Mode of Action of Aspirin

EFFECT OF DIETARY ASPIRIN ON MITOCHONDRIAL PYRUVATE METABOLISM IN NORMAL AND THIAMINE-DEFICIENT RATS*

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SUMMARY

Diets containing 0.2% acetylsalicylic acid were fed to control and thiamine-deficient rats. Acetylsalicylic acid caused a significant decrease in body weight and adipose tissue weight in thiamine-deficient acetylsalicylic acid-treated but not in normal acetylsalicylic acid-treated rats. A large decrease in the total blood ketone bodies in thiamine-deficient acetylsalicylic acid-treated rats was observed. A significant decrease in the blood β-hydroxybutyrate to acetoacetate ratio in both control and thiamine-deficient rats treated with aspirin was noted.

Rat liver mitochondria from both control, thiamine-deficient, and acetylsalicylic acid fed rats were incubated in the presence of ATP, Mg++, P, pyruvate, and radioactive bicarbonate, and synthesis of dicarboxylic acids was examined. It was found that in acetylsalicylic acid-treated rats, the pyruvate utilization and CO2 incorporation and organic acids formation were greatly decreased.

Addition of octanoylcarnitine greatly stimulated CO2 incorporation into organic acids in thiamine-deficient and acetylsalicylic acid-treated rats. The percentage of pyruvate carboxylated in the presence of octanoylcarnitine was significantly increased in mitochondria from all animals.

Acetylsalicylic acid is known to inhibit gluconeogenesis (1) and amino transferase enzymes which may lead to decreased gluconeogenesis (cf. Reference 2). Administration of salicylates to diabetic, adrenalectomized, and hypophysectomized animals resulted in a decrease in blood glucose levels (3-5). A depletion of muscle and liver glycogen in normal animals given salicylates was also reported (6, 7). It has been suggested that these effects of salicylates on carbohydrate metabolism are associated with stimulation of oxygen consumption (8-10), presumably through the uncoupling of oxidative phosphorylation (11, 12).

This paper reports the investigation on the sites and possible mode of action of aspirin. Thiamine-deficient animals were used in these studies as a model system since thiamine deficiency is known to cause well defined biochemical lesions of carbohydrate metabolism (13-17).

EXPERIMENTAL PROCEDURES

Treatment of Animals—Male albino Sprague Dawley rats weighing 235 to 245 g were housed in individual stainless steel cages with wire screen bottoms and kept at 25 ± 2°. All animals were fed ad libitum and had free access to tap water. A total of 24 animals were divided into four groups of 6 rats each, and fed two diets: (a) thiamine-deficient diet1 and (b) thiamine-deficient diet supplemented with 0.2% of aspirin.

Control animals, Group A without aspirin and Group B with 0.2% aspirin in the diet, were supplemented with 60 μg of thiamine hydrochloride administered orally three times a week. This dosage was found to be adequate for maintaining normal growth when rats were fed ad libitum. The deficient animals were fed the same diets unsupplemented with thiamine (Group C, without aspirin, Group D, with 0.2% aspirin in the thiamine-deficient diet). All experiments were performed on the 32nd day of thiamine deficiency after an overnight fast.

Procedures for Isolation of Mitochondria and Metabolite Analysis—The isolation of intact liver mitochondria, incubation, processing of samples, analysis for pyruvate, malate, citrate, CO2 incorporation, acetoacetate, β-hydroxybutyrate, and mitochondrial nitrogen were performed according to the methods previously cited (18, 19). The blood glucose was analyzed by the glucose oxidase procedure (Calbiochem).

Materials—The NaH14CO3 was purchased from Tracerlab and was diluted with unlabeled KHCO3 to give a final concentration of 0.22 μCi per μmol. The specific radioactivity of the solution used was determined as previously described (20). The enzymes

Salicylates are known to inhibit gluconeogenesis (1) and amino transferase enzymes which may lead to decreased gluconeogenesis (cf. Reference 2). Administration of salicylates to diabetic, adrenalectomized, and hypophysectomized animals resulted in a decrease in blood glucose levels (3-5). A depletion of muscle and liver glycogen in normal animals given salicylates was also reported (6, 7). It has been suggested that these effects of salicylates on carbohydrate metabolism are associated with stimulation of oxygen consumption (8-10), presumably through the uncoupling of oxidative phosphorylation (11, 12).

