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Diltiazem Inhibits Fatty Acid Oxidation in the Isolated Perfused Rat Liver

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The effects of diltiazem on fatty acid metabolism were measured in the isolated perfused rat liver and in isolated mitochondria. In the perfused rat liver diltiazem inhibited oxygen uptake and ketogenesis from endogenous substrates. Ketogenesis from exogenously supplied palmitate was also inhibited. The β-hydroxybutyrate/acetoacetate ratio in the presence of palmitate alone was equal to 3·2. When the fatty acid and diltiazem were present simultaneously this ratio was decreased to 0·93, suggesting that, in spite of the inhibition of oxygen uptake, the respiratory chain was not rate limiting for the oxidation of the reducing equivalents coming from β-oxidation. In experiments with isolated mitochondria, incubated in the presence of all intermediates of the Krebs cycle, pyruvate or glutamate, no significant inhibition of oxygen uptake by diltiazem was detected. Inhibition of oxygen uptake in isolated mitochondria was found only when palmitoyl CoA was the source of the reducing equivalents. It was concluded that a direct effect on β-oxidation may be a major cause for the inhibition of oxygen uptake caused by diltiazem in the perfused liver.

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INTRODUCTION

Diltiazem is a voltage-dependent Ca^{2+} channel blocker in cardiac or vascular smooth muscles which finds ample use because of its vasodilatory action.[1,2] In the liver it has been found that diltiazem is able to inhibit the stimulation of glycogenolysis and Ca^{2+} efflux induced by the Ca^{2+}-dependent agonist phenylephrine.[3] Furthermore, the compound has also been shown to prevent the alterations in Ca^{2+} fluxes caused by vasopressin.[4] Yamamoto *et al.*[5] revealed that diltiazem not only abolished the stimulation of glycogenolysis caused by Ca^{2+}-dependent agonists, but also that it abolished glycogenolysis and glycolysis stimulation caused by respiratory inhibitors such as atractyloside, whose effects are independent of intra- and extracellular calcium ions.[6]

Yamamoto *et al.*[5] also showed that diltiazem is an inhibitor of mitochondrial respiration in the intact liver: at a concentration of 500 μM the respiratory chain was approximately 90 per cent inhibited. This toxic effect of diltiazem on the respiratory chain in the intact liver has not been satisfactorily reproduced with isolated rat liver mitochondria. Branca *et al.*[7] found considerable decreases of the ADP/O ratios and of the respiratory control ratios by 1 mM diltiazem in rat liver mitochondria respiring at the expense of succinate. However, whereas the basal respiration was slightly increased, the ADP stimulated respiration was only 24 per cent inhibited. In the presence of an uncoupler, inhibition was increased to 46 per cent, either in the absence or presence of calcium ions.[7] It must be noted that 1 mM diltiazem in direct contact with isolated mitochondria represents a relatively drastic condition if one compares it with the infusion of 0·5 mM diltiazem in the intact liver. In intact cells, the plasma membrane, the numerous

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intracellular binding sites, and the biotransformation reactions, are important factors diminishing the availability of the drug to the mitochondrial membrane.[8,9] For this reason, the possibility exists that inhibition of respiration in the intact liver is not solely the consequence of a deleterious action on the mitochondrial membrane. Since respiration in the perfused liver is mainly the consequence of fatty acid oxidation, the possibility of a direct action of diltiazem on β-oxidation deserves to be analyzed. In the present work this question was analyzed by experiments done both with isolated perfused rat liver and isolated liver mitochondria.

MATERIALS AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the metabolite assays were products of the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The reagent grade chemicals were from Merck (Darmstad, FRG), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Liver Perfusion

Male albino rats (Wistar strain, 200–250 g) were allowed free access to water and to a standard laboratory diet (Purina®). Food was withdrawn 24 h before the surgical removal of the liver under pentobarbital anesthesia (50 mg kg^{-1}). The perfusion technique described by Scholz *et al.*[10] was used. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer at pH 7·4, saturated with an oxygen/carbon dioxide mixture (95/5 per cent) and containing fatty acid-free bovine serum albumin (0·015 mM). The fluid was pumped through a temperature-regulated (37°C) membrane oxygenator before entering the liver via a cannula inserted in the portal vein. The perfusion flow was constant in each individual experiment and it was adjusted to between 30 and 35 ml min^{-1}, depending on the liver weight. Samples of the effluent perfusion fluid were collected in 2- or 4-min intervals and analyzed for β-hydroxybutyrate and acetoacetate.

