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Inhibition of Mitochondrial Carnitine Acylcarnitine Translocase-Mediated Uptake of Carnitine by 2-(3-Methyl-cinnamyl-hydrazono)-propionate¹⁾ Hydrazonopropionic Acids, a New Class of Hypoglycaemic Substances, VI²⁾

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Summary: The rate of mitochondrial carnitine-carnitine exchange mediated by carnitine acylcarnitine translocase was measured by following the uptake of *L*-[methyl-¹⁴C]carnitine. It was demonstrated that the hypoglycaemic compound 2-(3-methyl-cinnamyl-hydrazono)-propionate causes a concentration-dependent decrease in the rate of the translocase-mediated transport of carnitine in guinea pig liver mitochondria. Apparent initial influx rates were decreased by 20% at 0.3 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate, 38% at 0.5 mmol/l, and 75% at 2.0 mmol/l of this compound. This finding may explain the previously observed inhibitory effects of this substance on long-chain fatty acid oxidation, ketone body production and gluconeogenesis.

Introduction

Hydrazones of pyruvate exert a powerful hypoglycaemic effect in various laboratory animals (1–6). In addition to their potential usefulness for the treatment of diabetes, such substances facilitate studies on the interrelationship between metabolic pathways, provided that their mode of action is known.

Among these hydrazones, 2-(3-methyl-cinnamyl-hydrazono)-propionate is a powerful hypoglycaemic agent, the metabolic effects of which have already been studied in considerable detail. Studies on the mode of action revealed that 2-(3-methyl-cinnamyl-hydrazono)-propionate inhibits gluconeogenesis in guinea pig liver (7, 8). Furthermore in the rat and in the guinea pig an increase in the concentration of free fatty acids in the plasma and a decrease in blood ketone body concentration was observed, which pointed to an inhibition of long-chain fatty acid oxidation (5). It has been suggested that this inhibition by 2-(3-methyl-cinnamyl-hydrazono)-propionate oc-

¹⁾ Preliminary results of this study were presented at the third meeting of the German Association for the Study of the Liver in Hannover 1987 (abstract: *Z. Gastroenterologie* (1987) 25, 63–64).

²⁾ For previous publications see l. c. (3–5), (8), (25).

curred at the site of fatty acid transport across the inner mitochondrial membrane (5, 8). It has been proposed that this transport is the rate limiting step in the degradation of fatty acids to acetyl-CoA (9).

The long chain acyl-group transfer presumably proceeds in the following manner (10, 11): acyl-CoA formed in the outer mitochondrial membrane reacts with carnitine and coenzyme A by means of the outer carnitine acyl-CoA transferase (EC 2.3.2.21). Acylcarnitine is transferred through the inner membrane by a process of exchange diffusion facilitated by a carrier called carnitine acylcarnitine translocase (11, 12). Acylcarnitine is converted again to acyl-CoA and carnitine at the inside of the inner membrane by the inner carnitine acyl-CoA transferase. Acyl-CoA is now available for β -oxidation and can be converted to acetyl-CoA.

Since previous investigations have shown that 2-(3-methyl-cinnamyl-hydrazono)-propionate does not inhibit carnitine acyl-CoA transferases (13), in the present study we investigated the influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate on the carnitine acylcarnitine translocase-mediated carnitine uptake. Sulphobetaine, a known inhibitor of this transport (14), was included for purposes of comparison.

Materials and Methods

Materials

L-[methyl- 14 C]carnitine hydrochloride was purchased from Amersham-Buchler (Braunschweig, FRG), *L*-carnitine hydrochloride and mersalyl acid from Sigma Chemie GmbH (Deisenhofen, FRG), mannitol, sucrose, EDTA, Tris, KCl, HCl and formic acid from Merck (Darmstadt, FRG) and oligomycin and antimycin A from Boehringer Mannheim GmbH (Mannheim, FRG). 2-(3-methyl-cinnamyl-hydrazono)-propionate sulphate was synthesized by Dr. R. Heerdt and was generously provided by Prof. Dr. F. Schmidt and Dr. H. Kühnle (Boehringer Mannheim GmbH). Silicon oil (density 1.04 kg/l) was obtained from Wacker Chemie (München, FRG). Dextran 100 (*M_r* 100 000–200 000) was purchased from Serva (Heidelberg, FRG). Aquasol was from NEN Du Pont (Boston, Mass., USA). Sulphobetaine (Zwittergent 3-08 detergent, N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) was obtained from Calbiochem GmbH (Frankfurt, FRG).

