondria

Coupled mitochondria were isolated from rat liver as previously described (Johnson & Lardy, 1967; Brandt *et al.* 1992), and suspended in solution containing 250 mM sucrose, 2 mM Tris and 1 mM EDTA (pH 7.4). Heart mitochondria were isolated along the lines described by Jacobus & Saks (1982). Diced ventricular tissue was minced and washed with a solution containing 300 mM sucrose, 10 mM Hepes and 0.2 mM EDTA (pH 7.2). The tissue was treated with trypsin (~0.1 mg ml^{-1}) for 15 min, and twice homogenised before adding soybean trypsin inhibitor (~0.3 mg ml^{-1}). The heart mitochondria were subsequently washed, centrifuged and resuspended in solution containing 300 mM sucrose, 10 mM Hepes, 0.2 mM EDTA and 1 mg ml^{-1} bovine serum albumin (pH 7.4). The isolation was performed at 4°C.

The rate of respiration was monitored at 25°C using an Oxygraph-2 k system (Oroboros, Innsbruck, Austria) with DatLab software. Mitochondria, used at a final concentration of 1.4–1.6 mg ml^{-1} (liver) or ~0.2 mg ml^{-1} (heart), were added to buffer containing various concentrations of sucrose and (mM): 10 potassium phosphate, 10 Tris-HCl, 10 $MgSO_4$ and 2 EDTA (pH 7.4). Substrates were tested in the presence of 0.5 mM malate (in heart and selected liver experiments), 0.5 mM ADP and, in the case of acyl-CoA esters (decanoyl-CoA, lauryl-CoA, oleoyl-CoA and 5-HD-CoA), 0.13–0.2 mM L-carnitine. Unless stated otherwise, the free fatty acids were added in the absence of ATP and CoASH, which are required for extramitochondrial activation.

The mitochondria had respiratory control ratios of 5–6 with 65 mM sucrose in the buffer solution (low osmolar conditions). At high osmolarity (440 mM sucrose), the respiratory control ratio was higher (~10), largely due to the lower basal activity under these conditions.

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Statistical analysis

An ANOVA was used to determine statistical significance at the 0.95 level of confidence. Data are expressed as means ± S.E.M.

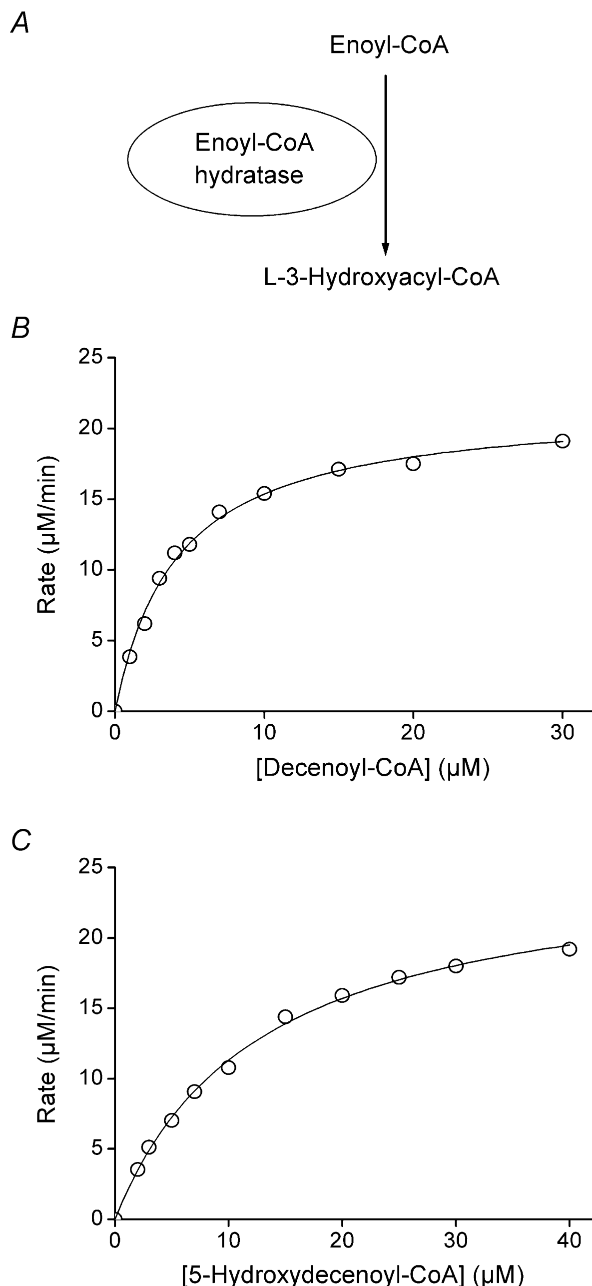

Figure 1. Kinetics of 5-hydroxydecanoyl-CoA (5-HD-CoA) metabolism at the second step of β-oxidation
A, the hydratase-catalysed reaction. *B* and *C*, steady-state activity of bovine liver enoyl-CoA hydratase, determined at various concentrations of decenoyl-CoA (*B*) or 5-hydroxydecenoyl-CoA (*C*). Data were fitted using the Michaelis–Menten equation.

