JPP Journal of Pharmacy and Pharmacology

Royal Pharmaceutical Society of Great Britain

JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 1697–1703

Received September 25, 2009
Accepted April 28, 2010
DOI
10.1111/j.2042-7158.2010.01136.x
ISSN 0022-3573

Research Paper

# Evaluation of a screening method by liquid chromatography-tandem mass spectrometry for estimating effect of drugs on the activation and β-oxidation of fatty acids in mitochondria

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## Abstract

**Objectives** Fatty acid metabolism is controlled not only by the acyl-coenzyme A (CoA) synthetases but by some enzymes in the β-oxidation cycle. Medium-chain and long-chain acyl-CoA esters are key metabolites in fatty acid metabolism. We have developed an enzymatic assay method for determining chain shortening of the acyl-CoAs via β-oxidation from palmitic and octanoic acids in liver mitochondria. We have evaluated the assay method for detecting whether drugs influence the activation or the β-oxidation of fatty acids.

**Methods** Liver mitochondria were used for investigating the effect of drugs on fatty acid metabolism. The drugs selected were salicylic acid, diclofenac, valproic acid and paracetamol. Each acyl-CoA formed was analysed by liquid chromatography–tandem mass spectrometry.

**Key findings** After less than 5 min of incubation, the levels of acyl-CoAs reflected the acyl-CoA synthetase activity, whereas after 60-min incubation they reflected the activity of some enzymes in the β-oxidation cycle. Salicylic acid, diclofenac and valproic acid inhibited the medium-chain acyl-CoA synthetases, whereas valproic acid only exhibited a weak inhibitory activity toward the β-oxidation of the medium-chain fatty acids. In the case of long-chain fatty acid metabolism, salicylic acid and diclofenac inhibited both the activation and β-oxidation, whereas valproic acid was a weak inhibitor for only the β-oxidation activity. Paracetamol showed hardly any influence on the metabolism of medium-chain and long-chain fatty acids.

**Conclusions** These findings suggest that salicylic acid, diclofenac, valproic acid and paracetamol exert a different influence on fatty acid metabolism depending on the length of the acyl chain. This assay allows sensitive and selective analysis for predicting the pathways by which drugs exert a greater influence over fatty acid metabolism.

**Keywords** acyl-coenzyme A esters; fatty acid metabolism; inhibition; β-oxidation; mitochondria

## Introduction

Fatty acids are first converted to acyl-coenzyme A (CoA) esters by the acyl-CoA synthetases. Acyl-CoAs formed can be utilized in two major metabolic pathways. The first pathway is the catabolic pathway of fatty acid β-oxidation. Fatty acids are oxidized in mitochondria and peroxisomes in the cells. Short-, medium- and long-chain fatty acids are primarily degraded in mitochondria, whereas very long-chain fatty acids are oxidized in peroxisomes. The mitochondrial and peroxisomal β-oxidation results in degradation of acyl-CoAs by two carbon units per the β-oxidation cycle. As a consequence, the levels of acyl-CoAs in tissues reflect the activity of the acyl-CoA synthetases and some enzymes in the β-oxidation cycle. The second pathway is the anabolic pathway converting fatty acids into cellular lipids. The changes in fatty acid composition of phospholipids in the membrane lipid produce the disturbances in membrane fluidity and function.[1–4] The production of the different varieties of membrane phospholipid has been reported to occur in neurological disorders.[1–3]

It has been speculated that the disturbances in fatty acid metabolism may play an important role in the pathology of Reye's syndrome or in influenza-associated acute

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encephalopathy. Aspirin, salicylate and valproic acid have been identified as being associated with the development of Reye's syndrome, which occurs after an infectious illness, typically chickenpox or influenza A or B.[5–9]. In Japan, it has been recognized that aspirin or diclofenac may serve a causal role in the development of influenza-associated acute encephalopathy.[10–12]. As a result, paracetamol is recommended as the antipyretic drug for an infectious illness, typically influenza A or B. Previously, we reported that salicylic acid and diclofenac exhibited the different mechanisms of inhibition of the acyl-CoA synthetases depending on the length of the acyl chain.[13] The medium-chain acyl-CoA synthetase purified from mitochondria of mouse liver and kidney, and bovine liver was also inhibited by the nonsteroidal anti-inflammatory drugs and quinolone antimicrobial drugs.[14–17]. Deschamps *et al*.[17] have shown that salicylic acid decreased the mitochondrial activation and $\beta$-oxidation of long-chain fatty acids, through a mechanism that involved the sequestration of extramitochondrial CoA and carnitine. Further, valproyl-CoA inhibited the purified acyl-CoA dehydrogenases with the exception of the long-chain acyl-CoA dehydrogenases, whereas valproic acid inhibited various acyl-CoA dehydrogenases only very weakly.[9,18,19] The drugs influencing mitochondrial fatty acid metabolism may be risk factors for Reye's syndrome or influenza-associated acute encephalopathy.

