INHIBITION OF CHOLESTEROL SYNTHESIS BY (−)-HYDROXYCITRATE IN PERFUSED RAT LIVER. EVIDENCE FOR AN EXTRAMITOCHONDRIAL MEVALONATE SYNTHESIS FROM ACETYL COENZYME A

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1. Introduction

(−)-Hydroxycitrate, an inhibitor of citrate cleavage enzyme (EC 4.1.3.8) [1] inhibits fatty acid synthesis in rat liver [2]. This effect is likely to result from a shortage of cytoplasmic acetyl-CoA. Whereas fatty acid synthesis is considered an extramitochondrial process, formation of HMG-CoA serving as precursor of mevalonate may be catalyzed by mitochondrial HMG-CoA synthase (EC 4.1.3.5) or by an extramitochondrial pathway, e.g. [3].

In this paper an inhibition of hepatic cholesterol and fatty acid synthesis by (−)-hydroxycitrate in the isolated perfused rat liver is reported; acetate reversed the effect on fatty acid synthesis. In cell-free extracts of rat liver (−)-hydroxycitrate is shown not to influence cholesterol formation itself but to be a potent stimulator of fatty acid from ^{14}C-acetate.

These experiments are taken as evidence of a physiologically important extramitochondrial pathway from acetyl-CoA to mevalonate in rat liver.

2. Methods

For measuring fatty acid [4] and cholesterol synthesis by the ^{3}HOH-method, livers of male Buffalo rats (150 g) were perfused as described extensively elsewhere [5]. The medium contained in 100 ml Krebs–Henseleit buffer: 2.5 g albumin reinst (Behringwerke AG, Frankfurt/Main, West Germany), 0.55 mmole glucose, 4 mg ampicillin and amino acids corresponding to steady state levels observed in liver perfusions [6]. Assays with cell-free extracts were performed according to [7]. ATP was determined after freeze-clamping of the perfused liver [8], citrate in deproteinized homogenates [9], and acetoacetate plus 3-hydroxybutyrate were analyzed in the perfusion medium [10]. Samples of (−)-hydroxycitric acid lactone were kindly supplied by Dr. Y.S. Lewis, Mysore, India, and by Dr. H. Lengsfeld (Hoffman-La Roche, Basel, Switzerland). The lactone was hydrolyzed according to [2] before use.

3. Results

3.1. *Perfusion experiments*

Fatty acid and cholesterol synthesis were inhibited in a similar manner by different (−)-hydroxycitrate concentrations (fig. 1). As *in vivo* [2], this compound appears to only partially inhibit fatty acid synthesis in the perfused organ.

ATP levels of liver tissue perfused for 60 min with different concentrations of (−)-hydroxycitrate remained in the control range. In the presence of 0.44, 1.1, and 2.2 mM (−)-hydroxycitrate, ATP contents of 2.8 ± 0.9, 2.61 ± 0.49, and 2.65 ± 0.07 μmoles/g wet weight (±S.D.), respectively, were found as compared to 2.88 ± 0.41 at the end of the control periods. Ketone body production (acetoacetate plus 3-hydroxybutyrate) by perfused liver was not changed in the presence of 1.1 mM (−)-hydroxycitrate. It amounted to 8.35 ± 5.15 μmoles/g liver, wet weight × hr (±S.D.) as compared to 9.25 ± 4.8 μmoles/g liver, wet weight × hr in control experiments.