Animals

Albino guinea pigs of 300–500 g body weight from Zentrales Tierlabor der Medizinischen Hochschule Hannover were used. They had access to water and food ad libitum.

Preparation of liver mitochondria

Guinea pigs were killed by decapitation or a blow to the neck, and mitochondria were isolated from guinea pig livers according to Parvin & Pande (15). The mitochondria were tested for respiratory control (16) at 25 °C with succinate as substrate. Electron microscopic examination and a respiratory control index of 6.7 confirmed the presence of intact mitochondria.

Assay of translocase-mediated transport of *L*-[methyl- 14 C]carnitine

Transport rates of *L*-[methyl- 14 C]carnitine were measured according to Parvin & Pande (15) with slight modifications. The reaction medium in a final volume of 200 μ l contained 100 mmol/l KCl, 50 mmol/l mannitol, 20 mmol/l Tris/HCl pH 7.4, 6.15 mg/l oligomycin, 2.23 μ mol/l of antimycin A, 0.50 mmol/l *L*-[methyl- 14 C]carnitine (specific activity: 147.6 GBq/mol). Uptake was initiated by adding 20 μ l of the mitochondrial preparation to the rapidly stirred incubation mixture. It was stopped after the indicated incubation time with 20 μ l of 25 mmol/l mersalyl in 200 mmol/l mannitol, 50 mmol/l Tris/HCl pH 7.4. The incubation was carried out at 2–4 °C. In the controls, mersalyl was added prior to mitochondria. Immediately after stopping the incubation, 150 μ l of the mixture was placed on a gradient in an 1.5 ml Eppendorf tube consisting of (from bottom to top): 100 μ l 900 ml/l formic acid, about 200 μ l silicon oil, 800 μ l of a solution containing 120 mmol/l KCl, 50 mmol/l mannitol, 5 mmol/l Tris/HCl pH 7.4, 60 g/l dextran 100. Mitochondria were separated by centrifugation: 4 min at 10 000 *g* using a Hettich centrifuge equipped with a swing out rotor. The layers above the silicon oil were removed by aspiration together with most of the silicon oil layer. Thoroughly mixed formic acid layer (80 μ l) was then transferred to the counting vials containing 10 ml of Aquasol. 14 C was counted in a LKB Wallac 1219 Rackbeta liquid scintillation counter. Protein was determined using the Biuret method (17). Uptake is expressed as nmol carnitine per mg protein.

Evaluation of the results

To determine apparent initial influx rates of *L*-[methyl- 14 C]carnitine into the mitochondria in exchange for internal *L*-carnitine, a sequence of incubations was performed over 5 to 20 seconds. The measured uptake expressed in nmol carnitine per mg protein was plotted against the incubation time. From the slopes of the lines the apparent initial transport rates were calculated. To minimize interassay deviations the same mitochondrial preparation was used in each experiment for controls and corresponding incubations with the test substance 2-(3-methyl-cinnamyl-hydrazono)-propionate or sulphobetaine. Two examples are given in figure 2, which refer to experiments No 16 and 17 in table 1.