Fatty acid metabolism is controlled not only by the acyl-CoA synthetases but also by some enzymes in the $\beta$-oxidation cycle. However, the $\beta$-oxidation reaction in many cases is expressed as the overall $\beta$-oxidation rate, including two steps of the activation and $\beta$-oxidation. The overall fatty acid oxidation is determined by the exhalation of $[^{14}C]CO_2$ or $[^{3}H]H_2O$ production from the labelled fatty acids.[17,19,20] However, it is difficult to elucidate the extent to which the drugs have an influence over the activation and $\beta$-oxidation of fatty acids, respectively. Acyl-CoA esters, as the substrate, are required for investigating the effect of drugs on the in-vitro $\beta$-oxidation activity.

To elucidate the mechanism by which the drugs cause the perturbation in fatty acid metabolism, it is important to investigate the dynamic situation of acyl-CoAs. The level of acyl-CoAs reflects the activity of the acyl-CoA synthetases and some enzymes in the $\beta$-oxidation cycle. Whether salicylic acid, diclofenac, valproic acid or paracetamol disrupted the activation or the $\beta$-oxidation of fatty acids has not been elucidated fully. To determine simultaneously the acyl-CoA synthetase and $\beta$-oxidation activity, we have developed a screening method by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) for detecting each acyl-CoA degraded via $\beta$-oxidation from octanoic and palmitic acids using mouse liver mitochondria, respectively. The assay was applied to evaluate the ability of salicylic acid, diclofenac, valproic acid and paracetamol to inhibit fatty acid metabolism in liver mitochondria.

## Materials and Methods

### Materials

ddY strain male mice (27–30 g, 6-weeks old) were purchased from SLC Co. Ltd (Shizuoka, Japan). Acetyl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradodecanoyl-CoA and palmitoyl-CoA were obtained from Sigma Chemical Co. (St Louis, MO, USA). Salicylic acid, valproic acid and paracetamol (acetaminophen) were purchased from Nacalai Tesque (Kyoto, Japan), diclofenac from Wako Pure Chemical Industries (Osaka, Japan), and CoA, ATP, FAD and $NAD^+$ from Kohjin Co. Ltd. (Tokyo, Japan). Other chemicals used were analytical grade and were used as received.

### LC-ESI-MS/MS analysis of Aacyl-CoAs

HPLC separation was performed with a Waters 2690 instrument (Micromass, Manchester, UK) having a Capcellpak $C_{18}$, UG120 column (2.0 mm i.d. × 150 mm). The mobile phases were A (10 mM ammonium acetate; pH 5.3) and B (acetonitrile). The eluting gradient was as follows: the column was equilibrated with 84% A, 84% A for 5 min, 84% A to 16% A in 30 min and 16% A to 84% A in 7 min. The flow rate was 0.2 ml/min. Quantitation was carried out on a Quattro Ultima (Micromass, Manchester, UK). The mass spectrometer was operated in the positive-ion mode with the source temperature set to 110°C, a corn voltage of 45 V and a capillary voltage of 3.5 kV. Collision energy was individually tuned at 30 eV. All positive-ion mass spectral data were obtained by scanning the mass range from m/z 100 to 1200.

The acyl-CoAs were determined by selective multireaction monitoring with a positive ionization mode according to a modification of the procedure described previously.[13] Briefly, the peak of each acyl-CoA was monitored by the product ion obtained from $[M+H]^+$ ion of each medium-chain and long-chain acyl-CoA ester (i.e. m/z 810 → 303 for acetyl-CoA, 838 → 331 for butyryl-CoA, m/z 866 → 359 for hexanoyl-CoA, m/z 894 → 387 for octanoyl-CoA, m/z 922 → 415 for decanoyl-CoA, m/z 950 → 443 for dodecanoyl-CoA, m/z 978 → 471 for tetradodecanoyl-CoA, m/z 1006 → 499 for palmitoyl-CoA).