Experiments with Isolated Rat Liver Mitochondria

Rat liver mitochondria were isolated by differential centrifugation according to Voss *et al.*[11] Oxygen consumption by mitochondria was measured polarographically as described by Voss *et al.*[11] using the incubation medium containing 5 mM disodium phosphate, 10 mM Tris–HCl (pH 7·4), 0·2 mM EDTA, 10 mM potassium chloride, 0·25 M mannitol and 50 mg per cent fatty acid-free bovine serum albumin. When required, substrates and ADP were added to the incubation medium. The ADP/O ratios and the respiratory control ratios were determined according to Chance and Williams.[12]

Analytical

β-Hydroxybutyrate and acetoacetate were measured by standard enzymatic methods, using β-hydroxybutyrate dehydrogenase.[13] The oxygen concentration in the venous perfusate was monitored continuously, employing a teflon-shielded platinum electrode and the rates of metabolite release were calculated from the venous concentrations and the flow rate. All metabolic rates were referred to the wet weight of the liver.

Treatment of Results

The statistical significance of the differences was evaluated by means of the Student's *t*-test. The test was done with the Primer program (version 1·0)[14] and the results are mentioned in the text as the *p* values. $p < 0{\cdot}05$ was adopted as a criterion of significance.

RESULTS AND DISCUSSION

In the first experiments, the action of diltiazem on ketogenesis was investigated. Substrate-free perfused livers from fasted rats are entirely dependent on the oxidation of endogenous fatty acids. Due to the high rates of fatty acid oxidation, the ketogenic activity of such livers is also pronounced. This is shown in Figure 1 which represents the rates of oxygen consumption and β-hydroxybutyrate and acetoacetate production from experiments in which 500 μM diltiazem was infused for 16 min. The introduction of diltiazem produced a rapid reduction of both β-hydroxybutyrate and acetoacetate production (more than 90 per cent inhibition). The time for the half-maximal effect was approximately 3 min for acetoacetate and 1·75 min for β-hydroxybutyrate production. The considerable decrease in oxygen consumption was similar to that already reported

by Yamamoto *et al.*[5] The time for the half-maximal effect was equal to 6·25 min. Thus inhibition of ketogenesis precedes oxygen uptake inhibition by 3 min or more.

Diltiazem also inhibited the oxidation of exogenously supplied palmitic acid. This is illustrated in Figure 2A,B. As revealed by Figure 2A, the introduction of 300 μM palmitate enhanced oxygen consumption (by 0·52 μmol min^{-1} g^{-1}), increased β-hydroxybutyrate production and decreased acetoacetate production. The increase in β-hydroxybutyrate production, however, exceeded the decrease in acetoacetate production, insuring a net increase in ketogenesis (0·7 μmol min^{-1} g^{-1}). Before palmitate infusion (basal conditions) the β-hydroxybutyrate/acetoacetate ratio was equal to $0·23 \pm 0·03$ ($n = 3$); upon palmitate infusion it was increased to $3·20 \pm 0·33$ ($n = 3$). Since the β-hydroxybutyrate dehydrogenase is a near-equilibrium enzyme, the increased β-hydroxybutyrate/acetoacetate ratio in the presence of palmitate reflects the high degree of reduction of the mitochondrial NAD^+-NADH couple.[15]