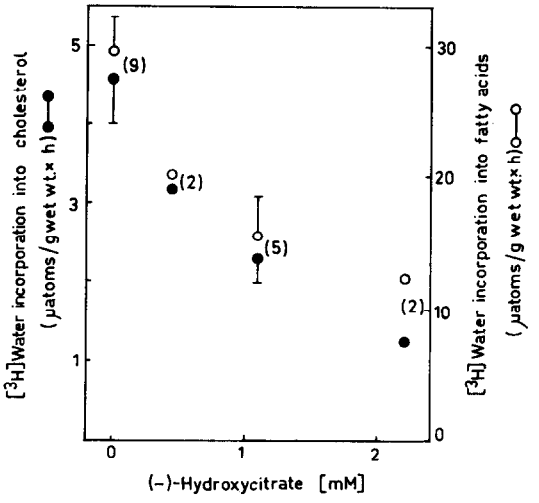

Fig. 1. Influence of (−)-hydroxycitrate on the rate of cholesterol and fatty acid synthesis in perfused rat liver. 40 min after operation (9:00 hr) ^{3}H-water was added to the perfusion medium (final specific activity 15,000 dpm/μatom hydrogen). 30 min thereafter a control liver sample was taken. (−)-Hydroxycitrate was added and perfusion continued for another 60 min, after which 2 additional liver samples were taken and saponified. The non-saponifiable fraction was washed twice (H_2O) and treated with digitonine (cholesterol). The fatty acids were extracted after acidification and washed with H_2O (1×) and 0.2 N CH_3COOH in 20% ethanol (2×). Ordinates: Controls represent averages of all control periods. The other values have been calculated from the increments of ^{3}H-incorporation during the (−)-hydroxycitrate periods. ^{3}H-incorporation into lipids was previously shown to be linear during 90 min. Vertical bars, S.E.M.

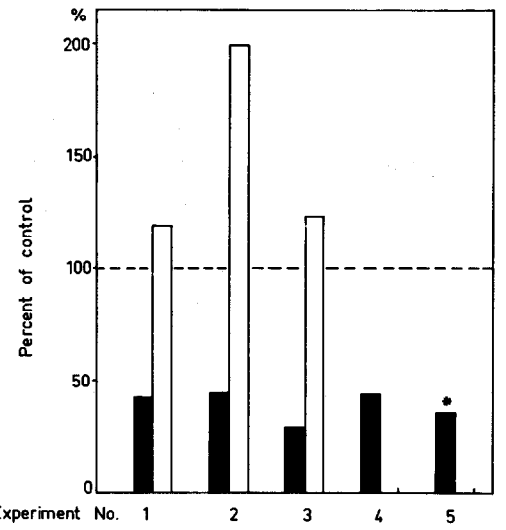

Fig. 2. Influence of acetate on (−)-hydroxycitrate-inhibition of cholesterol and fatty acid synthesis in perfused rat liver. Experimental design as in fig. 1. At 30 min, 10 mM acetate and 1.1 mM (−)-hydroxycitrate were added to the perfusion medium. This concentration of acetate was shown not to influence ^{3}H-incorporation into liver lipids [5]. The synthetic rates after acetate ± hydroxycitrate addition (30–90 min) are shown as percentages of control periods (0–30 min). White columns: fatty acid synthesis; black columns: cholesterol synthesis. *20 mM acetate.

3.2. *Acetate effect*

Studies on the subcellular localization of acetyl-CoA synthetase (EC 6.2.1.1) revealed that acetate is a citrate-independent precursor of cytoplasmic acetyl-CoA [11]. Acetate uptake by the perfused rat liver amounted to about 50 μmoles/g liver, wet weight × hr, if its medium concentration was higher than 10 mM [5].

Fig. 2 shows that the simultaneous addition of (−)-hydroxycitrate and 10 mM acetate increased fatty acid synthesis to 150% of the uninhibited rate. The reversion by acetate indicates that (−)-hydroxycitrate inhibition of fatty acid synthesis is mediated by a shortage of cytoplasmic acetyl-CoA. On the other hand, the inhibition of cholesterol synthesis was not relieved by acetate addition (fig. 2). Therefore, an additional effect of (−)-hydroxycitrate on cholesterol synthesis had to be considered.

3.3. *Experiments with cell-free extracts*

Addition of (−)-hydroxycitrate to a 10,000 *g* supernatant of rat liver increased the rate of 1-^{14}C-acetate incorporation into fatty acids considerably with a concomitant slight inhibition of its incorporation into cholesterol. If fatty acid synthesis was lowered by avidin to subnormal rates, however, formation of labeled cholesterol was not inhibited by (−)-hydroxycitrate (fig. 3). This was corroborated in other experiments where 1-^{14}C-acetate incorporation into cholesterol in the presence of both 1.33 mM (−)-hydroxycitrate and 1 U/ml avidin (7470 ± 168 dpm/assay) was the same as in the controls (7776 ± 211 dpm/assay) (n = 4; mean ±S.E.M.).

