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Effects of 2-(3-Methyl-cinnamyl-hydrazono)-propionate on Fatty Acid and Glucose Oxidation in the Isolated Rat Diaphragm Using ^{14}C-Labelled Substrates

Hydrazonopropionic Acids, a New Class of Hypoglycaemic Substances, VIII[1]

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Summary: The influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate on the utilization of various substrates in isolated rat hemidiaphragms was investigated in comparison with other hypoglycaemic compounds. The effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate was concentration-dependent. At a concentration of 0.5 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate, glucose utilization increased from 0.276 ± 0.043 μmol · $g^{-1} \cdot l^{-1}$ to 0.894 ± 0.303 μmol · $g^{-1} \cdot l^{-1}$ ($p < 0.05$). Pyruvate and lactate utilization were stimulated to a lesser extent, while acetate utilization remained nearly constant. At a concentration of 2 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate, the oxidation of palmitate decreased from 0.214 ± 0.017 μmol · $g^{-1} \cdot l^{-1}$ to 0.060 ± 0.005 μmol · $g^{-1} \cdot l^{-1}$, while the oxidation of octanoate was not decreased. These findings point to a stimulation of the glycolytic flux by inhibition of long-chain fatty acid oxidation.

Introduction

2-(3-Methyl-cinnamyl-hydrazono)-propionate and other hypoglycaemic substances with different sites of action in glucose and fatty acid metabolism can be used to study the interrelationships of fatty acid and glucose metabolism. Furthermore, 2-(3-methyl-cinnamyl-hydrazono)-propionate may be of potential use as an oral antidiabetic drug.

In previous studies it has been shown that hydrazonopropionic acids produce a strong hypoglycaemic effect in various laboratory animals (3, 4, 17, 18). In guinea pigs and rats 2-(3-methyl-cinnamyl-hydrazono)-propionate showed a reduction of gluconeogenesis in perfused liver and isolated liver cells (3, 16), inhibition of jejunal glucose uptake (2) and elevation of plasma free fatty acids (4).

In guinea pigs, a decrease of hepatic acetyl-CoA and ketone body concentration in serum (4), and a de-

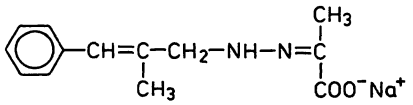
[1]) For previous publications see l.c. (2–5, 10, 17, 18)

crease of long-chain fatty acid oxidation in perfused liver (11, 15) have been observed. From measurements of metabolite concentrations in liver extracts it appears that the carnitine-dependent mitochondrial uptake of long-chain fatty acids is inhibited (3). An inhibition of carnitine-acylcarnitine translocase in liver mitochondria by 2-(3-methyl-cinnamyl-hydrazono)-propionate was recently reported (5).

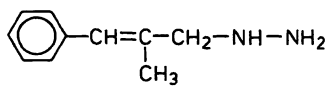
According to the concept of the "glucose-fatty acid cycle" (1, 6, 7, 8) inhibitors of fatty acid oxidation should increase glucose utilization and lower blood glucose concentration primarily when fatty acids are being used as the major substrates.

The effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on glycolysis and fatty acid oxidation in muscle has not previously been described; the purpose of the present study was to investigate this by measuring the conversion of ^{14}C-labelled substrates to carbon dioxide in hemidiaphragms of rats. Increased glucose consumption in muscle may make a large contribution to the hypoglycaemic effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate.

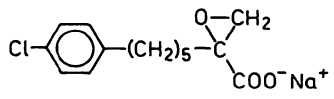
For purposes of comparison we tested three more substances (fig. 1): 2-bromopalmitate and clomoxir, two known inhibitors of long-chain fatty acid oxidation, and 3-methyl-cinnamyl-hydrazine, the corresponding hydrazine of 2-(3-methyl-cinnamyl-hydrazono)-propionate.



2-(3-Methyl-cinnamyl-hydrazono)-propionate



3-Methyl-cinnamyl-hydrazine



Clomoxir

Fig. 1. Structural formulae.