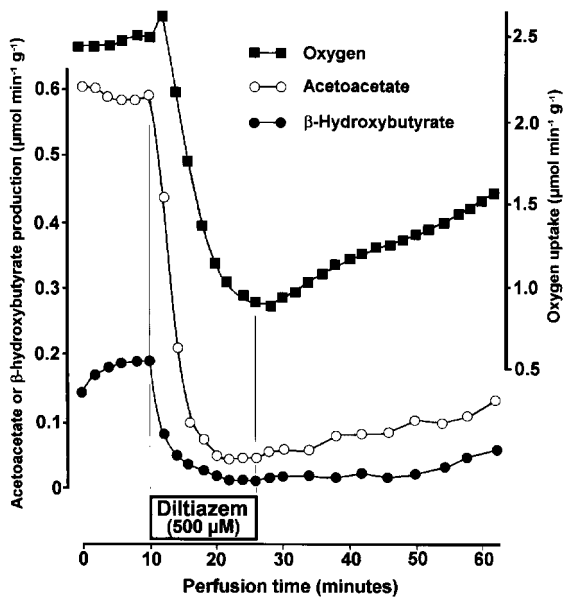

Figure 1. Effects of diltiazem on oxygen uptake and ketogenesis from endogenous substrates in the perfused rat liver. Livers from 24-h fasted rats were perfused. Diltiazem (500 μM) was infused as indicated by the horizontal bar. Samples of the effluent perfusate were collected for the measurement of β-hydroxybutyrate and acetoacetate. Both metabolites were measured enzymatically. The oxygen concentration in the venous perfusate was measured polarographically by means of a platinum electrode. The experimental points are the means of three liver perfusion experiments with identical protocol.

When palmitate was infused in the presence of diltiazem, as illustrated by Figure 2B, the changes caused by the fatty acid were much less pronounced, but there was still a clear increase in both oxygen uptake and β-hydroxybutyrate production. The acetoacetate production, which was decreased by palmitate in the absence of diltiazem, was increased in the presence of the drug. This phenomenon resulted in a β-hydroxybutyrate/acetoacetate ratio of $0·93 \pm 0·23$ ($n = 3$).

The β-hydroxybutyrate/acetoacetate ratios when palmitate was supplied to the liver in the absence of diltiazem were three times higher than those found in the presence of diltiazem (3·2 compared to 0·93). This means that, in the absence of diltiazem, a much greater fraction of the reducing equivalents that were generated by the oxidation of palmitate were exported by the liver in the form of β-hydroxybutyrate. Apparently, the normal activity of the respiratory chain is not able to oxidize all the reducing equivalents generated by palmitate oxidation so that it becomes the limiting factor. Paradoxically, in the presence of diltiazem, which inhibits oxygen uptake, a greater fraction of the reducing equivalents coming from palmitate were oxidized in the respiratory chain. If diltiazem were acting directly and solely on the respiratory chain, the β-hydroxybutyrate/acetoacetate ratio should be increased to values above 3. This is at least what happened in experiments in which an inhibitor of the respiratory chain (isosteviol) was introduced.[16] Conversely, the infusion of stevioside, a compound that inhibits palmitate transport across the plasma membrane without having access to the intracellular space,[17] also decreased both ketogenesis and the β-hydroxybutyrate/acetoacetate ratio (from 2·96 to 0·92).[18]

The subsequent experiments were planned in order to see if the inhibition of oxygen uptake can be reproduced with isolated mitochondria. Table 1 shows the results obtained when the effects of 500 μM diltiazem on the oxidation of pyruvate, glutamate and all intermediates of the Krebs cycle were tested. It is clear from Table 1 that the experiments with isolated mitochondria do not reproduce the oxygen uptake inhibition found in intact cells. Maximally one may conclude that diltiazem increased oxygen uptake under some conditions, for example, in state IV with pyruvate as substrate. Experiments with lower diltiazem concentrations (in the range of 50–200 μM) were equally unsuccessful in demonstrating inhibition of oxygen uptake. By virtue of these results, three