### Calibration of Aacyl-CoAs

Calibration curves of the medium-chain and long-chain acyl-CoA esters were obtained by adding known amounts of authentic medium-chain and long-chain acyl-CoA esters to mitochondria. The amount of each acyl-CoA ester was determined by linear regression of peak area representing each acyl-CoA ester vs concentration.

### Assay of fatty acid metabolism in mouse liver mitochondria

The activity of medium-chain acyl-CoA synthetases and some enzymes in the $\beta$-oxidation cycle was determined based on octanoyl-CoA formation from octanoic acid, followed by chain shortening of the acyl-CoAs via $\beta$-oxidation.[13]

Briefly, mouse liver was homogenized with four volumes of 0.25 M sucrose : 10 mM Tris-HCl buffer (pH 7.4). Homogenates were centrifuged at 700*g* for 15 min. The resulting supernatant was then centrifuged at 9200*g* for 15 min and the crude mitochondrial pellet obtained was used. For metabolism of the medium-chain fatty acid, the typical reaction mixture consisted of octanoic acid 0.16 μmol, ATP 5 $\mu$mol, CoA 1 $\mu$mol, FAD 1 $\mu$mol, $NAD^+$ 1 $\mu$mol, $MgCl_2$ 20 $\mu$mol, KCl 30 $\mu$mol and

mouse liver mitochondria (0.1–5.0 mg protein) in a final volume of 2 ml 0.2 M Tris-HCl buffer (pH 8.5) and incubated at 37°C for 5–60 min. At each reaction time, a 0.5-ml sample of the incubation mixture was removed and stopped by the addition of 0.3 ml cold acetonitrile and 0.2 ml 0.5 M sodium citrate-HCl buffer (pH 2.0). After centrifugation, the supernatant was subjected to LC-ESI-MS/MS analysis.

The formation of octanoyl-CoA from octanoic acid was linear up to 2 mg mitochondrial protein and 10 min incubation time. After this time, 0.2 mg mitochondrial protein and 5-min incubation were used. The addition of FAD, $NAD^+$ and carnitine to the 0.2 mg mitochondrial protein allowed us to proceed with the β-oxidation of octanoic acid for an incubation time of 60 min.

The activity of long-chain acyl-CoA synthetases and some enzymes in the β-oxidation cycle was determined as described above except for the use of a different substrate, palmitic acid, and an incubation time of 1–60 min.

### Inhibition assay of fatty acid metabolism in mouse liver mitochondria

Salicylic acid, diclofenac, valproic acid and paracetamol were selected for the ability to inhibit the enzymes involved in fatty acid metabolism using mouse liver mitochondria. The kinetics of the inhibition were determined as indicated above, using 80 μM of the substrates (octanoic or palmitic acids), and various concentrations of inhibitors. The inhibitor concentrations ranged from 10 μM to 50 mM for salicylic acid, from 500 μM to 50 mM for valproic acid and paracetamol, and from 10 μM to 10 mM for diclofenac, respectively. The protein concentration and the incubation times were used as described above.

### Protein analysis

Protein was determined in duplicate at 595 nm with a protein assay kit (Bio-Rad).

### Statistical analysis

The data were expressed as means ± SD and the results were analysed by the Kruskal–Wallis test. The significance of differences between the mean values of individual groups treated with each drug was determined by the Mann–Whitney test. A difference was considered to be significant when $P < 0.05$.

## Results

Table 1 shows the effect of salicylic acid on the activation and β-oxidation of palmitic and octanoic acids in mouse liver mitochondria. The activity of medium-chain and long-chain acyl-CoA synthetases was determined based on the formation of octanoyl-CoA and palmitoyl-CoA, respectively. Almost no β-oxidation of palmitic and octanoic acids was observed for the incubation times of 1 min and 5 min, respectively. The main acyl-CoA esters of $C_{2:0}$, $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ from palmitic acid, and the acyl-CoA esters of $C_{2:0}$, $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$ from octanoic acid in mouse liver mitochondria were detected by LC-ESI-MS/MS. In palmitic acid metabolism, the amounts of medium-chain acyl-CoAs ($C_{4:0}$, $C_{6:0}$ and $C_{8:0}$)

**Table 1** Effect of salicylic acid on the activation and β-oxidation of palmitic and octanoic acids in mouse liver mitochondria