Materials and Methods

Materials

Hydroxide of hyamine and Insta-Fluor were purchased from Packard Instruments International S. A. (Zürich, Switzerland); labelled compounds (*D*-[U-^{14}C]glucose, [1-^{14}C]palmitic acid, and sodium salt of [1-^{14}C]pyruvic acid, *D,L*-[1-^{14}C]lactic acid, [1-^{14}C]acetic acid) and [1-^{14}C]octanoic acid) from Amersham-Buchler (Braunschweig, W-Germany); fatty acid-free bovine serum albumin and sodium salt of palmitic and caprylic acid (octanoic acid) from Sigma Chemical Company (St. Louis, U. S. A.); sodium salt of *L*-lactic acid from Serva (Heidelberg, W-Germany); 2-bromopalmitate from Aldrich Chemical Company (Milwaukee, U. S. A.); unlabelled substrates and all further chemicals from E. Merck A. G. (Darmstadt, W-Germany). 2-(3-Methyl-cinnamyl-hydrazono)-propionate, and 3-methyl-cinnamyl-hydrazine were generously provided by Boehringer Mannheim GmbH (Mannheim, W-Germany). Clomoxir (sodium 2-[5-(4-chlorophenyl)pentyl)]oxirane-2-carboxylate) was made available by Byk Gulden GmbH (Konstanz, W-Germany).

Animals

Male Lewis rats weighing 180–300 g from Zentrales Tierlabor of the Medizinische Hochschule Hannover were used. Rats were fasted 48 hours before killing, except in one experiment with non-fasted animals.

Experimental procedure

During the preparation of rat diaphragms, Erlenmeyer flasks containing 2 ml of incubation medium (see below) were preincubated for 15 minutes at 37 °C.

Rats were killed by decapitation or a blow on the neck and diaphragms excised. After removal of residual blood and fat, hemidiaphragms were rinsed, and placed in ice-cold *Krebs-Ringer* phosphate buffer for 15 minutes. One hemidiaphragm of each rat was subsequently incubated with test substance (test diaphragm), the other was incubated without (control). Hemidiaphragms were gently blotted and weighed. An Erlenmeyer flask with 2 ml of incubation medium was prepared for each hemidiaphragm. The incubation medium was gassed with 100% oxygen and the hemidiaphragms were placed immediately in this gassed medium. The Erlenmeyer flasks were instantly closed with a rubber stopper, carrying a small glass trough. The trough contained 0.3 ml hydroxide of hyamine for the absorption of released carbon dioxide. The Erlenmeyer flasks were continuously incubated in a shaking incubator at 37 °C. After one hour shaking was interrupted, 0.6 ml of 0.6 mol/l perchloric acid was injected into the flasks (penetrating the rubber stopper with an injection needle) and shaking was continued for another hour. The flasks were carefully opened, and the trough, containing hyamine, was placed in a counting vial containing scintillation liquid Insta-Fluor (10 ml). The disintegrations per minute were registered in a beta counter (Tri-carb 460 CD, Liquid Scintillation System, Packard Instruments Company, Illinois, U. S. A.) and related to 1 g of diaphragm (wet weight). The quantity of ^{14}C-labelled substrate converted to carbon dioxide per 1 g diaphragm during 1 h incubation time was calculated from the specific activity.

Incubation medium

Control hemidiaphragms were incubated in *Krebs-Ringer* phosphate buffer gassed with 100% oxygen, containing one of the following substrates:

5.0 mmol/l *D*-[U-^{14}C]glucose (specific activity: 3.7 GBq/mol)
1.0 mmol/l *L*-[1-^{14}C]lactate (specific activity: 9.25 GBq/mol),
1.0 mmol/l [1-^{14}C]pyruvate (specific activity: 18.5 GBq/mol),
2.0 mmol/l [1-^{14}C]acetate (specific activity: 4.63 GBq/mol),
2.0 mmol/l [1-^{14}C]palmitate (specific activity: 18.5 GBq/mol), or
2.0 mmol/l [1-^{14}C]octanoate (specific activity: 18.5 GBq/mol).

In accordance with *Frayn* et al. (9) experiments were performed with 4 g/l fatty acid-free bovine serum albumin in the incubation medium, unless otherwise indicated. Incubations with palmitate or octanoate were performed in the presence of 40 g/l albumin, to ensure the dissolution of these fatty acids.

