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]

Y. YOSHIDA, M. FUJII, F. R. BROWN III, AND I. SINGH

Departments of Pediatrics and Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425

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.
Correspondence and reprint requests Inderjit Singh, Ph.D., Professor of Pediatrics and Cell Biology, Medical University of South Carolina, Charleston, SC 29425.
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 α-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 α-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 μg 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 μM CoASH, 30 mM KCl, 5 mM $MgCl_2$, and 2 mg of α-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 μM, 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 μM 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 μM 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 β-oxidation enzyme systems in mitochondria and peroxisomes (31, 32). Although both β-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 β-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 (μm)	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	9.5×10^{-2}	13.0

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

	Apparent Km (μm)	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	8.16×10^{-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 β-oxidation and uncoupling of oxidative phosphorylation) and it stimulates the ω-hydroxylation of fatty acids produced by lipolysis. These ω-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.

REFERENCES

1. Reye RDK, Morgan G, Baral J 1963 Encephalopathy and fatty degeneration of the viscera: a disease entity of childhood. Lancet 2:749–752
2. DeDivo DC 1978 Reye's syndrome: a metabolic response to an acute mitochondrial insult. Neurology 28:105–108
3. Brown RE, Foeman DT 1982 The biochemistry of Reye's syndrome. CRC Crit Rev Clin Lab Sci 17:247–257
4. Partin JC, Shubert WK, Partin JS, Jacobs R, Saalfeld K 1976 Isolation of influenza virus from liver and muscle biopsy specimens from a surviving case of Reye's syndrome. Lancet 2:599–602
5. Pollack JD 1976 Models of chemical and viral interaction and their relation to a multiple etiology of Reye's syndrome. Lancet 2:599–602
6. Safe S, Hutzinger O, Crocker JFS 1979 The role of chemicals in Reye's syndrome. In: Pollack JD (ed) Reyes Syndrome II. Grune and Stratton, New York, pp 281–309
7. Waldman RJ, Hall WH, McGee H, Van Amburg G 1982 Aspirin as a risk factor in Reye's syndrome. JAMA 247:3089–3094
8. Halpin TJ, Holtzhaver FJ, Cambell RJ, Hall LJ, Correa-Villasenor A, Lanese R, Rice J, Hurwitz ES 1982 Reye's syndrome and medication use. JAMA 248:687–691
9. Starko KM, Ray CG, Dominguez LB, Stromberg WL, Woodall DL 1980 Reye's syndrome and salicylate use. Pediatrics 66:859–864
10. Hurwitz ES, Barrett MJ, Bregman D, Gunn WJ, Pinsky P, Schomberger LB, Draga JS, Kaslow RA, Burlington B, Quinnan GV, La Montagne JR, Fairweather WR, Dayton D, Dowdle WR Public Health Service study of Reye's syndrome and medication. JAMA 257:1905–1911
11. Partin JS, Partin JC, Shubert WK, Hammond JA 1982 Serum salicylate concentration in Reye's syndrome. Lancet 1:191–192
12. Kang ES, Todd TA, Capaci MT, Schwenzer K, Jabbour JT 1983 Measurements of true salicylate concentration in serum from patients with Reye's syndrome. Clin Chem 29:1012–1014
13. Tousgaard JH, Huttenlocher PR 1981 Salicylates and Reye's syndrome. Pediatrics 68:747–748
14. Daugherty CC, McAdams AJ, Partin JS 1983 Aspirin and Reye's syndrome. Lancet 2:104
15. Mamunes P, DeVries GH, Miller CD, David RB 1979 Fatty acid quantitation in Reye's syndrome. In: Pollack JD (ed) Reye's Syndrome II. Grune and Stratton, New York, pp 217–235
16. Trauner D, Sweetman L, Holm J, Kulovich S, Nyhan W 1977 Biochemical correlates of illness and recovery in Reye's syndrome. Ann Neurol 2:238–241
17. Ogburn PL, Sharp H, Lloyd-Still JD, Johnson SB, Holman RT 1982 Abnormal polyunsaturated fatty acid patterns of serum lipids in Reye's syndrome. Proc Natl Acad Sci USA 79:908–911
18. Bourgeois C, Olson L, Comer D, Evans H, Keschamras N, Cotton R, Grossman R, Smith T 1971 Encephalopathy and fatty degeneration of the viscera: clinicopathological analysis of 40 patients. Am J Clin Pathol 56:558–571
19. Rochiccioli F, Cartier PH, Bougneres PF 1984 Mass spectroscopic identification of abnormal aromatic compounds in the urine of a child with Reye's syndrome. Biomed Mass Spectros 11:127–131
20. Tousgaard JH, Getz GS 1985 Effect of Reye's syndrome serum on isolated chinchila liver mitochondria. J Clin Invest 76:816–825
21. Hoshi M, Kishimoto Y 1973 Synthesis of cerebronic acid from lignoceric acid by rat brain preparation: Some properties and distribution of the α-hydroxylation system. J Biol Chem 218:4123–4130
22. Singh I, Moser AE, Goldfischer S, Moser HW 1984 Lignoceric acid is oxidized in peroxisomes: implications for the Zellweger's cerebro-hepato-renal syndrome and adrenoleukodystrophy. Proc Natl Acad Sci USA 81:4203–4207
23. Folch-Pi, J, Lees M, Sloane-Stanley GH 1957 A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
24. Singh I, Singh RP, Bhushan A, Singh AK 1987 Lignoceroyl-CoA ligase activity in rat brain microsomal functions: Topographical localization and effect of detergents and α-cyclodextrin. Arch Biochem 236:418–426
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
26. Yoshida Y, Singh I, Singh AK, Tecklenberg FW, Darby CP 1987 Fatty acid metabolism in Reye's syndrome. J Neurochem 48:163C
27. Groot PHE, Scholte HR, Hulsman WC 1976 Fatty acid activation: Specificity, localization, and function. Adv Lipid Res 14:75–125
28. Sindo Y, Hashimoto T 1978 Acyl-coenzyme A synthetase and fatty acid oxidation in rat liver peroxisomes. J Biochem (Tokyo) 84:1177–1181
29. Krisans SK, Mortensen RM, Lazarow PB 1980 Acyl-CoA synthetase in rat liver peroxisomes. J Biol Chem 255:9599–9607
30. Bronfman M, Inestrosa MC, Nervi FO, Leighton F 1984 Acyl-CoA synthetase and the peroxisomal enzymes of β-oxidation in human liver. Biochem J 224:709–720
31. Mannaerts GP, DeBeer L 1982 Mitochondrial and peroxisomal β-oxidation of