Results

Kinetics of 5-HD-enoyl-CoA and decenoyl-CoA metabolism

The substituted fatty acid 5-HD is widely used to block preconditioning. To clarify its mechanism of action we studied the metabolism of 5-HD by mitochondrial enzymes and compared it to the metabolism of the physiological substrate decanoate. The second step of β-oxidation is catalysed by enoyl-CoA hydratase (Fig. 1*A*). The kinetics of this reaction were determined using various concentrations of purified decenoyl-CoA (Fig. 1*B*) or 5-HD-enoyl-CoA (Fig. 1*C*). When decenoyl-CoA was used as substrate, a K_m of $4.1 \pm 0.3\ \mu$M and a V_{max} of $21.7 \pm 0.5\ \mu$M min^{-1} were obtained. In accord, K_m values in the range 3–8 μM have been previously reported for the decenoyl-CoA dependent hydratase reaction (Yang *et al.* 1985; Jiang *et al.* 1996). We found that enzyme kinetics were similar when 5-HD-enoyl-CoA was used as substrate; hydratase catalysed the hydration of 5-HD-enoyl-CoA (yielding 3,5-dihydroxydecanoyl-CoA) with a K_m of $12.7 \pm 0.6\ \mu$M and a V_{max} of $25.7 \pm 0.5\ \mu$M min^{-1}. This observation, together with the recent data of Hanley *et al.* (2003), indicates that 5-HD-CoA can be efficiently metabolised via the first two steps of the β-oxidation pathway, that is, medium-chain acyl-CoA dehydrogenase and enoyl-CoA hydratase.

We next tested whether 5-HD-CoA was metabolised via the last two steps of β-oxidation (Fig. 2*A*) catalysed by L-3-hydroxyacyl-CoA dehydrogenase (HAD) and L-3-ketoacyl-CoA thiolase (3-ketothiolase). In preliminary experiments (not shown) we found that addition of 90 μM 5-HD-enoyl-CoA stimulated HAD activity, albeit to a lesser extent than addition of 90 μM decenoyl-CoA. Addition of 3-ketothiolase shifted the reaction equilibrium to the right, indicating that 5-HD-CoA can be metabolised via all four enzymes of the β-oxidation pathway. Free CoASH concentration was reduced from 100 μM to 27 μM (maximal rate, 17 μM min^{-1}) when L-3-hydroxydecanoyl-CoA was used in the assay. In comparison, free CoASH concentration was only reduced from 100 μM to 63 μM (maximal rate, 1 μM min^{-1}) when 3,5-dihydroxydecanoyl-CoA was used as substrate.

As shown in Fig. 2*B* and *C*, there was a striking difference in the kinetics of the HAD-thiolase catalysed reactions when 3,5-dihydroxydecanoyl-CoA rather than L-3-hydroxydecanoyl-CoA was used as the substrate. With the naturally occurring substrate L-3-hydroxydecanoyl-CoA, HAD had a K_m of $7.5 \pm 0.6\ \mu$M and a V_{max} of $40.3 \pm 1.2\ \mu$M min^{-1} (Fig. 2*B*), whereas with 3,5-dihydroxydecanoyl-CoA (Fig. 2*C*), the K_m was about two times higher ($12.6 \pm 1.1\ \mu$M) and V_{max}

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was considerably slower ($7.7 \pm 0.2\ \mu$M min^{-1}). To shed light on the mechanism by which the 5-hydroxyl group of 3,5-dihydroxydecanoyl-CoA slowed kinetics at HAD, we used a molecular model based on X-ray crystallographic data.

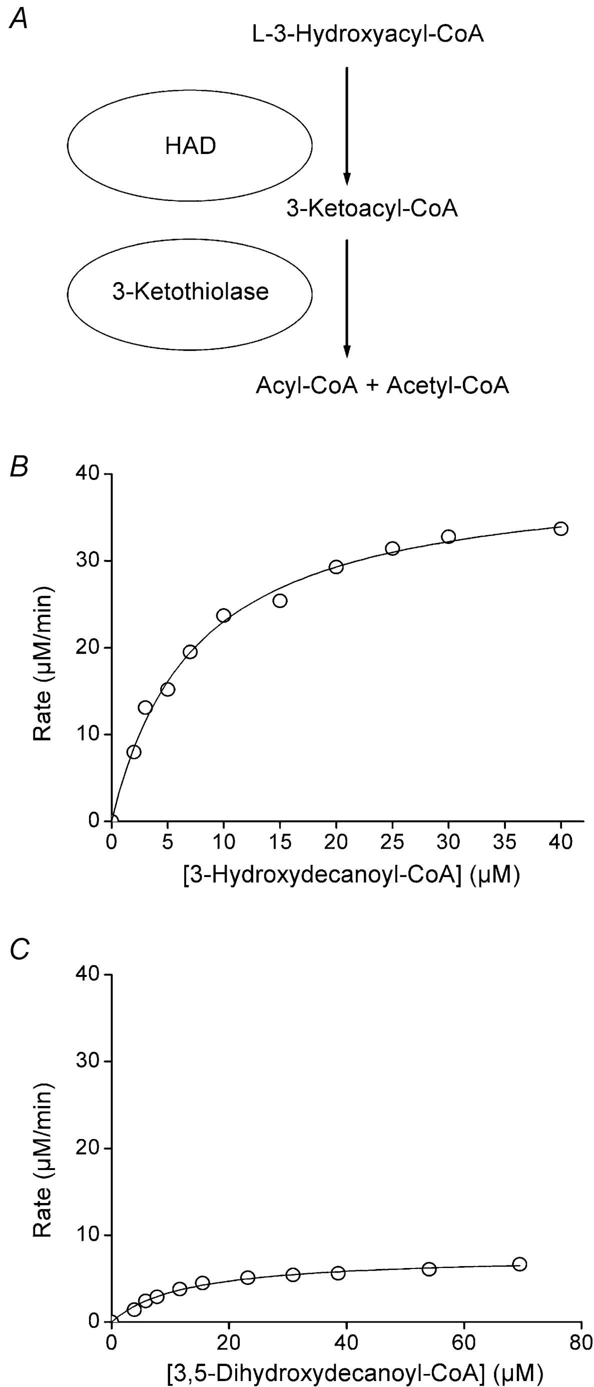

Figure 2. Kinetics of 5-HD-CoA metabolism at the last two steps of β-oxidation
A, the sequential reactions catalysed by L-3-hydroxyacyl-CoA dehydrogenase (HAD) and 3-ketoacyl-CoA thiolase (3-ketothiolase). *B* and *C*, kinetics of the coupled HAD-3-ketothiolase reactions determined as a function of the concentrations of 3-hydroxydecanoyl-CoA or 3,5-dihydroxydecanoyl-CoA.