In experiments with test hemidiaphragms 2-(3-methyl-cinnamyl-hydrazono)-propionate or another test substance was added to the medium described for controls. Concentrations are given in figures 2 and 3 and in tables 1 and 2.

Results

The effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on glucose oxidation in comparison with other hypoglycaemic substances is shown by table 1.

Tab. 1. Effect of various substances (2 mmol/l) on glucose oxidation in hemidiaphragms of rats. Incubations and measurement of glucose oxidation were performed as described under "Materials and Methods". Results of "glucose converted to CO_2" are presented as mean value ± standard deviation.
MCHP: 2-(3-methyl-cinnamyl-hydrazono)-propionate
MCH: 3-methyl-cinnamyl-hydrazine

Incubation medium	Glucose converted to CO_2 ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	Increase of glucose oxidation (%)	Number of hemi-diaphragms
Control	0.428 ± 0.068		4
MCHP	1.918* ± 0.232	348	4
Control	0.354 ± 0.050		5
MCH	0.610* ± 0.094	72	5
Control	0.301 ± 0.083		5
Clomoxir	1.255* ± 0.234	317	5

* Differs significantly from its corresponding control value ($p < 0.05$, t-test).

$$\% \text{ increase of glucose oxidation} = \frac{G_T - G_C}{G_C} \cdot 100$$

G_C: glucose oxidation of hemidiaphragms incubated in medium (control).
G_T: glucose oxidation of hemidiaphragms incubated in medium containing test substance.

Conversion of glucose to carbon dioxide depended upon the concentration of 2-(3-methyl-cinnamyl-hydrazono)-propionate. A significant enhancement of glucose utilization was observed at a concentration as low as 0.1 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate ($p < 0.005$) (fig. 2). The concentration-dependence of glucose conversion to carbon dioxide is shown in figure 2. There was a statistically significant difference between the effect of 0.1 and 0.5 mmol/l, 0.5 and 1.0 mmol/l, and between that of 1.0 and 2.0 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate on glucose oxidation ($p < 0.05$).

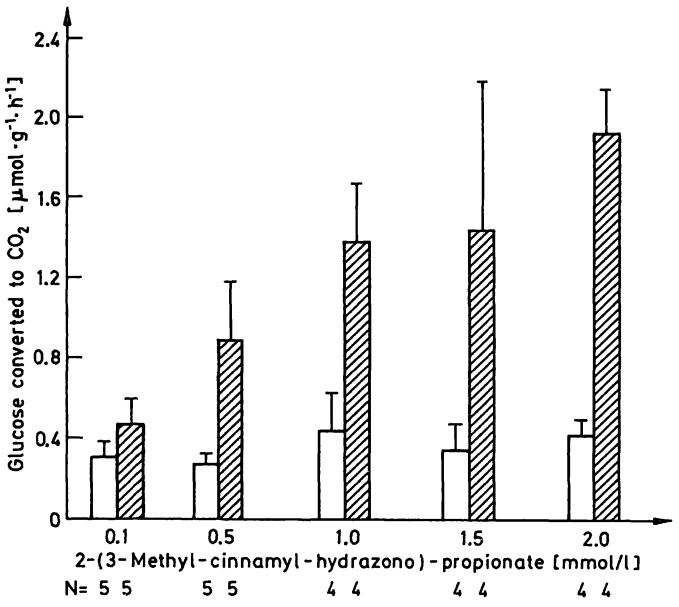


Fig. 2. Concentration-dependent effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on glucose utilization.
Incubations and measurement of glucose utilization were performed as described under "Materials and Methods".
Unshaded columns: controls, $\bar{x} \pm s$
Shaded columns: various concentrations of 2-(3-methyl-cinnamyl-hydrazono)-propionate, $\bar{x} \pm s$

In four experiments with 2-(3-methyl-cinnamyl-hydrazono)-propionate, glucose oxidation was compared with the oxidation of lactate, pyruvate, and acetate; the results are shown in figure 3.