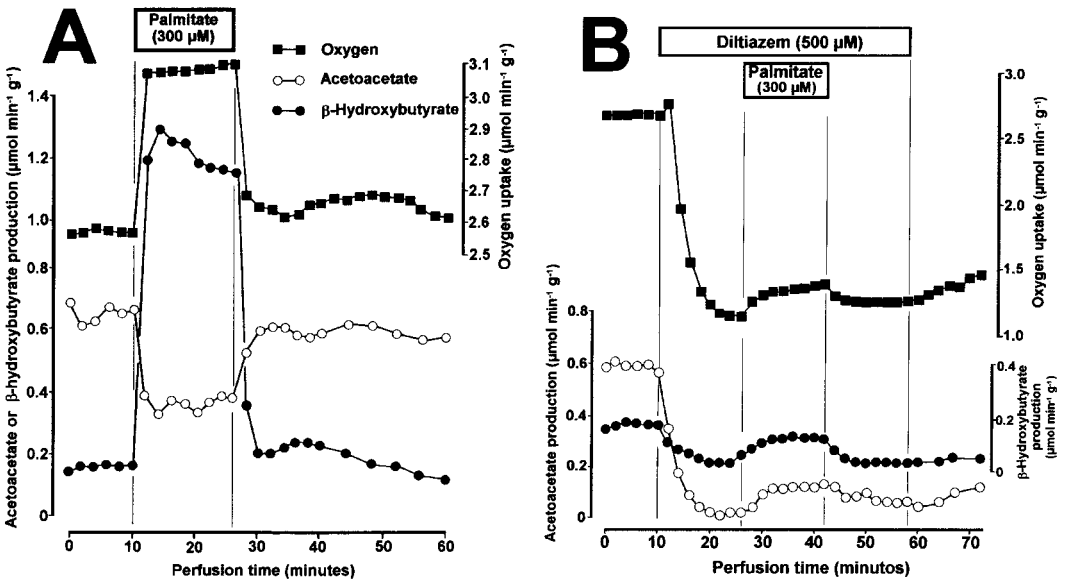

Figure 2. Effects of diltiazem on the oxidation of palmitate in the perfused rat liver. Livers from 24 hours fasted rats were perfused as described in Materials and Methods. Palmitate (300 μM; panels A and B) and diltiazem (500 μM; panel B) were infused as indicated by the horizontal bars. Palmitate was solubilized by 50 μM fatty acid-free bovine serum albumin. Samples of the effluent perfusate were collected for the measurement of β-hydroxybutyrate and acetoacetate. Both metabolites were measured enzymatically. The oxygen concentration in the venous perfusate was measured polarographically by means of a platinum electrode. The experimental points are the means of three liver perfusion experiments with identical protocol.

possibilities can be considered: (1) oxygen uptake inhibition depends on intracellular conditions or factors that are not reproduced in the isolated mitochondria system; (2) in the intact liver cell diltiazem inhibits processes that require metabolic energy so that the lowered ATP demands automatically result in decreased respiratory rates; (3) alternatively, inhibition of oxygen uptake occurs at a step that precedes the oxidation of the intermediates of the Krebs cycle and the other substrates listed in Table 1 (pyruvate and glutamate), a possibility that is also suggested by the changes in the β-hydroxybutyrate/acetoacetate ratios described above. It is difficult to reproduce the cellular environment, so that it is also very difficult to analyze the first alternative listed above. Alternative (2) looks unlikely, because the energy demands of any single biosynthetic process in a substrate-free perfused liver are far below the decreases in oxygen uptake that occurred when diltiazem was infused. For example, diltiazem caused an approximate decrease of 1·5 μmol min^{-1} g^{-1} in oxygen uptake (Figure 1). If one assumes a mean P/O ratio of 2·0 in the intact cell, this means a reduction of 6·0 μmol min^{-1} g^{-1} in ATP production within the mitochondria. No single anabolic process in the liver is known to occur at such high rates in the absence of exogenously supplied substrates.