**(a) Acyl-CoA formation of fatty acids (nmol/min/mg protein)**

| | Salicylic acid (mM) | | | | | Salicylic acid (mM) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 50 | | Control | 0.5 | 1.0 | 10 | 50 |
| Acyl-CoA ($C_{16:0}$) | 234.5 ± 20.4 | 169.0 ± 19.7 | 84.8 ± 15.8* | 39.8 ± 2.9* | Acyl-CoA ($C_{8:0}$) | 13.8 ± 1.6 | 6.0 ± 1.3 | 3.6 ± 0.6* | 1.9 ± 0.6* | 1.2 ± 0.4* |

**(b) β-Oxidation of fatty acids (nmol/mg protein)**

| | Salicylic acid (mM) | | | | | Salicylic acid (mM) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 10 | 20 | 50 | | Control | 0.5 | 1.0 | 10 | 50 |
| Acyl-CoA ($C_{16:0}$) | 255.0 ± 13.0 | 268.0 ± 14.5 | 280.8 ± 14.0 | 320.0 ± 12.2* | Acyl-CoA ($C_{8:0}$) | 79.7 ± 6.4 | 69.0 ± 2.6 | 65.0 ± 3.3 | 58.1 ± 4.7* | 40.3 ± 3.2* |
| ($C_{14:0}$) | 117.5 ± 12.5 | 95.7 ± 10.6 | 72.5 ± 14.1* | 40.3 ± 7.0* | ($C_{6:0}$) | 23.8 ± 4.4 | 12.6 ± 3.5 | 10.4 ± 2.5 | 9.5 ± 2.8* | 5.8 ± 1.5* |
| ($C_{12:0}$) | 90.5 ± 9.5 | 66.3 ± 7.4 | 40.0 ± 3.6* | 10.5 ± 2.7* | ($C_{2:0}$) | 16.7 ± 2.5 | 14.5 ± 2.0 | 12.3 ± 1.5 | 9.5 ± 1.0* | 3.5 ± 0.5* |
| ($C_{10:0}$) | 49.5 ± 6.9 | 20.0 ± 4.5 | 12.8 ± 3.3* | 6.6 ± 1.5* | | | | | | |
| ($C_{2:0}$) | 45.2 ± 6.6 | 17.1 ± 4.7 | 10.3 ± 2.5* | 3.5 ± 0.5* | | | | | | |

(a) Activation; (b) β-oxidation. The activity of long-chain and medium-chain acyl-CoA synthetases was determined for 1 and 5 min incubation times, respectively. The β-oxidation activity towards palmitic and octanoic acids, as in myristoyl-CoA formation from palmitoyl-CoA and hexanoyl-CoA from octanoyl-CoA, was determined for 60 min, respectively. Activity of the acyl-CoA synthetases and β-oxidation was determined using 80 μM octanoic acid or palmitic acid, respectively. The concentration of salicylic acid ranged from 0.5 mM to 50 mM. Data are means ± SD, $n = 6$. *$P < 0.05$ compared with the control group using a Kruskall–Wallis test.

