0031-3998/88/2303-0338$02.00/0
PEDIATRIC RESEARCH

Vol. 23, No. 3, 1988
*Printed in U.S.A.*

# Effect of Salicylic Acid on Mitochondrial-Peroxisomal Fatty Acid Catabolism[1]

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**ABSTRACT. To understand the possible role of salicylic acid in the pathogenesis of Reye's syndrome, we examined its effect on the oxidative metabolism of fatty acids in the rat liver mitochondrial-peroxisomal fraction. Fatty acids of different chain lengths are oxidized in different organelles. Octanoic acid is oxidized in mitochondria, lignoceric acid in peroxisomes, and palmitic acid in both mitochondria and peroxisomes. Salicylic acid (up to 1 mM concentration) had no effect on the oxidation of [1-$^{14}$C]lignoceric acid. However, at the same concentration it inhibited the oxidation of [1-$^{14}$C]palmitic acid by 26% and [1-$^{14}$C]octanoic acid by 42%. The apparent Ki for the oxidation of [1-$^{14}$C] octanoic acid, [1-$^{14}$C]palmitic acid and [1-$^{14}$C]lignoceric acid were 0.27, 6.0, and 14.8 mM, respectively. This selective inhibition of mitochondrial oxidation of medium-chain (octanoic acid) and long-chain (palmitic acid) fatty acids by salicylic acid may potentiate the accumulation of fatty acids in plasma in Reye's syndrome patients. (*Pediatr Res* 23: 338–341, 1988)**

**Abbreviations**

**RS, Reye's syndrome**
**CoASH, coenzyme A**
**FAD, flavin adenine dinucleotide**
**NAD, nicotinamide adenine dinucleotide**
**MOPS, 2-(N-morpholino)-2hydroxypropane sulfonic acid**
**Km, Michaelis constant**
**Ki, Inhibiton constant**
**Vmax, maximum velocity**

RS, a serious disease of infants and children, is a metabolic disorder characterized by mitochondrial injury (1–3). Interactions between viral illness and environmental agents (pesticides, solvents, and drugs) have been purported to be possible etiologic factors in the development of RS (4–10). Because of the high levels of salicylic acid in the plasma of many RS patients, the role of aspirin in the RS disease process has received particular attention (11–14). Mitochondrial defects and an accumulation of free fatty acids (15–18) and dicarboxylic acids (19, 20) in RS suggest an abnormality in fatty acid metabolism. Herein we report the effect of salicylic acid on mitochondrial-peroxisomal fatty acid catabolism.

Received August 17, 1987; accepted November 17, 1987.
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Supported by grants from the National Reye's Syndrome Foundation and National Institutes of Health Grant NS 22576.
[1]Presented in part at the Annual Meeting of the Society for Pediatric Research, Anaheim, CA (Pediatr Res 350A, 1987).

## MATERIALS AND METHODS

[1-$^{14}$C]Labeled palmitic acid (58.0 mCi/mmol), [1-$^{14}$C]octanoic acid (53.5 mCi/mmol), and K[$^{14}$CN] (56.0 mCi/mmol) were purchased from New England Nuclear, Boston, MA. [1-$^{14}$C]lignoceric acid was synthesized from K[$^{14}$CN] and tricosanoyl bromide (21). Carnitine, ATP, CoASH, FAD, and $\alpha$-cyclodextrin were obtained from PL-Biochemicals, Milwaukee, WI.

*Preparation of mitochondrial-peroxisomal fractions.* Livers were removed from male rats (Sprague-Dawley) fasted overnight, washed with cold saline solution, and homogenized in 10 volumes (w/v) of buffer containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris HCl, pH 7.3. The mitochondrial-peroxisomal fraction was prepared by differential centrifugation between 8,000 × *g* min and 250,000 × *g* min and washed with 0.25 M sucrose solution. Specific activity of marker enzymes for mitochondria (cytochrome c oxidase), peroxisomes (catalase), and microsomes (NADPH-cytochrome c reductase) in this fraction were 20.8, 1.35, and 1.0 mU/mg protein/min, respectively.