Previous dialysis of the homogenate did not change the response of fatty acid synthesis to (−)-hydroxycitrate (fig. 3). As less than 0.01 mM citrate was

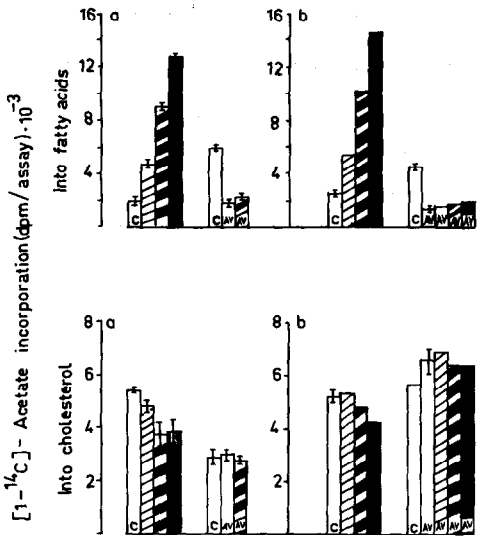

Fig. 3. Influence of (−)-hydroxycitrate and avidin on 1-^{14}C-acetate incorporation into cholesterol and fatty acids by 10,000 *g* supernatant of rat liver. In each of the 4 experiments, livers from 2 male Wistar rats (200 g) were forced through a sieve. Homogenization: 6 strokes (300 rpm) in a loose-fitting Potter-Elvehjem homogenizer with 2.5 vol/g, wet weight of a medium, containing 81 mM potassium phosphate, pH 7.4; 5 mM $MgCl_2$; 37 mM nicotinamide. Assay mixture, see Methods. Start by addition of 10 mM 1-^{14}C acetate (specific activity 150,000 dpm/μmole) after gassing 10 min at 0° with 95% O_2:5% CO_2. For analytical methods see fig. 1. Columns: AV denotes 10 min preincubation with 1 U/ml avidin. ⊓: controls without additions. Addition of (−)-hydroxycitrate: 0.53 mM, ▨; 1.33 mM, ▨; 2.66 mM, ■. Vertical bars: range of duplicate assays. a) 10,000 *g* supernatant; b) supernatant dialysed twice (30 min each) against 500 ml of the above medium + 20 mM cysteine.

present at 0 and 60 min of the incubation period, it is concluded that (−)-hydroxycitrate rather than endogenous citrate is responsible for the stimulatory effect.

4. Discussion

The similarity of the (−)-hydroxycitrate effect on cholesterol and fatty acid synthesis is suggestive of a common mechanism of inhibition, e.g. lowering of the cytoplasmic acetyl-CoA level. This assumption requires that both effects be abolished by replenishment of the acetyl-CoA pool by exogenous acetate. While this was verified for fatty acid synthesis, ^{3}H-incorporation into cholesterol was not normalized by acetate addition. Experiments with cell-free extracts, however, demonstrated that ^{14}C-acetate incorporation into cholesterol was not inhibited if acetyl-CoA consumption by fatty acid synthesis was blocked by avidin (fig. 3). Moreover, they revealed a considerable stimulation of fatty acid synthesis by (−)-hydroxycitrate; this effect may be explained most readily by an activation of acetyl-CoA carboxylase (EC 6.4.1.2) as known from other tricarboxylates [12]. The (−)-hydroxycitrate stimulation of fatty acid synthesis was also observed in the perfused liver if acetate was added (fig. 2). It appears that the stimulated fatty acid synthesis drains acetyl-CoA generated by acetyl-CoA synthetase off from other acetyl-CoA requiring reactions in the cytoplasm, e.g. mevalonate synthesis.

The reported effect of (−)-hydroxycitrate on cholesterol synthesis cannot be explained by an impairment of intramitochondrial acetyl-CoA metabolism including HMG-CoA synthesis because ketone body production was found unchanged.

These observations suggest that acetyl-CoA must be available in the cytoplasm for an unimpaired cholesterol synthesis in rat liver; this implies the existence of an extramitochondrial pathway from acetyl-CoA to mevalonate. The fact that cholesterol synthesis was not inhibited by avidin nor stimulated by (−)-hydroxycitrate does not support a pathway involving the known acetyl-CoA carboxylase reaction.

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