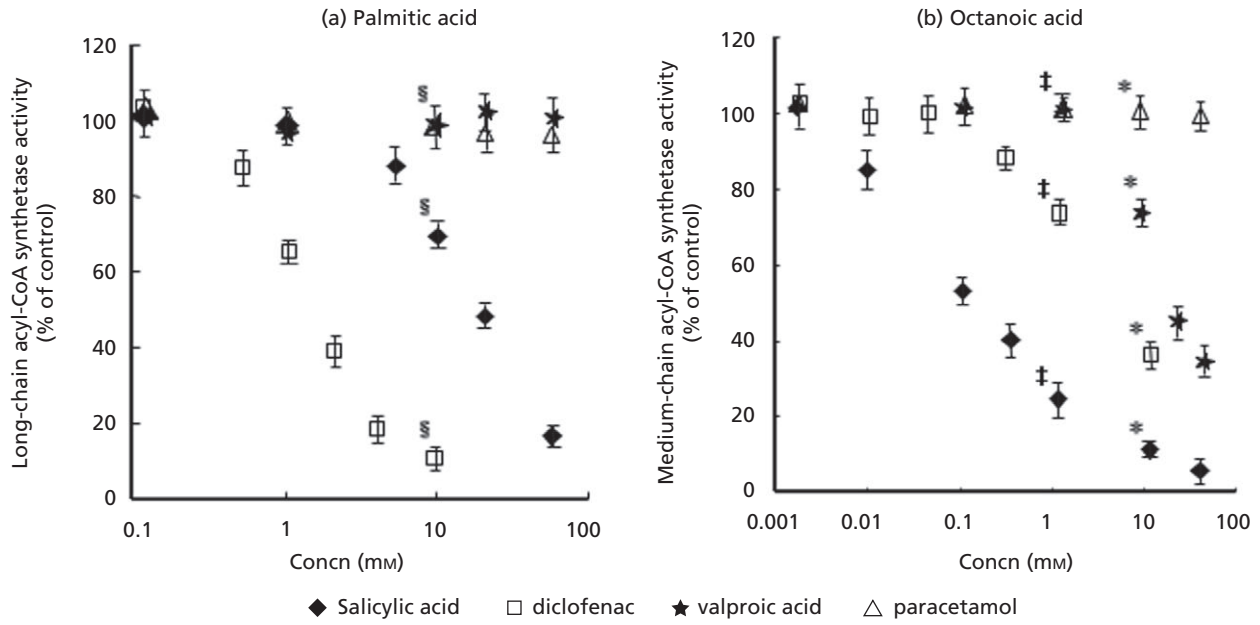


**Figure 1** Inhibition of long-chain and medium-chain acyl-CoA synthetases by salicylic acid, valproic acid, diclofenac and paracetamol. (a) Long-chain acyl-CoA synthetases; (b) medium-chain acyl-CoA synthetases. The kinetics of the inhibition was determined using 80 $\mu$M octanoic acid or palmitic acid, respectively. Salicylic acid 10 $\mu$M–50 mM; diclofenac 10 $\mu$M–10 mM; valproic acid 500 $\mu$M–50 mM; paracetamol 500 $\mu$M–50 mM. Activity has been expressed as percent of the specific activity of octanoic acid or palmitic acid in the absence of inhibitors. The specific activities for octanoic and palmitic acids were 11.4 and 201 nmol/min/mg protein, respectively. The data have been expressed as means ± SD, $n = 6$. For (a) $^{\S}P < 0.05$ between salicylic acid and diclofenac at 10 mM. For (b) $^{\ddagger}P < 0.05$ between salicylic acid and diclofenac at 1 mM; $^{\ddagger}$between diclofenac, and valproic acid and paracetamol at 1 mM. $^{*}P < 0.05$ between salicylic acid and diclofenac at 10 mM; *between valproic acid and diclofenac at 10 mM; *between valproic acid and paracetamol at 10 mM.

(corresponding to a few nmol/mg protein) were much less than acetyl-CoA and long-chain acyl-CoAs ($C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$). This finding showed that palmitoyl-CoA formed from palmitic acid was further metabolized via $\beta$-oxidation to acetyl-CoA. In the case of the $\beta$-oxidation of octanoic acid, the formation of hexanoyl-CoA from octanoyl-CoA could be fully detected. When the effect of salicylic acid on metabolism of palmitic and octanoic acids was examined, the acyl-CoA synthetase activity toward 80 $\mu$M palmitic and octanoic acid was significantly inhibited by salicylate concentration-dependently, respectively (Table 1a). In the $\beta$-oxidation step, the level of palmitoyl-CoA was increased with increases in salicylate concentration, whereas the formation of myristoyl-CoA ($C_{14:0}$) was significantly decreased (Table 1b). This finding indicated that salicylic acid inhibited the enzymes involved in the $\beta$-oxidation of long-chain fatty acids, followed by accumulation of palmitoyl-CoA. A different tendency on the inhibitory activity of salicylic acid for the $\beta$-oxidation was shown in the case of palmitic and octanoic acids. The slight decrease of octanoyl-CoA concentration in the $\beta$-oxidation step may have result in reflecting a strong inhibition over the activation of octanoic acid by salicylic acid (Table 1b).

Inhibition of long- and medium-chain acyl-CoA synthetases by salicylic acid, valproic acid, diclofenac and paracetamol is shown in Figure 1. Salicylic acid had an inhibitory activity (IC50 = 25.5 mM) for the formation of palmitoyl-CoA from palmitic acid (Table 1a and Figure 1a). In contrast, salicylic acid was a strong inhibitor of medium-chain acyl-CoA synthetases toward 80 $\mu$M octanoic acid with an IC50 value of 0.12 mM (Table 1a and Figure 1b).