The third alternative listed above can be more easily analyzed in experimental terms. In the liver, the main substrates for respiration are the long-chain fatty acids. In several experiments with the perfused liver, as illustrated by Figure 2, palmitate was used as the substrate and, for this reason, we decided to investigate the effect of diltiazem on the oxidation of palmitoyl CoA by isolated mitochondria. The results of these investigations are shown in Figure 3. Mitochondria were incubated in the presence of 100 μM palmitoyl CoA and the oxygen concentration in the incubation medium was recorded polarographically. Carnitine was also added in order to allow rapid transport of the palmitoyl residues into the mitochondria. In contrast to what happened with the other substrates listed in Table 1, oxygen consumption with palmitoyl CoA was not constant in time. As shown in Figure 3B, the rates of oxygen consumption, which were calculated from the first derivative of the polynomial of the fourth degree that was fitted to the concentration versus time curves, were maximal initially with a subsequent progressive

Table 1. Diltiazem and the respiratory activity of isolated rat liver mitochondria. The mitochondria (0.6–1.0 mg protein) were added to a reaction medium containing 250 mM mannitol, 5 mM phosphate (pH 7.4), 10 mM TRIS, 10 mM KCl, 0.2 mM EDTA and 8 μM bovine serum albumin. Additions were performed in the following sequence: (1) diltiazem (final concentration: 500 μM), when required; (2) substrates as indicated (basal rates); (3) ADP (500 nmol; state III and state IV respiration). Oxygen concentration was measured polarographically. Temperature of the incubation system was 37°C. RC is the respiratory control ratio (state III/state IV). The data represent the mean values of at least three mitochondrial preparations. Statistical analysis is given as the p value, determined by applying Student's t test.

Substrate	Diltiazem	Basal rate	State III	State IV (nmol min^{-1} ml^{-1})	RC	ADP/O
Pyruvate	−	5·61 ± 0·56	16·18 ± 1·06	5·16 ± 0·43	3·14 ± 0·14	2·44 ± 0·31
(10 mM)	+	6·83 ± 0·98	15·38 ± 0·44	7·57 ± 0·44	2·06 ± 0·32	2·07 ± 0·29
p		0·38	0·71	0·017	0·037	0·42
Citrate	−	8·78 ± 1·70	15·54 ± 1·71	11·26 ± 0·90	1·38 ± 0·04	1·54 ± 0·10
(10 mM)	+	11·03 ± 1·92	16·21 ± 2·06	11·26 ± 1·19	1·44 ± 0·10	1·66 ± 0·28
p		0·43	0·80	1·00	0·59	0·70
Isocitrate	−	12·09 ± 0·55	39·03 ± 3·85	11·55 ± 0·01	3·38 ± 0·33	2·32 ± 0·06
(5 mM)	+	15·39 ± 1·46	43·43 ± 2·91	17·59 ± 1·98	2·49 ± 0·14	2·39 ± 0·04
p		0·10	0·41	0·038	0·139	0·39
Fumarate	−	10·07 ± 0·51	16·47 ± 1·59	12·40 ± 1·69	1·34 ± 0·06	1·29 ± 0·19
(10 mM)	+	11·04 ± 1·21	16·85 ± 0·89	13·37 ± 0·89	1·26 ± 0·02	0·99 ± 0·11
p		0·50	0·84	0·64	0·28	0·24
α-Ketoglutarate	−	5·96 ± 0·22	25·32 ± 2·61	6·52 ± 0·63	4·04 ± 0·75	3·39 ± 0·30
(10 mM)	+	12·19 ± 0·92	32·22 ± 1·69	14·10 ± 1·25	2·29 ± 0·08	2·99 ± 0·16
p		0·003	0·091	0·006	0·081	0·31
L-Glutamate	−	5·13 ± 1·38	33·64 ± 11·7	4·88 ± 1·23	6·99 ± 1·42	3·20 ± 0·03
(10 mM)	+	8·43 ± 2·26	31·50 ± 10·4	7·62 ± 2·40	4·09 ± 0·09	3·03 ± 0·19
p		0·28	0·89	0·37	0·11	0·43
Succinate	−	27·76 ± 6·17	88·14 ± 18·1	21·56 ± 7·28	4·49 ± 0·69	2·19 ± 0·23
(10 mM)	+	32·12 ± 8·74	86·85 ± 21·4	32·18 ± 11·6	3·00 ± 0·75	1·90 ± 0·05
p		0·70	0·97	0·48	0·22	0·29
Oxaloacetate	−	9·01 ± 0·45	11·49 ± 0·68	9·46 ± 0·01	1·21 ± 0·07	0·97 ± 0·12
(5 mM)	+	8·56 ± 0·45	10·36 ± 0·45	9·01 ± 0·45	1·15 ± 0·01	0·98 ± 0·09
p		0·52	0·24	0·37	0·41	0·36
DL-Malate	−	8·91 ± 1·03	17·05 ± 0·77	12·59 ± 1·7	1·40 ± 0·18	1·02 ± 0·04
(10 mM)	+	8·14 ± 0·33	15·11 ± 1·34	12·40 ± 1·0	1·22 ± 0·02	0·79 ± 0·09
p		0·51	0·38	0·93	0·36	0·08