This paper reports the investigation on the sites and possible mode of action of aspirin. Thiamine-deficient animals were used in these studies as a model system since thiamine deficiency is known to cause well defined biochemical lesions of carbohydrate metabolism (13-17).

1 Diet composition: vitamin-free casein, 18%; sucrose, 67%; corn oil, 18%; salt mixture, 6%. Salt mixture (No. 2 USP XIII) contained: calcium biphosphate, 12.95%; calcium lactate·2H2O, 2.7%; potassium phosphate (dibasic), 23.98%; sodium diphosphate·2H2O, 8.72%; sodium chloride, 4.33%. Vitamin mixture contained in grams per 100 pounds of diet: vitamin A (200,000 units per g), 4.5; vitamin D (400,000 units per g), 0.25; a-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine HCl, 1.0; calcium pantothenate, 3.0; in milligrams per 100 pounds of diet: biotin, 20; folie acid, 90; vitamin B-12, 1.35. Diet was obtained from Nutritional Biochemicals.
were purchased from Boehringer Mannheim. All other reagents were of the highest purity commercially available. L-Octanoylcarnitine was a gift of Dr. Yuzo Kawashima of Otsuka Pharmaceutical Factory, Naruto, Japan. Aspirin was a gift of Dr. R. Moe of Hoffmann-La Roche, Inc., Nutley, New Jersey.

RESULTS

Effect of Feeding Dietary Aspirin on Body Weight and Adipose Tissue Weight of Normal and Thiamine-deficient Rats—In normal animals, 0.2% aspirin in the diet had no effect on growth. On the other hand, Vaughan, Steele, and Korty (21) showed that normal animals fed 0.5% aspirin in the diet for 2 weeks significantly reduced weight gain. Animals fed deficient diet started to lose weight after the 18th day on the diet. The rate of weight loss was significantly larger (p < 0.01) in the deficient rats fed aspirin (Table I).

Adipose tissue is highly responsive to metabolic and hormonal (22) alterations of the animals. Results in Table I show that there was a significant decrease (p < 0.02) in the adipose tissue weight of thiamine-deficient rats. The decrease in adipose tissue weight in thiamine-deficient rats is consistent with the requirement for utilization of additional lipid for energy, because of impairment in carbohydrate metabolism. In deficient animals fed aspirin, the decrease in adipose tissue weight was more severe (p < 0.01). When adipose weights were expressed as grams per 100 g of body weight, a significant difference in weight among normal, deficient, and deficient fed aspirin was observed. Feeding of aspirin to normal rats had no effect on the adipose tissue weight.

Effect of Feeding Dietary Aspirin on Blood Glucose and Blood Ketone Bodies of Normal and Thiamine-deficient Rats—Results in Table II show the concentrations of blood glucose and ketone bodies of normal and deficient rats fed aspirin. A significant (p < 0.01) hypoglycemic effect of normal animals fed aspirin was observed. There were no differences in the blood glucose levels among normal, thiamine-deficient, and deficient aspirin-fed rats.

Modulations in the blood levels of acetoacetate of β-hydroxybutyrate can reflect changes in the liver intramitochondrial oxidation-reduction state of pyridine nucleotides or the rate of metabolism of ketone bodies (23). A significant increase (p < 0.01) in the acetocetate levels in normal aspirin-treated rats were observed (Table II).

The oxidation-reduction state of pyridine nucleotides in mitochondria, as represented by the β-hydroxybutyrate to acetocetate ratios, is significantly decreased (p < 0.01) in both normal (from 3.64 ± 1.54 to 1.27 ± 0.30) and deficient (from 2.40 ± 0.27 to 1.33 ± 0.35) rats treated with aspirin. In the deficient rats, the ratio of β-hydroxybutyrate to acetocetate was also significantly (p < 0.01) lower than that of normal rats (from 3.60 ± 0.54 to 2.40 ± 0.27). In deficient aspirin-treated but not in normal aspirin-treated rats, there was a highly significant decrease (from 1.430 ± 0.097 to 0.522 ± 0.009 μmoles per ml) in the total ketone bodies (p < 0.005).