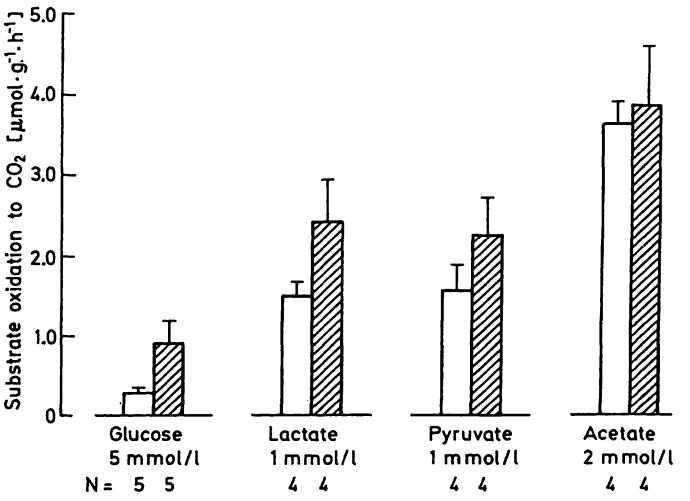


Fig. 3. Utilization of glucose, lactate, pyruvate, and acetate in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate.
Incubations and measurement of glucose utilization were performed as described under "Materials and Methods".
Unshaded columns: controls, $\bar{x} \pm s$
Shaded columns: 0.5 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate, $\bar{x} \pm s$

The influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate and other hypoglycaemic substances on long-chain fatty acid oxidation is shown in table 2.

Tab. 2. Effect of various hypoglycaemic substances (2 mmol/l) on palmitate oxidation in hemidiaphragms of rats. Incubations and measurement of palmitate oxidation were performed as described under "Materials and Methods". Results of "palmitate converted to CO_2" are presented as mean value ± standard deviation.
MCHP: 2-(3-methyl-cinnamyl-hydrazono)-propionate
2-Br-palmitate: 2-bromopalmitate

Incubation medium	Palmitate converted to CO_2 ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	Decrease of palmitate oxidation (%)	Number of hemi-diaphragms
Control	0.214 ± 0.017		5
MCHP	0.060* ± 0.005	72	5
Control	0.191 ± 0.007		4
2-Br-palmitate	0.081* ± 0.009	58	4
Control	0.141 ± 0.025		5
Clomoxir	0.028* ± 0.005	80	5

* Differs significantly from its corresponding control value ($p < 0.05$, t-test).

$$\% \text{ decrease of palmitate oxidation} = \frac{P_C - P_T}{P_C} \cdot 100$$

P_C: palmitate oxidation of hemidiaphragms incubated in medium (control).
P_T: palmitate oxidation of hemidiaphragms incubated in medium containing test substance.

In one experiment diaphragms were incubated in a medium with octanoate. The carbon dioxide production from octanoate was 0.066 ± 0.007 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in controls, compared with 0.077 ± 0.004 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in test diaphragms incubated in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate. While 2-(3-methyl-cinnamyl-hydrazono)-propionate induced a decrease of palmitate oxidation there was a slight increase of octanoate oxidation.

In a further experiment with fed rats, glucose utilization was enhanced from 0.460 ± 0.151 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in controls to 1.182 ± 0.670 $\mu mol \cdot g^{-1} \cdot h^{-1}$ in test diaphragms by 2-(3-methyl-cinnamyl-hydrazono)-propionate.

Discussion

2-(3-Methyl-cinnamyl-hydrazono)-propionate is a compound that lowers blood glucose levels by multifactorial action. The contribution of accelerated glycolysis in muscle to the decrease in blood glucose after treatment with 2-(3-methyl-cinnamyl-hydrazono)-propionate was not investigated so far.

Data from our study show that this substance causes a concentration-dependent increase of glucose utilisation in rat diaphragms (fig. 2). *Haeckel* et al. (10) found a concentration-dependent effect on glycolysis in human leukocytes.

Clomoxir, which was compared with 2-(3-methyl-cinnamyl-hydrazono)-propionate under these experimental conditions, showed a similar enhancement of glucose oxidation (tab. 1). However, the effect of the corresponding hydrazine (3-methyl-cinnamyl-hydrazine) on glucose utilization was much weaker (tab. 1). In this context, it has to be considered that the protein binding of 3-methyl-cinnamyl-hydrazine (protein-bound fraction: 0.05) was even lower than that of 2-(3-methyl-cinnamyl-hydrazono)-propionate (protein-bound fraction 0.20) (unpublished results). This corresponds to results from experiments with fasted guinea pigs (4), where significant hypoglycaemic effects were achieved only in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate. With 3-methyl-cinnamyl-hydrazine no effect on blood glucose level could be observed in the latter study (4).