*Fatty acid oxidation to acetate (water-soluble products).* The oxidation of lignoceric acid was measured according to procedures previously described (22). The reaction mixture, total volume 0.5 ml, contained [1-$^{14}$C]lignoceric acid coated on celite, 20 mM MOPS-HCl buffer, pH 7.8, 30 mM KCl, 1 mM $MgCl_2$, 10 mM ATP, 0.25 mM NAD, 0.17 mM FAD, 2.5 mM L-carnitine, 0.08 mM CoASH, and 2 mg of $\alpha$-cyclodextrin. Salicylic acid was added as indicated with each experiment. The reaction mixture was preincubated for 10 min and then the reaction was started by the addition of 20–300 $\mu$g of the mitochondrial-peroxisomal fraction and stopped with 0.5 ml of ice cold 0.6 M perchloric acid. After centrifugation the supernatant was transferred to another tube, partitioned by the procedure of Folch-Pi *et al.* (23), and the radioactivity in the upper layer was measured. The assay conditions for oxidation of [1-$^{14}$C]palmitic acid were the same as those used for lignoceric acid, but the reaction was stopped by the addition of 1.25 ml of 1 N KOH. The reaction mixture was allowed to stand for 1 h at room temperature and then centrifuged at 3000 × *g* for 10 min. The supernatant was transferred to another set of tubes and 0.4 ml of 3 N HCl was added to 1.25 ml of the reaction supernatant and partitioned by the procedure of Folch-Pi *et al.* (23). The upper layer was measured for radioactivity. For the oxidation of [1-$^{14}$C]octanoic acid, octanoic acid was added to the incubation mixture without using celite. The assay conditions were the same as for palmitic acid except that the upper Folch partition was washed once with organic solvents composed of the lower layer of the Folch partition and radioactivity was measured in the aqueous layer. The amount of octanoic, palmitic, and lignoceric acids in the assay mixture were 20.0, 6.0, and 2.4 nmol, respectively.

*Assay for Acyl-CoA ligases.* The assays for palmitoyl-CoA and lignoceroyl-CoA ligase activities were carried out as described previously (24). The reaction mixture of 0.5 ml contained 6.0

nmol of [1-$^{14}$C]palmitic acid or 2.4 nmol of [1-$^{14}$C]lignoceric acid coated on celite, 10 mM ATP, 80 $\mu$M CoASH, 30 mM KCl, 5 mM $MgCl_2$, and 2 mg of $\alpha$-cyclodextrin. The pH of the MOPS-HCl buffer of the assay system for palmitoyl-CoA ligase was 7.8 and for lignoceroyl-CoA ligase 8.3. The assay conditions for octanoyl-CoA ligase activity was the same as described above, except the reaction was stopped by the addition of 4.0 ml of chloroform-methanol-heptane (1.25:1.41:1.00). Then 0.75 ml of 0.3 M sodium acetate (pH 4.0) was added, mixed, and centrifuged. The upper layer was washed once with chloroform-heptane (1.25:1.00) and 1.0 ml of upper layer was measured for radioactivity. The proteins were measured according to the procedure of Lowry *et al.* (25).

## RESULTS

Addition of salicylic acid inhibited both the activation of octanoic acid to octanoyl-CoA as well as oxidation of octanoic acid to acetyl-CoA (Table 1). At a concentration of 300 $\mu$M, salicylic acid inhibited the oxidation and activation of octanoic acid to 66 and 38% of the control, respectively. Similarly, salicylic acid inhibited the oxidation and activation of palmitic acid, but to a lesser degree (Table 2). The parallel inhibition of activation and oxidation of both octanoic (Table 1) and palmitic acids (Table 2) suggests that the enzymatic step(s) for activation, as well as oxidation of these fatty acids, are sensitive to inhibition by salicylic acid. As shown in Table 3, salicylic acid had apparently no effect on either the activation of lignoceric acid to lignoceroyl-CoA or on the oxidation of lignoceric acid (Table 3).

The kinetic parameters for activation and oxidation of different chain length fatty acids were also examined. The apparent Km for activation of octanoic, palmitic, and lignoceric acids were 3.2, 2.0, and 2.6 $\mu$M and the apparent Vmax was 5.4, 31.5 and 9.5 $\times$ $10^{-2}$ nmol/mg protein/min, respectively (Table 4). The apparent Ki for activation of octanoic, palmitic, and lignoceric acids was 0.13, 16.0, and 13.0 mM, respectively. The apparent Km for oxidation of octanoic, palmitic, and lignoceric acids was 1.7, 4.0, and 1.64 $\mu$M and apparent Vmax was 2.2, 1.08, and 8.16 $\times$ $10^{-3}$ nmol/mg protein/min, respectively (Table 5). The apparent Ki for octanoic, palmitic, and lignoceric acids was 0.27, 6.0, and 14.8 mM, respectively. Salicylic acid inhibition of octanoic acid oxidation was competitive whereas palmitic acid and lignoceric acid inhibition was noncompetitive and uncompetitive, respectively.