Results and Discussion

The inhibitory effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on the carnitine acylcarnitine translocase-mediated carnitine-carnitine exchange was investigated by following the mitochondrial uptake of *L*-[methyl- 14 C]carnitine. The time course of the *L*-[methyl- 14 C]carnitine uptake in exchange for unlabelled carnitine from inside the mitochondrion approaches an equilibrium after about twenty minutes of incubation (fig. 1). During the first 20 s the relationship between uptake and time can be regarded as linear (fig. 2). Therefore the apparent initial reaction velocities have been determined as described by Ramsay & Tubbs for efflux experiments (18), by calculating the slopes of the lines in linear plots of *L*-[methyl- 14 C]carnitine uptake versus time. A different model for the evaluation of the results from influx experiments has been proposed by Parvin & Pande (15) which involves pseudo first order kinetics and

Fig. 1. Time course of *L*-carnitine-uptake by mitochondria of guinea pig liver by exchange with internal *L*-carnitine. Incubations and measurement of *L*-[methyl- 14 C]carnitine uptake were performed as described under 'Materials and Methods'.

Fig. 2. Apparent initial rate of *L*-carnitine uptake in mitochondria of guinea pig liver. Incubations and measurement of *L*-[methyl- 14 C]carnitine uptake were performed as described under 'Materials and Methods', in the presence and absence of:
2-(3-methyl-cinnamyl-hydrazono)-propionate
(○—○ controls
◇—◇ 2-(3-methyl-cinnamyl-hydrazono)-propionate 2.0 mmol/l)
or sulphobetaine:
(●—● controls
◆—◆ sulphobetaine 25 mmol/l)
Values represent means of one to three determinations.

maximum uptake estimation. In our study this model has not been used, as it is still unresolved whether maximum uptake estimation as described by these authors results in a valid parameter for the assessment of initial transport rates. In this context it has to be considered that a microcompartmentation of carnitine may exist inside the mitochondrion, as discussed by Murthy & Pande in more recent publications (19, 20). Furthermore a slow uniport may occur which would affect the uptake in longer periods of incubation (18).

The observed kinetics of the carnitine-carnitine exchange in guinea pig liver mitochondria (fig. 1) are similar to those reported by Parvin & Pande for rat liver mitochondria (15). In their experiments, however, a lower maximal uptake was observed and the equilibrium was reached about ten to twenty minutes earlier than in our study. A reason for this difference might be a lower carnitine content of rat liver mitochondria and/or a different microcompartmentation of carnitine inside the mitochondrion. Comparable data for guinea pig mitochondria are so far not available from other authors.

Table 1 summarizes the apparent initial influx rates in incubations with test substance and in the corresponding controls. The results of one experiment shown in figure 2 (No 16, table 1) revealed that 2.0 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate produced a 78% decrease in the rate of carnitine-carnitine exchange of mitochondria from livers of fed guinea pigs.

In controls, initial influx rates of carnitine ranged from 0.17 to 0.59 nmol/min per mg protein (mean value: 0.35 nmol/min per mg protein). With 2-(3-methyl-cinnamyl-hydrazono)-propionate a concentration-dependent decrease of initial influx rates was found (tab. 1, fig. 3). Sulphobetaine (25 mmol/l), a known inhibitor of the carnitine acylcarnitine translocase (14), decreased the initial transport rate for *L*-carnitine by 84–97% compared with the corresponding control (fig. 2, tab. 1).

Inhibition of the carnitine acylcarnitine translocase-mediated transport could be the explanation for the previously observed 2-(3-methyl-cinnamyl-hydrazono)-propionate-induced alterations in metabolite concentrations (5, 8, 21). The resulting decrease of long-chain acyl-CoA degradation to acetyl-CoA leads to a reduced ketone body production (5). As we have demonstrated previously, gluconeogenesis from pyruvate, lactate or alanine is decreased in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate (8). This can be explained by the observed decrease of acetyl-CoA, the positive modulator of pyruvate carboxylase (22), in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate (5, 8). When hexanoic acid was added, glucose formation from alanine was not inhibited by 2-(3-methyl-cinnamyl-hydrazono)-propionate (8). It appears that under the latter experimental conditions, pyruvate carboxylase might be activated despite an inhibition of translocase; the transport of medium-chain fatty acids is translocase-independent, so that acetyl-CoA formation from medium-chain fatty acids is not prevented.