2-(3-Methyl-cinnamyl-hydrazono)-propionate was shown to be an inhibitor of fatty acid oxidation (11, 3, 5) in guinea pig liver. In the present study various experiments were performed to determine whether the increase of glucose oxidation in muscle could be explained by the effect on fatty acid oxidation.

In fasted or diabetic animals fatty acids are used as the major substrates for energy metabolism. If the effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on glucose oxidation is mediated by a reduction of fatty acid metabolism it should be more pronounced in fasted than in fed rats. This was confirmed by our experiments, since glucose oxidation was increased by 360% in diaphragms of fasted (tab. 1), but only by 157% in diaphragms of fed rats.

To investigate directly, whether 2-(3-methyl-cinnamyl-hydrazono)-propionate influences the metabolism of long-chain fatty acids in muscle, diaphragms were incubated in a medium containing palmitate. Like clomoxir and 2-bromopalmitate (6, 7), tested for reasons of comparison, 2-(3-methyl-cinnamyl-hydrazono)-propionate induced a highly significant decrease of palmitate utilization (tab. 2). However, the oxidation of short- or medium-chain fatty acids like acetate or octanoate was not decreased by 2-(3-methyl-cinnamyl-hydrazono)-propionate. According to *Pande* (1975), *Ramsey* et al. (1975), and *Murthy* et al. (1984), carnitine acylcarnitine translocase is involved in the carnitine-dependent import of long-chain acyl groups like palmitate into mitochondria.

On the other side the transport of short-chain fatty acids and octanoate does not require the participation of carnitine (19) or carnitine acylcarnitine translocase. So the finding that this hydrazone decreases the oxidation of long-chain but not of short- or medium-chain fatty acids points to an inhibition of long-chain fatty acid transport into mitochondria. An inhibition of carnitine acylcarnitine translocase by 2-(3-methyl-cinnamyl-hydrazono)-propionate has already been demonstrated with mitochondria from guinea pig liver (5).

To obtain further insight into the mechanism of glucose utilization in fasted rats under the influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate, various substrates were employed. Highly significant increases of pyruvate ($p < 0.01$) and lactate ($p < 0.05$) utilization were observed, but there was only an insignificant enhancement of acetate consumption. This points to a direct or indirect activation of the pyruvate dehydrogenase complex or to a decrease of a preexisting inhibition of this enzyme.

As demonstrated in figure 3, the stimulation of glucose oxidation is much higher than that of lactate or pyruvate. This suggests that an additional enzyme, regulating the glycolytic flux, may be influenced by 2-(3-methyl-cinnamyl-hydrazono)-propionate. A decrease of hepatic citrate and acetyl-CoA concentrations has been shown in experiments with fasted guinea pigs (3, 4). It can be speculated that under conditions of increased metabolism of long-chain fatty acids 2-(3-methyl-cinnamyl-hydrazono)-propionate may lower elevated concentrations of citrate and acetyl-CoA in muscle also. In this way the inhibition of phosphofructokinase and pyruvate dehydrogenase may be decreased and glucose utilization be accelerated.

Our results suggest that the blood glucose lowering effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate may be based not only on inhibition of hepatic gluconeogenesis, and jejunal resorption of glucose, but also on a marked increase of the glucose oxidation in muscle. Like some other hypoglycaemic substances (6, 8, 12, 13, 14) 2-(3-methyl-cinnamyl-hydrazono)-propionate may increase glucose utilization in muscle by decreasing the oxidation of long-chain fatty acids according to the "glucose-fatty acid cycle" (1).

2-(3-Methyl-cinnamyl-hydrazono)-propionate has been shown to inhibit hepatic carnitine-acylcarnitine translocase (5). It remains to be established whether the described effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on palmitate oxidation in muscle is also due to an inhibition of translocase-mediated transport of long-chain fatty acids.

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