Figure 2 shows the inhibition of the $\beta$-oxidation of octanoic and palmitic acids by salicylic acid, valproic acid, diclofenac and paracetamol. A similar tendency on the inhibitory activity for the $\beta$-oxidation was observed in the case of valproic and salicylic acids. Palmitoyl-CoA formation was increased with increases in valproate concentration, whereas the formation of myristoyl-CoA ($C_{14:0}$) was decreased. Valproic acid did not inhibit the activation but the $\beta$-oxidation of palmitic acid (figure 1a and 2a). However, valproic acid inhibited both the activation (IC50 = 11.1 mM) and $\beta$-oxidation of octanoic acid (figure 1b and 2b).

No information is available about the effect of diclofenac on fatty acid metabolism. Diclofenac showed a weak inhibitory activity (IC50 = 4.4 mM) for the formation of octanoyl-CoA from octanoic acid (figure 1b), whereas it inhibited the formation of palmitoyl-CoA with an IC50 value of 1.4 mM (figure 1a). In the case of $\beta$-oxidation, diclofenac had no inhibitory ability for the $\beta$-oxidation of octanoyl-CoA, whereas it inhibited that of palmitoyl-CoA (IC50 = 7.4 mM) (Figure 2a, b).

However, paracetamol had no or little effect on either the activation or $\beta$-oxidation of octanoic and palmitic acids (Figure 1 and 2).

## Discussion

The acyl-CoA synthetases produce the acyl-CoAs, which are the important intermediates for the degradation of fatty acids via $\beta$-oxidation. The mitochondrial $\beta$-oxidation resulted in degradation of acyl-CoAs by two carbon units per $\beta$-oxidation