Influence of Dietary Aspirin on Pyruvate Metabolism of Normal and Thiamine-deficient Rats—The incorporation of radioactive carbon dioxide into tricarboxylic acid cycle intermediates, mainly malate and citrate, in the presence of octanoylcarnitine from pyruvate, H14CO3, ATP, inorganic phosphate, and magnesium ions by intact liver mitochondria from normal, deficient, and aspirin-treated rats is shown in Table III.

The major intermediates formed by liver mitochondria are malate and citrate, in the presence of octanoylcarnitine, β-hydroxybutyrate, and acetoacetate. In normal aspirin-treated rats there was a significant (p < 0.01) decrease in utilization of pyruvate. The mitochondria from deficient rats used less than half the amount of pyruvate of normal rats. In the deficient aspirin-treated rats the utilization of pyruvate was greatly impaired (p < 0.001).

The incorporation of radioactive bicarbonate was significantly decreased (p < 0.05) in normal aspirin-treated rats. Pyruvate utilization was also decreased (p < 0.001). In the mitochondria from the deficient and deficient aspirin-treated rats, 14CO2 incorporation was significantly decreased (from 1705 ± 180 to 780 ± 48 μmoles per mg of phosphate per hour) (p < 0.005). The formation of products, citrate and malate, was decreased in normal and deficient mitochondria from aspirin-treated rats. The percentage of pyruvate carboxylated in the absence of fatty acids was the same in normal (65.7%) and deficient (65.3%) mitochondria and was significantly decreased in mitochondria from normal (45.5%) and deficient (41.3%) aspirin-treated rats.

Fatty acids are known to inhibit oxidation of pyruvate in mitochondria from liver (18, 20, 24, 25). In the presence of 0.67 μmol octanoylcarnitine, the utilization of pyruvate was significantly decreased in mitochondria from normal and normal aspirin-treated rats (Table III). In the mitochondria from deficient animals, especially in the deficient aspirin-treated group, pyruvate

<table>
<thead>
<tr>
<th>Parameters examined</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Without aspirin, Group A</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>327 ± 16</td>
</tr>
<tr>
<td>Adipose tissue wet weight (g)</td>
<td>4.16 ± 0.05</td>
</tr>
<tr>
<td>Adipose tissue (g/100 g)</td>
<td>1.27 ± 0.26</td>
</tr>
</tbody>
</table>

* Group A compared with Group C.

** Group C compared with Group D.
**Table II**

<table>
<thead>
<tr>
<th>Parameters examined</th>
<th>No. of rats</th>
<th>Normal</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without aspirin, Group A</td>
<td>With aspirin, Group B</td>
<td>Without aspirin, Group C</td>
</tr>
<tr>
<td>Blood glucose (mg per 100)</td>
<td>6</td>
<td>127 ± 10</td>
<td>90 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>Acetoacetate (wmoles per ml)</td>
<td>6</td>
<td>0.236 ± 0.085</td>
<td>0.410 ± 0.057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>β-Hydroxybutyrate (wmoles per ml)</td>
<td>6</td>
<td>0.765 ± 0.185</td>
<td>0.520 ± 0.130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.02)</td>
<td></td>
</tr>
<tr>
<td>Total ketone (wmoles per ml)</td>
<td>6</td>
<td>1.001 ± 0.091</td>
<td>0.929 ± 0.061</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(NS)</td>
<td></td>
</tr>
<tr>
<td>Ratio β-hydroxybutyrate to acetoacetate</td>
<td>6</td>
<td>3.64 ± 0.54</td>
<td>1.27 ± 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
</tbody>
</table>

*a Group A compared with Group B.

*b Group A compared with Group C.

*c Group C compared with Group D.

*d NS, not significant.

**Table III**

Effect of dietary aspirin on pyruvate metabolism in rat liver mitochondria from normal and thiamine-deficient rats

The reaction mixture was made isotonic with 0.154 M KCl and contained, in 3.0 ml, 3.3 mM ATP, 10 mM MgSO4, 13.3 mM potassium phosphate, and 13.3 mM triethanolamine buffers, pH 7.3, 10 mM KH14CO3 (specific activity 185,000 dpm per μmole) and 9.7 mM pyruvate. Mitochondria from 0.5 g of liver contained 3.2 mg of nitrogen and was suspended in 0.25 M sucrose. Incubation time was 7 min at 37°C.