Molecular modelling of HAD–substrate complexes

Figure 3 shows NAD$^+$ and 3,5-dihydroxydecanoyl-CoA bound to the active site of HAD. Note that the carbon at position 3 is modelled in an *sp2* configuration, as was observed in previously described structures of HAD in complex with either 3-hydroxybutyryl-CoA or acetoacetyl-CoA (Barycki *et al.* 2000). In the L configuration, the C5 hydroxyl group would be located between V212 and another active site residue, N208 (Fig. 3). It is important to note that the exact configuration of the side chain of N208 cannot be assigned unequivocally. The D configuration (hydroxyl on left; Fig. 3) would have a tighter fit. The hydroxyl group would be wedged between the side chains of I261 and the active site serine, S137, which is critical for activity.

D-3-Hydroxyoctanoyl-CoA does not inhibit HAD

When racemic 5-HD-CoA enters the β-oxidation pathway, and becomes shortened by two carbon atoms at the ultimate step, L-3-hydroxyoctanoyl-CoA and D-3-hydroxyoctanoyl-CoA should emerge. The L-isomer is a normal substrate for HAD, whereas the D-isomer is not a normal substrate for the enantiospecific enzyme HAD, and may even inhibit this enzyme. In order to test this possibility, we synthesised pure isomers of 3-hydroxyoctanoyl-CoA. Figure 4 shows that the l-isomer was good substrate for human heart HAD (Fig. 4*A*), whereas the D-isomer did not stimulate enzyme activity (Fig. 4*B*). More importantly, the presence of the D-isomer did not inhibit HAD-catalysed oxidation of the l-isomer (Fig. 4*C*).

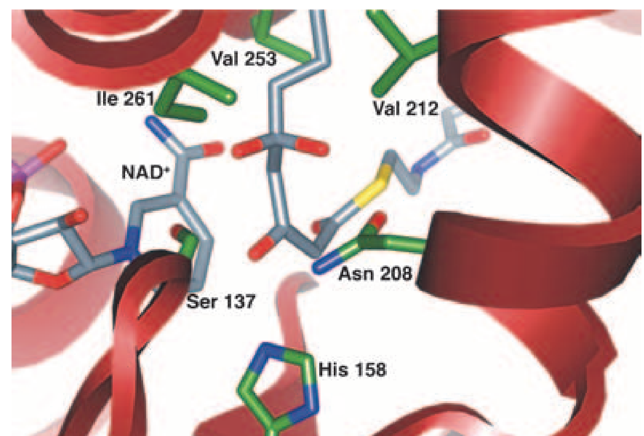

Figure 3. Model of HAD in complex with NAD$^+$ and 3,5-dihydroxydecanoyl-CoA
The hydroxyl group at C5 is depicted in both the D (nearest to NAD$^+$) and in the L configuration. Oxygen atoms are shown in red, nitrogen in blue, phosphorus in purple and sulphur in yellow.

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Metabolism of 5-HD and 5-HD-CoA by rat liver mitochondria

Oxidation of free fatty acids in the presence of respiring mitochondria was studied in the absence of ATP and CoASH so that their respective CoA-esters could not be synthesised at the mitochondrial outer membrane. To optimise fatty acid oxidation, a solution with low osmolarity (115 mosmol l^{-1}) was used (Halestrap & Dunlop, 1986; Halestrap, 1987). Addition of decanoate to coupled mitochondria potently stimulated state 3 respiration by a factor of between five and six (Fig. 5*A*), whereas 5-HD evoked a much weaker response (Fig. 5*B*). The addition of 0.5 mM malate did not affect the rate of respiration stimulated by 5-HD (not shown). Our observations are consistent with the data of Lim *et al.* (2002) and indicate that 5-HD can be activated in the matrix. At high osmolarity (490 mosmol l^{-1}), neither decanoate nor 5-HD stimulated respiration (not shown), consistent with the earlier work (Otto & Ontko, 1982; Halestrap & Dunlop, 1986; Halestrap, 1987).

To test whether extramitochondrially synthesised 5-HD-CoA can enter mitochondria via carnitine palmitoyltransferases, we added purified 5-HD-CoA as substrate to isolated mitochondria. As expected, decanoyl-CoA (or oleoyl-CoA; not shown), strongly stimulated oxygen consumption in the presence of ADP (state 3) (Fig. 5*C*). 5-HD-CoA also stimulated state 3 respiration (Fig. 5*D*), indicating that 5-HD-CoA is indeed a substrate for the carnitine-dependent acyl-transfer system.