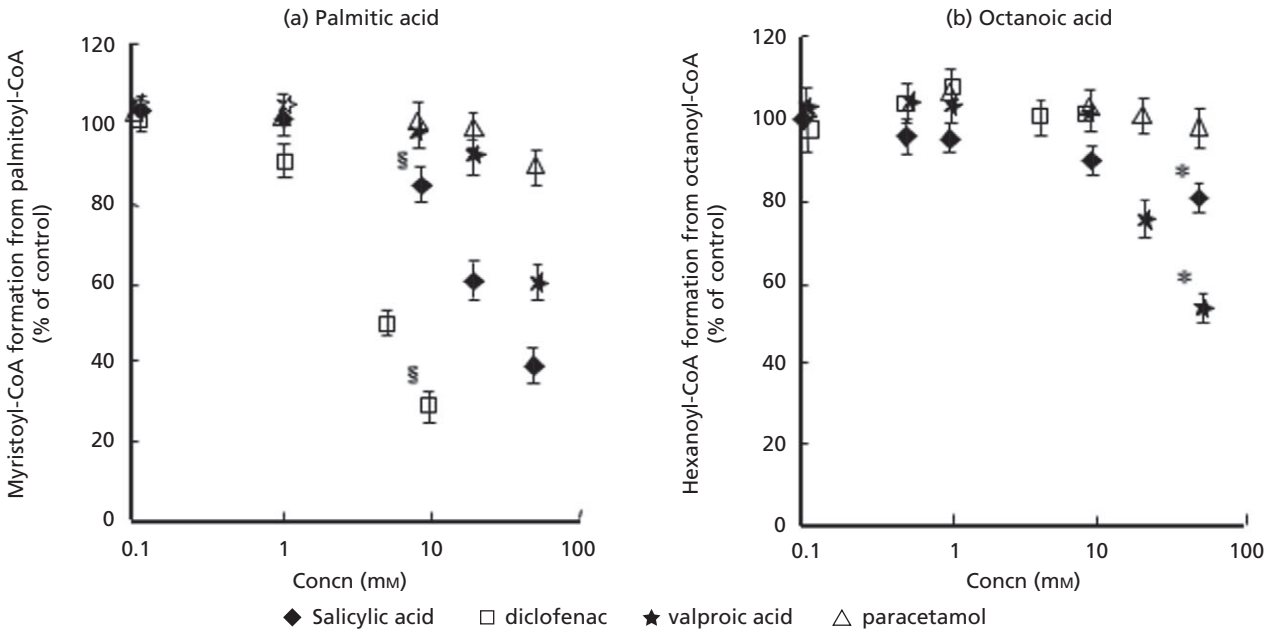


**Figure 2** Inhibition of the β-oxidation of octanoic and palmitic acids by salicylic acid, valproic acid, diclofenac and paracetamol. The kinetics of the inhibition was determined using 80 μM octanoic acid or palmitic acid, respectively. The concentrations of salicylic acid and diclofenac ranged from 10 μM to 50 mM and 10 μM to 10 mM, respectively. Valproic acid and paracetamol ranged from 500 μM to 50 mM, respectively. Activity has been expressed as percent of the specific activity of octanoic acid or palmitic acid in the absence of inhibitors. The specific activities for octanoic and palmitic acids were 70 and 255 nmol/mg protein, respectively. The data have been expressed as means ± SD, $n = 6$. For (a) $^{\S}P < 0.05$ between salicylic acid and diclofenac at 10 mM. For (b) $^{*}P < 0.05$ between salicylic acid and valproic acid at 50 mM.

cycle. As a consequence, the levels of acyl-CoAs in liver mitochondria reflected the activity of the acyl-CoA synthetases and some enzymes in the β-oxidation cycle.

Firstly, octanoic and palmitic acids were used as the substrates and the activity of medium- and long-chain acyl-CoA synthetases was determined based on the formation of octanoyl-CoA and palmitoyl-CoA, respectively. Almost no β-oxidation of palmitic acid and octanoic acid was observed for the incubation times of 1 and 5 min, respectively.

Secondly, the β-oxidation activity was determined based on chain shortening of the acyl-CoA by the oxidative removal of two carbon units. The addition of FAD, $NAD^+$ and carnitine allowed us to proceed with the β-oxidation of palmitic and octanoic acids for a 60-min incubation time. The main acyl-CoA esters of $C_{2:0}$, $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$ in mouse liver mitochondria were detected by LC-ESI-MS/MS. Eight acyl-CoA esters were well separated and eluted in the order of acyl-CoAs of $C_{2:0}$, $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$. In palmitic acid metabolism, the abundance of the product ion of each acyl-CoA of $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$ was much less than that of $C_{12:0}$, $C_{14:0}$ and $C_{16:0}$. However, the abundance of the product ion of acetyl-CoA ($C_{2:0}$) was considerably higher than that of $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$. This finding showed that palmitoyl-CoA formed from palmitic acid was further metabolized via β-oxidation to acetyl-CoA. The amounts of medium-chain acyl-CoAs ($C_{4:0}$, $C_{6:0}$ and $C_{8:0}$) (corresponding to a few nmol/mg protein) were very low (Table 1). This was consistent with the observation that the levels of medium-chain acyl-CoAs extracted from mouse liver and brain were less than the long-chain acyl-CoAs.[13,21] The brain especially contained only a total of 91 pmol medium-chain acyl-CoAs ($C_6$, $C_8$ and $C_{10}$)/g wet tissue (0.6% of the amounts of long-chain acyl-CoAs found in brain).[13] The detection limits of (S/N = 3) of LC-ESI-MS/MS were 98 fmol for palmitoyl-CoA and 105 fmol for octanoyl-CoA in multireaction monitoring mode, respectively.

The present enzyme assay was applied to examine the pathways by which the activation and the β-oxidation of fatty acids were influenced by drugs. To elucidate the mechanism by which the drugs cause the perturbation in fatty acid metabolism, salicylic acid, diclofenac, valproic acid and paracetamol were tested for their inhibitory effects on fatty acid metabolism in mouse liver mitochondria.

Salicylic acid was a strong inhibitor of the liver mitochondrial medium-chain acyl-CoA synthetases (IC50 = 0.12 mM), whereas it was a weak inhibitor of the activation (IC50 = 25.5 mM) and β-oxidation of long-chain fatty acids. Depending on the length of the acyl chain of thefatty acid, salicylic acid exhibited strong inhibition of the activation of medium-chain fatty acids and decreased the mitochondrial activation and β-oxidation of long-chain fatty acids, through mechanisms that involved the sequestration of extramitochondrial coenzyme A (CoA) and carnitine.[13,17]. However, there were no reports concerning the effect of salicylic acid on the β-oxidation of medium-chain fatty acids. This study indicated that the extent to which salicylic acid exerted an influence over the enzymes involved in the β-oxidation of octanoic acid remains unclear. Medium-chain acyl-CoA synthetase activity activating salicylate in liver of man and mouse is very low, suggesting that salicylic acid may directly inhibit the