fatty acids in rat liver. NY Acad Sci 386:30–39
32. Hashimoto T 1982 Individual peroxisomal β-oxidation enzymes. NY Acad Sci 386:5–12
33. Aprille JR, Kelley RT, Brown CA 1980 Salicylates and mitochondrial metabolism. J Natl Reye's Syndrome Found 6:65–93
34. Roe CR, Willington DS, Maltby DA, Kinnebrew P 1986 Recognition of medium-chain acyl-CoA dehydrogenase deficiency in asymptomatic siblings of children dying of sudden infant death or Reye-like syndrome. J Pediatr 103:13–18
35. Bougneres PF, Rocchiccioli F, Klvraa S, Hadchouel M, Lalau-Keraly J, Chaussain JL, Wadman SK, Gregersen N 1985 Medium chain acyl-CoA dehydrogenase deficiency in two siblings with Reye-like syndrome. J Pediatr 106:918–921
36. Taubman B, Hale DE, Kelley RI 1987 Familial Reye-like syndrome: a presentation of medium-chain acyl-coenzyme A dehydrogenase deficiency. Pediatrics 79:382–385
37. Okita R 1986 Effect of acetylsalicylic acid on fatty acid hydroxylation. Pediatr Res 20:1221–1224
38. Bjorkhem I 1973 Omega oxidation of stearic acid in the normal starved and diabetic rat liver. Eur J Biochem 40:415–422