Table 1. *Effect of salicylic acid on catabolism of [1-$^{14}$C]octanoic acid**

| Salicylic acid (mM) | Rate of formation of octanoyl-CoA | Rate of oxidation of octanoic acid |
|---|---|---|
| 0.00 | 6.2 ± 0.62 (5) nmol/mg protein/min 100% | 2.9 ± 0.12 (3) nmol/mg protein/min 100% |
| 0.05 | 60 ± 2 (5) | 102 ± 5 |
| 0.15 | 47 ± 1 (3) | 98 ± 4 |
| 0.30 | 34 ± 1 (3) | 62 ± 12 |
| 0.50 | 28 ± 2 (3) | |
| 1.00 | | 58 ± 6 |

* Results are expressed as the mean of ($n$) experiments ± SEM.

Table 2. *Effect of salicylic acid on catabolism of [1-$^{14}$C]palmitic acid**

| Salicylic acid (mm) | Rate of formation of palmitic-CoA | Rate of oxidation of palmitic acid |
|---|---|---|
| 0.00 | 69.7 ± 4.7 (3) nmol/mg protein/min 100% | 1.41 ± 0.08 (4) nmol/mg protein/min 100% |
| 0.10 | 97 ± 2 (3) | 80 ± 4 (4) |
| 0.30 | 90 ± 6 (3) | 77 ± 4 (4) |
| 0.60 | 88 ± 13 (3) | 70 ± 6 (4) |
| 1.0 | 77 ± 4 (3) | 74 ± 9 (4) |

* Results are expressed as the mean ($n$) experiments ± SEM.

Table 3. *Effect of salicylic acid on catabolism of [1-$^{14}$C] lignoceric acid**

| Salicylic acid (mM) | Rate of formation of lignoceroyl-CoA | Rate of oxidation of lignoceric acid |
|---|---|---|
| 0.00 | 1.24 ± 0.2 $\times$ $10^{-1}$ (3) nmol/mg protein/min 100% | 5.10 ± 0.5 $\times$ $10^{-3}$ (5) nmol/mg protein/min 100% |
| 0.10 | 104 ± 6 (3) | 104 ± 2 (3) |
| 0.15 | | |
| 0.30 | 103 ± 1 (3) | 112 ± 2 (3) |
| 0.60 | 91 ± 1 (3) | 112 ± 4 (3) |
| 1.00 | 91 ± 6 (7) | 117 ± 2 (3) |

* Results are expressed as the mean of ($n$) experiments ± SEM.

## DISCUSSION

We have recently observed deficient oxidation of octanoic acid in a homogenate of leukocytes from a RS patient with a high serum level (22.2 mg/dl) of salicylic acid (26). The strong association between ingestion of salicylate during the antecedent viral illness and the onset of RS, and the accumulation of fatty acids in RS (15–18), prompted us to examine the effect of salicylic acid on fatty acid metabolism. The salicylate concentrations used herein are similar to the serum levels observed in RS patients (11–14).

Activation of fatty acids is the initial and obligatory step in their degradation. At the subcellular level acyl-CoA ligases, enzymes for activation of fatty acids, are localized in mitochondria, peroxisomes, and microsomes (27–30). Salicylic acid inhibited the activation of octanoic acid more than the activation of palmitic acid and it had no effect on the activation of lignoceric acid. Mitochondria contain various acyl-CoA ligases for different chain length fatty acids and they are localized both in the outer as well as the inner matrix membrane (27). Fatty acids are converted to CoA-derivatives by acyl-CoA ligase present in the outer membrane, which in turn are converted to acyl-carnitine derivatives by acyl-carnitine transferase and are transported through the mitochondrial wall. These acyl-carnitine derivatives inside the mitochondria are again converted to acyl-CoA derivatives by inner matrix acyl-CoA ligases. The effect of salicylic acid on the microsomal acyl-CoA ligases was also examined, but the effect was not as great as that observed with the mitochondrial-peroxisomal fraction. Salicylic acid (0.3 mM) inhibited the microsomal octanoyl-CoA ligase activity by only 20%. This differential effect suggests the presence of different acyl-CoA ligases for the activation of lignoceric, palmitic, and octanoic acids.