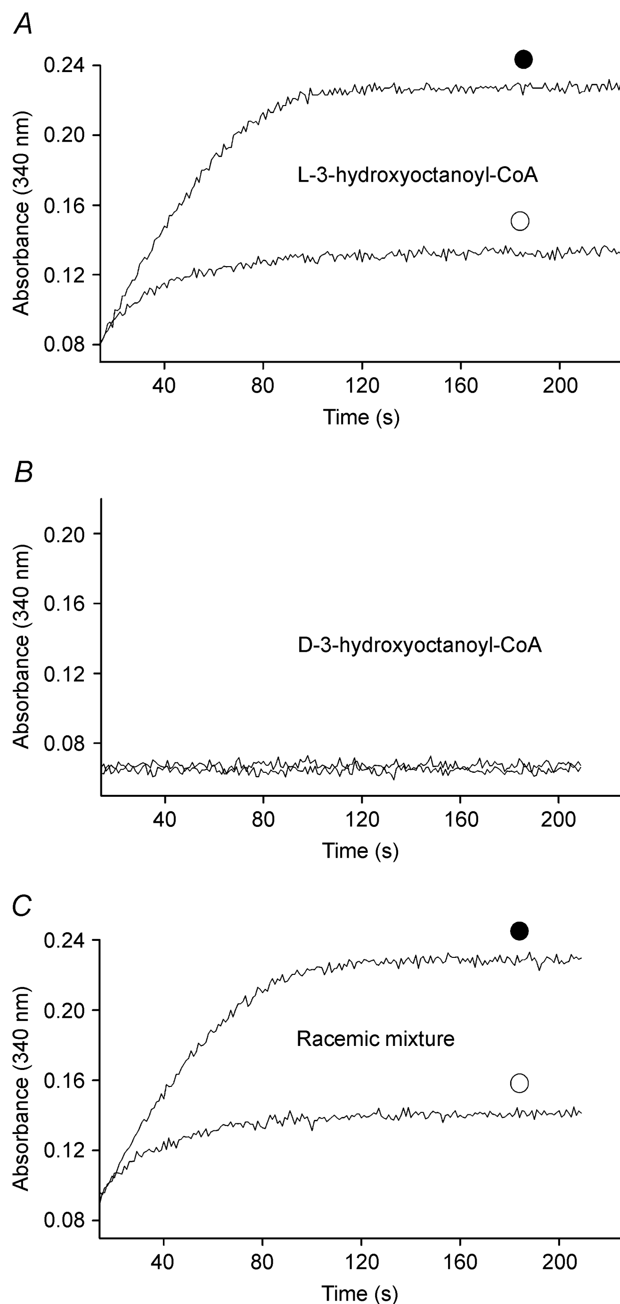

Figure 4. D-3-Hydroxyoctanoyl-CoA is neither a substrate nor an inhibitor of HAD
A, the pure isomer L-3-hydroxyoctanoyl-CoA (40 μM) was a good substrate for the human heart HAD (open circle, lower trace). In parallel experiments, 3-ketoacyl-CoA thiolase was added to circumvent product inhibition of HAD (filled circle, upper trace). *B*, the pure isomer D-3-hydroxyoctanoyl-CoA (40 μM) did not act as substrate for HAD (even at concentrations as high as 400 μM; not shown). *C*, the presence of D-3-hydroxyoctanoyl-CoA (40 μM) did not inhibit HAD-catalysed oxidation of L-3-hydroxyoctanoyl-CoA (40 μM).

Metabolism of 5-HD-CoA, but not 5-HD, by rat heart mitochondria

In preliminary experiments using heart mitochondria, we found that malate stimulated the rate of metabolism of medium-chain acyl-CoA esters and medium-chain acyl-carnitine; therefore, we performed all subsequent experiments in the presence of 0.5 mM malate. Figure 6*A* shows that decanoate strongly stimulated state 3 respiration in heart mitochondria in the absence of L-carnitine, suggesting that it is readily activated in the matrix. Under the same conditions, 5-HD did not stimulate respiration (Fig. 6*B*), suggesting that it is a poor substrate for heart-type matrix acyl-CoA synthetase. On the other hand, both decanoyl-CoA (Fig. 6*C*) and 5-HD-CoA (Fig. 6*D*) were significantly metabolizsd by heart mitochondria (as was the case with liver mitochondria; Fig. 5*D*). However, in the presence of 5-HD-CoA, the maximal rate of oxygen consumption of heart mitochondria was about threefold slower than with decanoyl-CoA.

5-HD-CoA acts both as substrate and inhibitor of β-oxidation

The slow kinetics of 5-HD-CoA metabolism at HAD (Fig. 2*C*) suggested that it could act both as substrate and as an inhibitor of the β-oxidation pathway. To test this possibility, we examined the effect of 5-HD-CoA

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on respiration supported by decanoyl-CoA (Fig. 7*A*). Addition of decanoyl-CoA (10 μM) strongly stimulated respiration, but when, in addition, 5-HD-CoA (100 μM) was present, the maximal rate was reduced by ~40%. In principle, 5-HD-CoA could limit the rate of decanoyl-CoA metabolism by either decreasing its rate of uptake into the matrix (via CPT-I/CPT-II) and/or by attenuating the activity of matrix enzymes. In support of the latter possibility, the metabolism of lauryl-carnitine (C12-carnitine), downstream of CPT-I, was similarly inhibited by ~40% in the presence of 5-HD-CoA (Fig. 7*B*).

Discussion

Metabolism of 5-HD

5-HD reliably blocks all forms of preconditioning, and has been assumed to be a specific blocker of mitochondrial K_{ATP} channels. In fact, blockage by 5-HD was often used as evidence for the involvement of mitochondrial K_{ATP} channels. In the present study, we have shown at the level of individual enzymes and in intact mitochondria that 5-HD is metabolised by the fatty acid oxidation machinery. 5-HD is activated (Hanley *et al.* 2002*a*), transported into the matrix and efficiently metabolised via the first two steps of the β-oxidation pathway. However, in the coupled HAD-thiolase reaction (penultimate and ultimate steps), the V_{max} with 3,5-dihydroxydecanoyl-CoA was fivefold lower than with L-3-hydroxydecanoyl-CoA. Two different mechanisms could be responsible for the slower kinetics observed with the 5-HD-CoA intermediate. First, the 5-hydroxyl group may impair the catalytic mechanism of HAD. Second, the D-isomer product of the thiolase reaction, D-3-hydroxyoctanoyl-CoA, may inhibit HAD in a negative-feedback fashion. To shed light on the former possibility we used modelling, whereas the latter possibility was tested directly using specially synthesised isomers.