The role of acetyl-CoA in the regulation of pyruvate carboxylase, however, is not certain (23). Alterna-

Tab. 1. Influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate MCHP and sulphobetaine on apparent initial influx rates of *L*-carnitine.

Experiment number		Apparent initial influx rate (v) $\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$	Relative decrease in v (%)
1	0.3 mmol/l MCHP control	0.32 0.44	27
2	0.3 mmol/l MCHP control	0.24 0.29	17
3	0.3 mmol/l MCHP control	0.18 0.21	14
4	0.3 mmol/l MCHP control	0.33 0.43	23
5	0.5 mmol/l MCHP control	0.28 0.53	47
6	0.5 mmol/l MCHP control	0.26 0.44	41
7	0.5 mmol/l MCHP control	0.13 0.22	41
8	0.5 mmol/l MCHP control	0.20 0.34	41
9	0.5 mmol/l MCHP control	0.29 0.42	31
10	0.5 mmol/l MCHP control	0.12 0.17	29
11	2.0 mmol/l MCHP control	0.11 0.34	68
12	2.0 mmol/l MCHP control	0.09 0.24	63
13	2.0 mmol/l MCHP control	0.06 0.41	85
14	2.0 mmol/l MCHP control	0.11 0.38	71
15	2.0 mmol/l MCHP control	0.10 0.59	83
16	2.0 mmol/l MCHP control	0.0 0.27	78
17	25 mmol/l sulphobetaine control	0.03 0.19	84
18	25 mmol/l sulphobetaine control	0.01 0.37	97

$$\% \text{ relative decrease in } v = \frac{v_{\text{control}} - v_{\text{test substance}}}{v_{\text{control}}} \cdot 100$$

tively it has been suggested that the ATP/ADP-ratio may play a role in the regulation of pyruvate carboxylase activity. Although 2-(3-methyl-cinnamyl-hydrazono)-propionate (0.04 mmol/l) did not influence the overall ATP/ADP-ratio of perfused guinea pig livers (8), the intramitochondrial ATP/ADP-ratio could be decreased due to the diminished long-chain fatty acid

Fig. 3. Concentration-dependent decrease of apparent initial influx-rates of *L*-carnitine by 2-(3-methyl-cinnamyl-hydrazono)-propionate. Percent relative decrease in v calculated as described in table 1.

oxidation. This would result in a reduction of pyruvate carboxylase activity (23) and a decrease of gluconeogenesis. In the presence of hexanoic acid, however, ATP production would not be reduced by 2-(3-methyl-cinnamyl-hydrazono)-propionate because this medium-chain fatty acid can enter the mitochondrion where it is oxidized. The activity of pyruvate carboxylase therefore will not be decreased under the latter conditions so that an inhibition of gluconeogenesis by 2-(3-methyl-cinnamyl-hydrazono)-propionate is avoided (8).

It is to be expected that an inhibition of long-chain fatty acid oxidation by 2-(3-methyl-cinnamyl-hydrazono)-propionate will result in an acceleration of glucose utilisation. Current investigations with rat haemidiaphragms support this hypothesis (24).

Haeckel et al. (25) reported that the uptake of glucose in the jejunum is inhibited by 2-(3-methyl-cinnamyl-hydrazono)-propionate. Investigations are required to determine whether this is due to an inhibition of the Na^+/K^+ -ATPase by acylcarnitine, which has been shown to be a relatively strong inhibitor of this enzyme in the heart (26). Acylcarnitine may accumulate when the carnitine acylcarnitine translocase is inhibited by 2-(3-methyl-cinnamyl-hydrazono)-propionate.

The results of our study show that 2-(3-methyl-cinnamyl-hydrazono)-propionate causes a concentration-dependent inhibition of mitochondrial carnitine uptake mediated by carnitine acylcarnitine translocase. Thus from the evidence so far available it appears reasonable to conclude that the metabolic effects of 2-(3-methyl-cinnamyl-hydrazono)-propionate mainly result from the observed inhibition of the translocase.

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