decline. After 15 min the rate of oxidation had already been reduced by 50 per cent. It is clear, however, that 500 μM diltiazem inhibits palmitoyl CoA oxidation. The phenomenon can be more easily appreciated in Figure 3A, where the inhibition degree was represented against time. Initially the rate of palmitoyl CoA oxidation was only inhibited by 20 per cent. However, the inhibition degree increased with time: at 10 min it was equal to 70 per cent, at 15 min it was 85 per cent and at 20 min it was virtually 100 per cent.

In conclusion, it is apparent that diltiazem exerts a direct effect on β-oxidation. This is indicated by measurements in the perfused liver as well as in experiments with isolated mitochondria. This direct effect on β-oxidation is likely to contribute, partly at least, to the strong inhibition of respiration caused by the drug. One cannot completely exclude, however, that diltiazem also exerts effects that depend on the presence of factors (compounds or ATP demanding metabolic pathways) that are normally present in the intact cell, but absent in an incubation containing isolated mitochondria.

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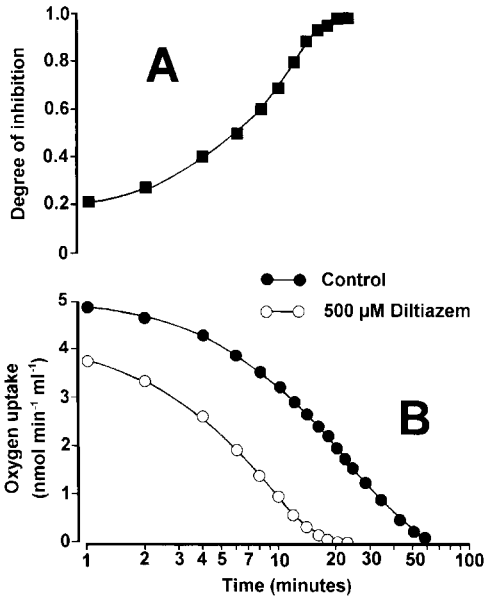

Figure 3. Effects of diltiazem on the rates of oxygen consumption of rat liver mitochondria incubated in the presence of palmitoyl CoA. Rat liver mitochondria (0·6 mg ml^{-1} protein) were incubated in reaction medium supplemented with 5 mM carnitine, 5 mM oxaloacetate and 100 μM palmitoyl CoA. The decrease in oxygen concentration was recorded polarographically. Polynomials of the fourth degree were fitted to the concentration versus time curves and the rates of oxygen uptake, that are represented against time in panel B, were calculated as the first derivatives of the optimized regression curves. For the control curve (●—●) the polynomial was $\hat{y}_{control} = 0{\cdot}283 + 5{\cdot}109t - 0{\cdot}113t^2 + 0{\cdot}00131t^3 - 0{\cdot}00000677t^4$; for the curve obtained when diltiazem was present (○—○) the polynomial was $\hat{y}_{diltiazem} = -0{\cdot}276 + 4{\cdot}209t - 0{\cdot}227t^2 + 0{\cdot}00441t^3 - 0{\cdot}00000898t^4$. In panel A the degree of inhibition caused by diltiazem is represented against the incubation time.

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