Our modelling studies of NAD^+ and 3,5-DHD-CoA complexed with HAD suggest that the 5-hydroxyl group, either in the D or L configuration, can bind at the enzyme active site with relatively minor structural perturbations. However, in the D configuration, the hydroxyl group is wedged between I261 and the critical residue S137,

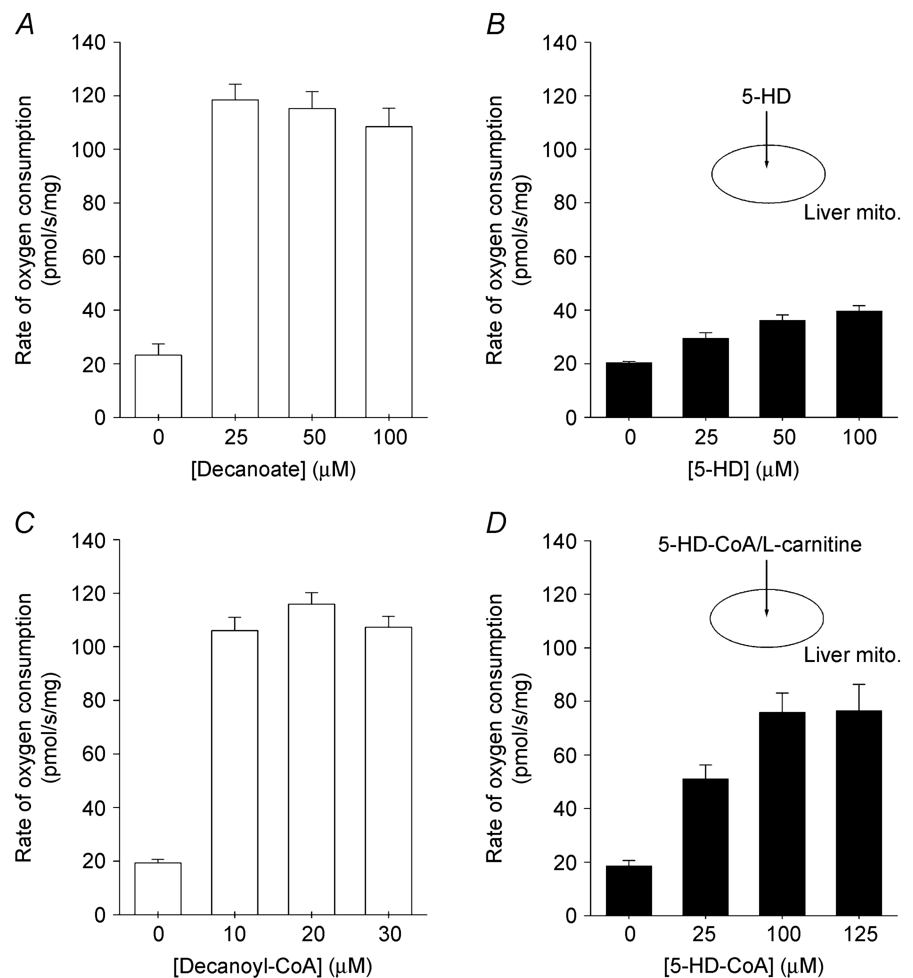

Figure 5. Metabolism of decanoate, 5-hydroxydecanoate (5-HD) and their CoA esters by liver mitochondria
A and *B*, effects of decanoate or 5-hydroxydecanoate on the rate of oxygen consumption of coupled rat liver mitochondria in the presence of 0.5 mM ADP, but in the absence of ATP and CoASH. Note the lower rate of oxygen consumption when 5-HD was the substrate. At least $n = 3$ experiments were performed at each concentration. *C* and *D*, effects of decanoyl-CoA and 5-HD-CoA on the rate of oxygen consumption of coupled mitochondria. Five experiments were performed at each concentration. In control experiments (not shown), addition of malate did not enhance the rate of oxygen consumption.

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critical in the sense that mutation of S137 to an alanine virtually abolishes activity (J. J. Barycki and L. J. Banaszak, unpublished data). On this basis, we infer that the D configuration (L-3,D-5-dihydroxydecanoyl-CoA derived from the D-isomer of 5-HD) may be metabolised slowly by HAD, and thus may be capable of inhibiting the enzyme in the presence of other substrates. This interpretation is supported by our finding that state-3 respiration induced by decanoyl-CoA or lauryl-CoA was reduced by ≈40% after addition of 100 μM 5-HD-CoA (Fig. 7). In contrast, the L configuration (L-3,L-5-dihydroxydecanoyl-CoA) can be nicely accommodated between V212 and N208, the latter of which is less critical for activity.

One of the end products of β-oxidation of racemic 5-HD-CoA, L-3-hydroxyoctanoyl-CoA, could bypass the first two steps of the next cycle and be further metabolised by the last two enzymes, HAD and 3-ketoacyl-CoA thiolase (Fig. 8). In principle, the D-isomer should not be metabolised by the enantiospecific enzyme HAD (Lehninger & Greville, 1953; Wakil & Mahler, 1954), although molecular modelling suggests that there is room for D-3-hydroxyl substrates at the enzyme active site with minor perturbations of the side chain positions of H158 and N208. In line with the original studies (Lehninger & Greville, 1953; Wakil & Mahler, 1954) we found that D-3-hydroxyoctanoyl-CoA did not act as substrate for HAD and, more importantly, we showed that this isomer did not inhibit enzyme activity. Hence, we conclude that 5-hydroxyl- and not the 3-hydroxyl-intermediates of 5-HD-CoA are responsible for the observed slow kinetics at HAD.