activation and β-oxidation of fatty acids rather than coenzyme A sequestration in liver mitochondria.[13,22,23] Aspirin and salicylate have been identified as being associated with the development of Reye's syndrome. In particular, higher levels of octanoic acid were observed in plasma and urine of patients with Reye's syndrome.[24] Salicylic acid was a strong inhibitor of the liver mitochondrial medium-chain acyl-CoA synthetases (IC50 = 0.12 mM), whereas it was a weak inhibitor of the activation (IC50 = 25.5 mM) and β-oxidation of long-chain fatty acids. Although long-chain acyl-CoA dehydrogenase deficiency causes a Reye's syndrome-like disease, the most commonly diagnosed metabolic disorder associated with a Reye's syndrome-like disease has been medium-chain acyl-CoA dehydrogenase deficiency.[25] Therefore, it has been speculated that the disturbances in the metabolism of medium-chain fatty acids may play a particularly important role in the pathology of Reye's syndrome.

Valproic acid inhibited the activation of only the medium-chain fatty acids (IC50 = 11.1 mM), whereas it had very weak inhibitory activity for the β-oxidation of medium-chain and long-chain fatty acids. Silva *et al*[19] reported that the effect of 1 mM valproic acid on the overall β-oxidation rate (including the activation and β-oxidation) of palmitic acid was pronounced. Our finding was consistent with the fact that 5 mM valproic acid inhibited various purified acyl-CoA dehydrogenases onlyvery weakly.[18] Results reported previously had been highly variable.

No information is available about the effect of diclofenac on fatty acid metabolism. We reported previously that diclofenac showed a weak inhibitory activity (IC50 = 4.4 mM) for the liver mitochondrial formation of hexanoyl-CoA from hexanoic acid, whereas it inhibited long-chain acyl-CoA synthetases with an IC50 value of 1.4 mM.[13] This study indicated that diclofenac inhibited only the β-oxidation of long-chain fatty acids (IC50 = 7.4 mM).

However, paracetamol showed hardly any influence on the metabolism of medium-chain and long-chain fatty acids. Chi *et al*.[26] reported that accumulation of long-chain acylcarnitines as well as triglycerides and free fatty acids was observed with fasting mice treated with a paracetamol overdose of 400 mg/kg. A paracetamol overdose of 400 mg/kg can cause irreversible inhibition of fatty acid oxidation when glucose metabolism is blocked or suppressed, such as occurs during fasting. Therefore, paracetamol may have little influence on fatty acid metabolism in therapeutic dosage. This finding may be the reason that paracetamol is recommended as an antipyretic drug for influenza A or B.

The significant differences between the mean values of individual groups treated with each drug at 1, 10 or 50 mM were determined by the Mann–Whitney test and were confirmed with $P < 0.05$. Order of inhibitory activity depended on the length of the acyl chain as follows: salicylic acid > diclofenac > valproic acid > paracetamol for the activation of medium-chain fatty acids; valproic acid > salicylic acid, diclofenac, paracetamol for the β-oxidation of medium-chain fatty acids; diclofenac > salicylic acid, valproic acid, paracetamol for the activation and β-oxidation of long-chain fatty acids.

There have been many reports suggesting that the pathology of Reye's syndrome and the influenza-associated acute encephalopathy might be associated with the inhibition of many mitochondrial enzymes involved in metabolism of fatty acids and branched-chain amino acids.[6-8,27] On the other hand, dynamic intracellular changes of acyl-CoAs may contribute to the production of the different varieties of membrane phospholipids, which results in changes in membrane fluidity and ion permeability. These alterations have been reported to occur in neurological disorders also.[1–3]. It is important to elucidate the mechanism linking drugs, disease and fatty acid metabolism.

Cultured fibroblasts and leucocytes obtained from patients were used for the diagnosis of genetic deficiency of medium-chain acyl-CoA dehydrogenase.[20,28,29] Although the isolated hepatocytes were frequently used for the in-vitro fatty acid metabolism, liver mitochondria were useful for the present assay. To elucidate the mechanism by which the drugs caused the perturbation in fatty acid metabolism, the assay was useful for estimating whether the drugs influenced the activation or the β-oxidation of fatty acids.

## Conclusions

Salicylic acid, diclofenac, valproic acid and paracetamol exerted different influences on the activation and β-oxidation of fatty acids depending on the length of the acyl chain. This assay allowed sensitive and selective analysis for predicting the pathways of which the drugs exerted a greater influence over fatty acid metabolism.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

### Funding

This research was supported by Grant in Aid for ″Academic Frontier″ Project from MEXT(2006–2010).

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