The acyl-CoA derivatives are catabolized primarily by $\beta$-oxidation enzyme systems in mitochondria and peroxisomes (31, 32). Although both $\beta$-oxidation systems are functionally similar with respect to degradation of fatty acids by two carbon units per cycle, they are structurally different (32). Very long-chain fatty acids (>$C_{22}$) are degraded predominantly in peroxisomes and short- and medium-chain fatty acids (<$C_{12}$) in mitochondria. Long-chain ($C_{12-20}$) fatty acids are oxidized both in mitochondria as well as in peroxisomes.

Herein we demonstrate that salicylic acid has no effect on the *in vitro* enzyme assay for the activation and oxidation of lignoceric acid, thus suggesting that the peroxisomal fatty acid $\beta$-oxidation system is not inhibited by salicylic acid. However, the inhibition of octanoic acid and palmitic acid oxidation suggests that salicylic acid does inhibit the mitochondrial fatty acid oxidation system. Moreover, the enzyme system for the oxidation

Table 4. *Effect of salicylic acid on kinetic parameters of different chain length fatty acid activation*

| | Apparent Km ($\mu$m) | Apparent Vmax (nmol/mg protein/ min) | Apparent Ki (mM) |
|---|---|---|---|
| [1-$^{14}$C]octanoic acid | 3.2 | 5.4 | 0.13 |
| [1-$^{14}$C]palmitic acid | 2.0 | 31.5 | 16.0 |
| [1-$^{14}$C]lignoceric acid | 2.6 | $9.5 \times 10^{-2}$ | 13.0 |

Table 5. *Effect of salicylic acid on kinetic parameters of different chain length fatty acid oxidation*

| | Apparent Km ($\mu$m) | Apparent Vmax (nmol/mg protein/ min) | Apparent Ki (mM) |
|---|---|---|---|
| [1-$^{14}$C]octanoic acid | 1.7 | 2.20 | 0.27 |
| [1-$^{14}$C]palmitic acid | 4.0 | 1.08 | 6.0 |
| [1-$^{14}$C]lignoceric acid | 1.64 | $8.16 \times 10^{-3}$ | 14.8 |

of medium-chain fatty acids (octanoic acid) is relatively more sensitive to salicylic acid inhibition. Salicylic acid is known to cause uncoupling of mitochondrial oxidative phosphorylation activity; however, the observed inhibition of octanoic acid oxidation by salicylic acid is not due to the ATP deficiency because enzyme activity was measured in the presence of 10 mM of exogenously added ATP. These studies clearly demonstrate that salicylic acid inhibits mitochondrial fatty acid oxidation but has no effect on peroxisomal fatty acid oxidation.

A genetic disorder, medium-chain acyl-CoA dehydrogenase deficiency, also manifests symptoms similar to RS, thus suggesting that some of the cases previously reported as RS may, in fact, be disorders of medium-chain acyl-CoA dehydrogenase deficiency (34–36). Although salicylic acid inhibition of octanoic acid oxidation in *in vitro* described herein represents a situation similar to medium-chain acyl-CoA dehydrogenase deficiency, salicylic acid may not be the primary cause of illness in RS. It may rather play a role in pathogenesis of RS by potentiation of the cellular toxicity. Salicylic acid inhibits mitochondrial functions (*e.g.* fatty acid $\beta$-oxidation and uncoupling of oxidative phosphorylation) and it stimulates the $\omega$-hydroxylation of fatty acids produced by lipolysis. These $\omega$-hydroxy fatty acids are converted to dicarboxylic acids by cytosolic alcohol and aldehyde dehydrogenases (37, 38), and dicarboxylic acids have been found to be toxic to mitochondrial structure and function (20). The cumulative effects of excessive amounts of free fatty acids, dicarboxylic acids, and lysophospholipids may perturb the membrane properties and metabolic capability of mitochondria.

*Acknowledgments.* The authors thank Mr. Glenn Goudy for technical help and Ms. Louise Richter for typing the manuscript.

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