Recently, Lim *et al.* (2002) observed that 5-HD could be metabolised by liver and heart mitochondria, but was a weak substrate compared to decanoate. In addition, these authors found that 5-HD could not be metabolised in the presence of CoASH, ATP and L-carnitine, suggesting that extramitochondrial 5-HD-CoA could not access the matrix. In contrast, we found that both 5-HD and 5-HD-CoA (in the presence of L-carnitine) could be metabolised by liver mitochondria. The rates of 5-HD or 5-HD-CoA oxidation were slower than their straight-chain counterparts (decanoate and decanoyl-CoA). These

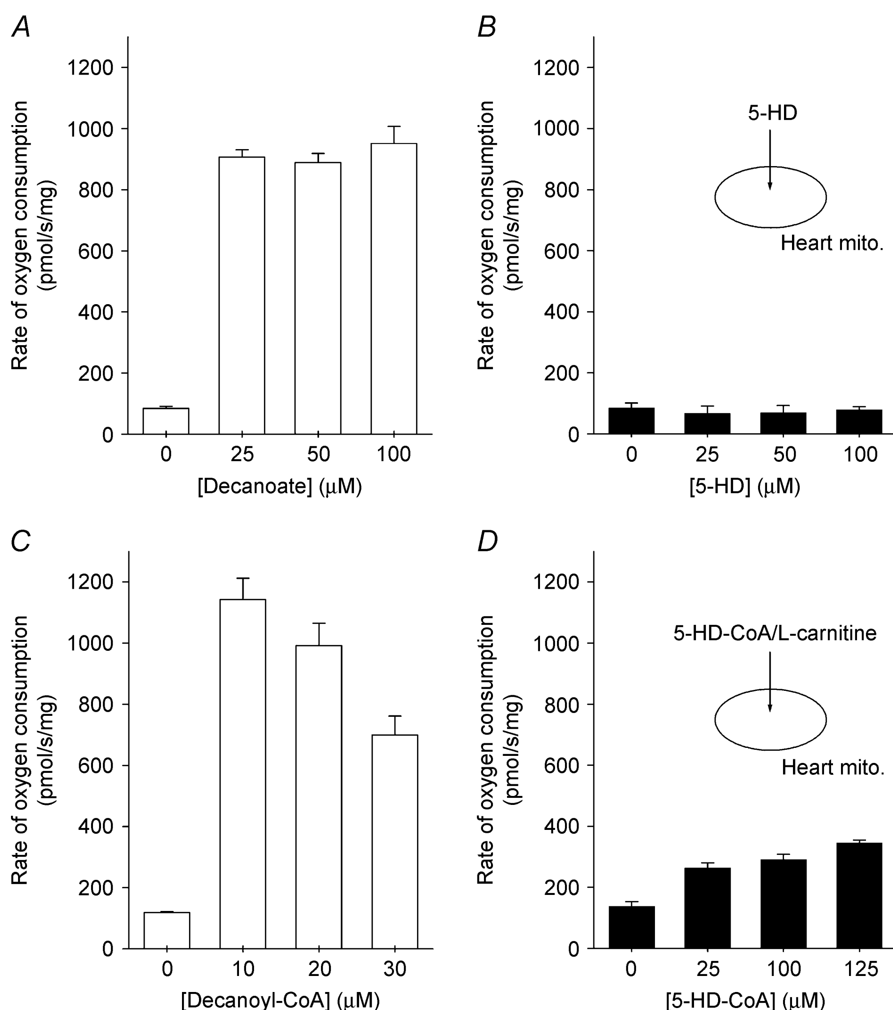

Figure 6. Metabolism of decanoate, 5-HD and their CoA esters by heart mitochondria
A and *B*, effects of decanoate or 5-HD on the rate of oxygen consumption of coupled rat heart mitochondria in the presence of 0.5 mM ADP and 0.5 mM malate, but in the absence of l-carnitine. Note that 5-HD did not stimulate respiration. Four experiments were performed at each concentration. *C* and *D*, effects of decanoyl-CoA and 5-HD-CoA on the rate of oxygen consumption of coupled mitochondria in the presence of 0.5 mM ADP, 0.5 mM malate and 0.2 mM L-carnitine. Three experiments were performed at each concentration.

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findings indicate that, at least in the liver, mitochondria can metabolise 5-HD in either case, after activation in the matrix or after activation at the outer membrane (Fig. 5). The efficient metabolism of extramitochondrial 5-HD-CoA by liver mitochondria suggests that carnitine palmitoyltransferases are not rate limiting.

In heart mitochondria, however, 5-HD did not stimulate respiration (Fig. 6*B*). The most plausible interpretation of these data is that 5-HD is a poor substrate for heart-type matrix acyl-CoA synthetase, an enzyme which activates selectively short- and medium-chain fatty acids. However, 5-HD can be activated on the outer membrane by heart mitochondria (Lim *et al.* 2002), and in this form it may be taken up via CPT-I, carnitine acylcarnitine translocase and CPT-II, and metabolised (Fig. 6*D*). The slower metabolism of 5-HD-CoA by heart mitochondria compared to liver mitochondria may be due to less-efficient oxidation of 3,5-dihydroxydecanoyl-CoA by heart-type HAD or, alternatively, heart-type HAD may be more susceptible to inhibition by the D-isomer.

Implications for the mechanisms of preconditioning

The current findings, together with recent work (Hanley *et al.* 2002*a*, 2003; Lim *et al.* 2002), establish that 5-HD is readily activated, taken up into the matrix and metabolised by the β-oxidation pathway, albeit with slow kinetics (for overview, see Fig. 8). As a consequence, application of 5-HD in experimental studies initiates a complex sequence of biochemical reactions, some of which may play a role in the ability of this drug to block preconditioning. One possibility is that 5-HD, acting as a weak substrate (Figs 5 and 6), may bypass inhibition of the electron transport chain at either complex I or complex II during pharmacological preconditioning (Hanley *et al.* 2002*a*,*b*; Dzeja *et al.* 2003). The resulting supply of redox equivalents to the electron transport chain may counteract inhibition of complex I by volatile anaesthetics or pinacidil (Berman *et al.* 1974; Hanley *et al.* 2002*a*) or inhibition of complex II by diazoxide (Schafer *et al.* 1969; Ovide-Bordeaux *et al.* 2000).