<table>
<thead>
<tr>
<th>Addition to system</th>
<th>No. of rats</th>
<th>Pyruvate used</th>
<th>14CO2 incorporated</th>
<th>Malate found</th>
<th>Citrate found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without octanoyl-carnitine</td>
<td>With octanoyl-carnitine</td>
<td>Without octanoyl-carnitine</td>
<td>With octanoyl-carnitine</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
<td>2.14 ± 0.08</td>
<td>2.19 ± 0.13</td>
</tr>
<tr>
<td>None, Group A</td>
<td>4</td>
<td>4.40 ± 0.46</td>
<td>4.40 ± 0.46</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td>With aspirin, Group B</td>
<td>4</td>
<td>4.40 ± 0.46</td>
<td>4.40 ± 0.46</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td>Deficient</td>
<td>4</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
<td>2.14 ± 0.08</td>
<td>2.19 ± 0.13</td>
</tr>
<tr>
<td>None, Group C</td>
<td>4</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
<td>2.14 ± 0.08</td>
<td>2.19 ± 0.13</td>
</tr>
<tr>
<td>With aspirin, Group D</td>
<td>4</td>
<td>3.12 ± 0.23</td>
<td>3.12 ± 0.30</td>
<td>2.14 ± 0.08</td>
<td>2.19 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.26 ± 0.27</td>
<td>4.13 ± 0.38</td>
<td>1.71 ± 0.19</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(NS)</td>
<td>(p &lt; 0.005)</td>
<td>(NS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.26 ± 0.27</td>
<td>4.13 ± 0.38</td>
<td>1.71 ± 0.19</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(NS)</td>
<td>(p &lt; 0.005)</td>
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<td>3.26 ± 0.27</td>
<td>4.13 ± 0.38</td>
<td>1.71 ± 0.19</td>
<td>3.12 ± 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(NS)</td>
<td>(p &lt; 0.005)</td>
<td>(NS)</td>
</tr>
</tbody>
</table>

*a Group A compared with Group B.

*b NS, not significant.

*c Group A compared with Group C.

*d Group C compared with Group D.

**utilization and carboxylation were greatly increased. There was no difference in pyruvate utilization in the presence of octanoylcarnitine between normal and deficient rats. The incorporation of 14CO2 into organic acids and formation of malate was the same for all groups in the presence of octanoylcarnitine.

There were large significant decreases in the amounts of citrate formed in normal and deficient aspirin-treated rats.**

Results in Table IV show that when pyruvate carboxylation was estimated by summing up all products by the method previously described (26) there was a 50% decrease in normal aspirin-treated rats (from 4930 ± 191 to 2436 ± 129 mpmoles per mg of protein per hour). In the deficient aspirin-treated rats the pyruvate carboxylation was decreased by 63% when compared to deficient rats (from 2110 ± 154 to 573 ± 69 mmoles
Effect of dietary aspirin on pyruvate carboxylation in rat liver mitochondria from normal and thiamine-deficient rats

Table IV

<table>
<thead>
<tr>
<th>Addition to system</th>
<th>No. of rats</th>
<th>Pyruvate carboxylation</th>
<th>Pyruvate carboxylated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without octanoylcarnitine (0.67 mM)</td>
<td>With octanoylcarnitine (0.67 mM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µmoles/mg protein/hr</td>
<td>% control</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
<td></td>
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<tr>
<td>None, Group A</td>
<td>4</td>
<td>4.93 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.005)</td>
<td>(p &lt; 0.01)</td>
</tr>
<tr>
<td>With aspirin, Group B</td>
<td>4</td>
<td>2.44 ± 0.13</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None, Group C</td>
<td>4</td>
<td>2.1 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>With aspirin, Group D</td>
<td>4</td>
<td>0.57 ± 0.07</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p &lt; 0.001)*</td>
<td></td>
</tr>
</tbody>
</table>

* Group A compared with Group B.
* Group A compared with Group C.
* Group C compared with Group D.

per mg of protein per hour). Also, in thiamine deficiency, pyruvate carboxylation was decreased by approximately 57% from normal rats (from 4390 ± 191 to 2110 ± 154 μmoles per mg of protein per hour).

The pyruvate carboxylation in the presence of octanoylcarnitine in control aspirin-treated rats was decreased by 30% (from 4253 ± 94 to 3000 ± 176 μmoles per mg of protein per hour). There was no difference between the activities of normal and deficient rats. In the deficient aspirin-treated rats there was 40% decrease in pyruvate carboxylation (from 3997 ± 402 to 2375 ± 94 μmoles per mg of protein per hour). The percentage of pyruvate carboxylated was greatly increased in the presence of octanoylcarnitine in all groups. In the aspirin-treated groups the increase in the percentage of pyruvate carboxylated was smaller than that of untreated groups. In the presence of fatty acid in both deficient (from 65.3 ± 1.3 to 92 ± 2.8%) and untreated groups, the percentage of pyruvate carboxylated was greatly increased (Table IV).

Discussion

The results presented in this study show that aspirin affects pyruvate metabolism. The importance of pyruvate metabolism in rat liver, kidney, and adipose tissue mitochondria for providing precursors for gluconeogenesis has been studied in great detail (18–20, 25–33). These studies in rats demonstrate that malate and aspartate formed in mitochondria from pyruvate carboxylation are transported to cytosol where they are used for gluconeogenesis.

Thiamine is a known important cofactor for metabolism of pyruvate and other α-keto acids (12–17). In addition fatty acids are known to exert "thiamine sparing effect" (34–37); but the exact mechanism by which fatty acids spare thiamine requirement still remains obscure. However, it is known that thiamine as thiamine pyrophosphate is involved in the dehydroxylation of pyruvate to acetyl-CoA. Acetyl-CoA is a required allosteric activator of pyruvate carboxylase (EC 6.4.1.1, pyruvate:carbon dioxide ligase (ADP)) (38–40). In 1940, Krebs and Egggeston (41) observed that the carboxylation of pyruvate was decreased in thiamine-deficient rats and these effects could be due to decrease in acetyl-CoA concentration which is required for pyruvate carboxylation. Results in Table III show that addition of fatty acids to mitochondria from thiamine-deficient rats greatly stimulated carboxylation of pyruvate. This stimulation of pyruvate carboxylation may be attributed to (a) increase in the acetyl-CoA levels from oxidation of fatty acid, and (b) to the production of reducing equivalent from fatty acid oxidation which may be required for conversion of oxalacetate to malate. This was further supported by the decrease in the oxidation-reduction potential of pyridine nucleotide of mitochondria in the deficient rats as shown in Table II, where the ratio of β-hydroxybutyrate to acetoacetate were shown to be decreased, a value which is used to measure the NADH to NAD+ ratio in mitochondria (44). The decrease in ratio (β-hydroxybutyrate to acetoacetate) in blood of aspirin-treated and thiamine-deficient rats (Table II) may explain the decrease in the mitochondrial pyruvate metabolism. Williamson et al. (42–44) showed that gluconeogenesis from pyruvate is greatly increased in perfused liver by an increase in the NADH to NAD+ ratio produced by metabolism of fatty acids or ethanol. Addition of fatty acids greatly stimulated pyruvate carboxylation in both aspirin-treated and deficient rats (Table IV). This is presumably due to an increase in the mitochondrial NADH to NAD+ ratio from oxidation of fatty acids, which would result in decreased inhibition of pyruvate carboxylase by the mechanism suggested by Fung and Utter (45).

The effect observed with aspirin in this study on blood ketone bodies and pyruvate metabolism are similar to that of thiamine-deficient animals. The more severe alterations in the carbohydrate metabolism of thiamine-deficient rats treated with aspirin may be due to increased excretion of thiamine in urine, similar to that stimulated by salicylates (46), in humans.

Thus, it may be possible to conclude that some of the sites of
action of aspirin on carbohydrate metabolism are in the mitochondria at the pyruvate carboxylation step, due to alteration of mitochondrial oxidation-reduction potential of pyridine nucleotides that can be controlled by fatty acids.

REFERENCES
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