Another implication of our study is that L-3,D-5-dihydroxydecanoyl-CoA may accumulate in the matrix and create a bottleneck for β-oxidation of endogenous fatty acids at HAD. In support of this notion, Lim *et al.* (2002) reported that 5-HD inhibited the rate of oxidation of fatty acid substrates in heart mitochondria. In a precedent case, the potent 3-ketoacyl-CoA thiolase inhibitor trimetazidine (Kantor *et al.* 2000) has recently been shown to block ischaemic and pharmacological preconditioning as effectively as 5-HD (Minners *et al.* 2000). Interestingly, glibenclamide, a nonselective K_{ATP} channel blocker and inhibitor of preconditioning, has also been shown to inhibit fatty acid metabolism, in this case by an inhibitory action at CPT-I (Lehtihet *et al.* 2003).

Our results suggest that application of 5-HD may have profound effects on cardiac energy metabolism *in vivo*. (i) Inhibition of β-oxidation of fatty acids by metabolites of 5-HD at the level of HAD could exacerbate

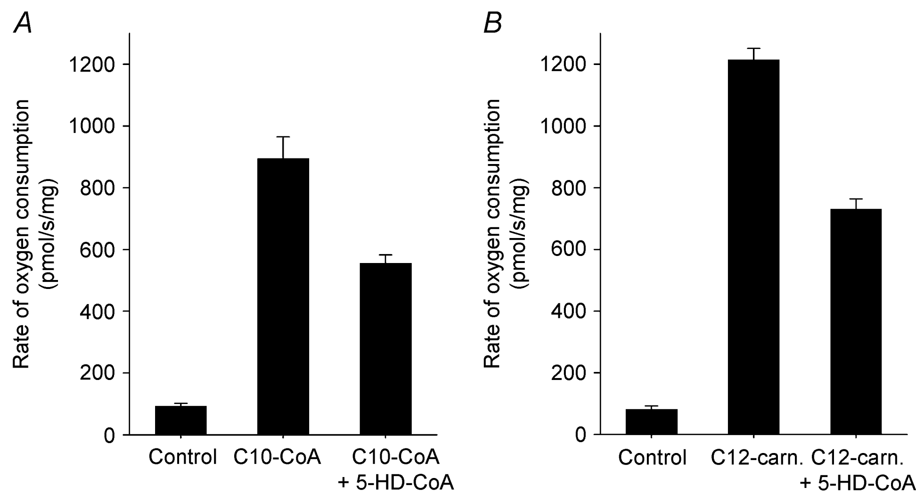

Figure 7. 5-HD-CoA acts as an inhibitor of fatty acid oxidation in heart mitochondria
The rate of oxygen consumption of coupled heart mitochondria was measured in the presence of 0.5 mM ADP, 0.5 mM malate, 0.2 mM L-carnitine and various added fatty acid substrates. *A*, addition of 10 μM decanoyl-CoA (C10-CoA) ($n = 4$) greatly stimulated respiration compared to control conditions ($n = 8$). In the presence of 100 μM 5-HD-CoA, the rate of respiration stimulated by decanoyl-CoA was significantly reduced ($n = 4$). *B*, lauryl-carnitine (C12-carn.) strongly stimulated respiration ($n = 4$) compared to control conditions ($n = 8$). In the presence of 100 μM 5-HD-CoA this effect was significantly reduced ($n = 4$). The ordinate in both *A* and *B* corresponds to the maximal rate of oxygen consumption.

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the postischaemic accumulation of acyl-CoA esters, acylcarnitine and fatty acids (van der Vusse *et al.* 1992; Zammit, 1999; De Windt *et al.* 2001; Bartlett & Eaton, 2004). (ii) Mitochondrial accumulation of acyl-CoA esters could lead to a decrease of free CoA since the mitochondrial CoA pool is of limited size. This would inhibit both β-oxidation (at 3-ketoacyl-CoA thiolase) and glucose oxidation (at pyruvate dehydrogenase) (Randle, 1998; Bartlett & Eaton, 2004). (iii) Accumulation of 5-HD-CoA and other acyl-CoA derivatives, both in the matrix and in the cytosol, may impair oxidative phosphorylation by inhibiting adenine nucleotide translocase (Shrago, 2000). (iv) 5-HD-CoA accumulating in the cytosol may compete with endogenous acyl-CoA esters at metabolically important regulatory enzymes such as acetyl-CoA carboxylase or CPT-I. All of these effects of 5-HD on cardiac energy metabolism are potentially detrimental for postischaemic recovery of the heart (van der Vusse *et al.* 1992; Dyck & Lopaschuk, 2002; Dyck *et al.* 2004), and may therefore contribute to the inhibitory effects of 5-HD on preconditioning.

The complex metabolic effects of 5-HD severely limit its usefulness as a 'selective' blocker of mitochondrial K_{ATP} channels. Furthermore, since their identification heavily relies on the use of diazoxide as a specific opener and 5-HD as a specific blocker, the very existence of mitochondrial K_{ATP} channels may be questioned. This sceptical view is supported by a recent study in which no changes in mitochondrial matrix volume induced by diazoxide or 5-HD could be detected (Das *et al.* 2003).

In conclusion, 5-HD is metabolised by mitochondria at concentrations employed to block preconditioning. The novel findings and implications of this study include those in the following list. (i) Extramitochondrial 5-HD-CoA can enter the matrix via carnitine palmitoyltransferases. (ii) The kinetics of

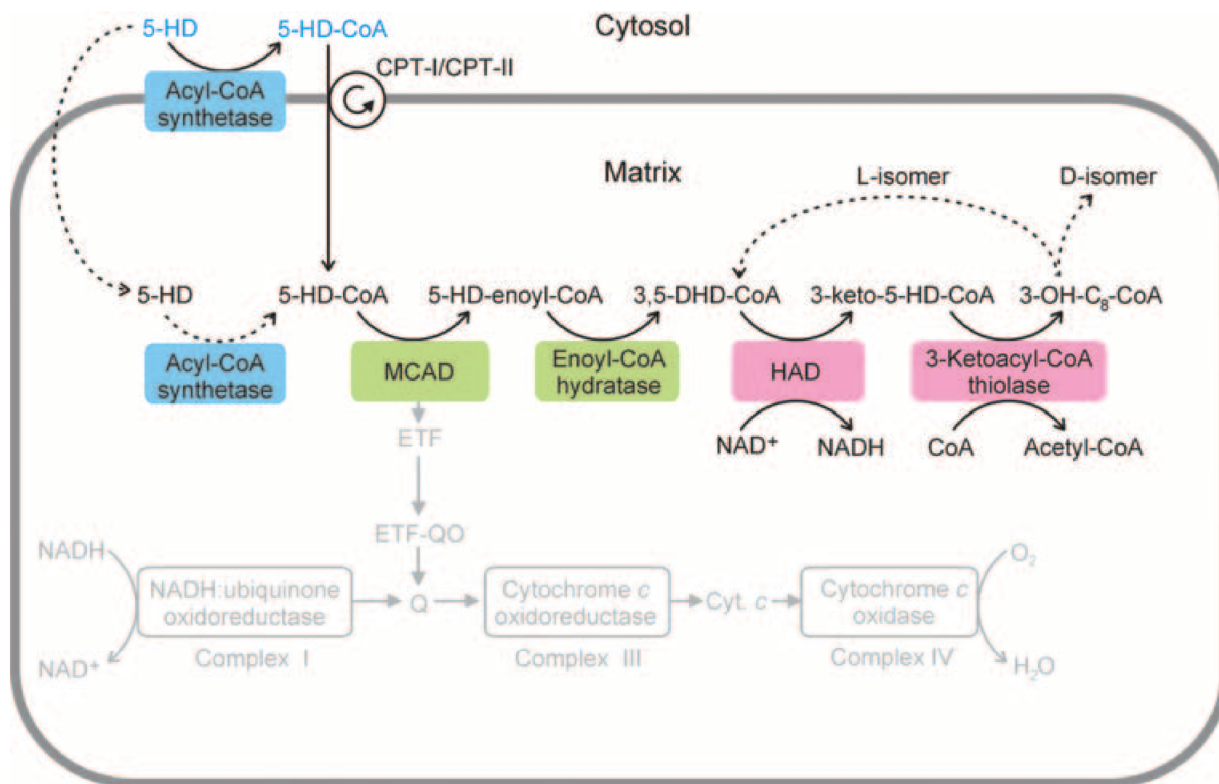

Figure 8. Overview of the metabolism of 5-HD in mitochondria
In both heart and liver mitochondria, 5-HD can be activated at the mitochondrial outer membrane and subsequently enters the matrix via carnitine palmitoyltransferases (CPT-I and CPT-II). In liver, but not in heart, 5-HD can also be activated in the matrix. The activated form of 5-HD, 5-HD-CoA, is a substrate for the β-oxidation spiral, which consists of four enzymes: medium-chain acyl-CoA dehydrogenase (MCAD), enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase (HAD) and 3-ketoacyl-CoA thiolase. Compared to the normal substrate decanoyl-CoA, the kinetics of 5-HD-CoA metabolism are dramatically slowed at the penultimate step. After one spiral of β-oxidation, racemic 5-hydroxydecanoyl-CoA would be reduced by two carbons atoms, yielding racemic 3-hydroxyoctanoyl-CoA, that is, the 5-hydroxyl group becomes a 3-hydroxyl group. The dotted line indicates that L-3-hydroxyoctanoyl-CoA (the L-isomer) serves as normal substrate for HAD. The other isomer, D-3-hydroxyoctanoyl-CoA, is neither a substrate nor an inhibitor of HAD. The points in β-oxidation that are slowed down in the presence of 5-HD are highlighted in red.

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5-HD-enoyl-CoA and decenoyl-CoA metabolism are similar at the second step of β-oxidation, catalysed by enoyl-CoA hydratase. (iii) β-Oxidation of 5-HD-CoA is slowed at the penultimate step, catalysed by HAD, which secondarily impairs the metabolism of other fatty acids. (iv) L-3,D-5-Dihydroxydecanoyl-CoA is mainly responsible for reducing the catalytic efficiency of HAD. (v) D-3-Hydroxyoctanoyl-CoA, generated from β-oxidation of D-5-hydroxydecanoyl-CoA, acts neither as substrate nor blocks HAD. (vi) Finally, in view of its complex metabolic effects, 5-HD should no longer be considered a useful tool for studying the role of mitochondrial K_{ATP} channels in preconditioning.

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Errata

The paper by Hanley *et al.* (2005) contained a number of errors throughout the paper. These are corrected below.

On page 308, Synthesis and purification of β-oxidation intermediates, the components of Tris buffer solution were incorrectly listed. The first occurrence of EDTA should have appeared as $MgCl_2$. The sentence should have read:

> In brief, acyl-CoA synthetase (EC 6.2.1.3; Sigma) was used to synthesise 5-HD-CoA from 5-HD and CoASH in tris(hydroxymethyl)aminomethane (Tris) buffer solution containing (mM): 1.2 Na_2ATP, 100 Tris, 9.1 $MgCl_2$ and 1.8 EDTA (pH 7.5 with HCl).

On page 314, in the second complete sentence of the page, lauryl-carnitine was incorrectly written as lauryl-CoA. The sentence should have read:

> This interpretation is supported by our finding that state-3 respiration induced by decanoyl-CoA or lauryl-carnitine was reduced by ≈40% after addition of 100 μM 5-HD-CoA (Fig. 7).

Two references, cited on pages 307 (Garlid *et al.* 1996) and 309 (Kawaguchi *et al.* 1981), were omitted from the list. The full references are given in the Reference list below.

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