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EFFECTS OF CLOFIBRATE, *IN VITRO*, ON MITOCHONDRIAL RESPIRATION AND OXIDATIVE PHOSPHORYLATION

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Abstract—The effects of clofibrate on mitochondrial respiration and oxidative phosphorylation were studied *in vitro*. Clofibrate inhibited state 3 oxidation, stimulated state 4 oxidation and lowered ADP:O ratio when 3-hydroxybutyrate, succinate and ascorbate plus tetramethylphenylenediamine (TMPD) were used as substrates. The inhibitions of state 3 oxidation were not reversed by dinitrophenol. In mitochondrial fragments, both NADH- and succinate-linked oxidations were inhibited by clofibrate but ascorbate plus TMPD oxidation was not. A higher concentration of clofibrate was required to inhibit succinate oxidation than NADH oxidation. In mitochondrial fragments, 50 per cent inhibitions of respiration, with NADH and succinate as substrates, were elicited with concentrations of 0·09 and 0·4 μmoles of clofibrate/mg of protein, respectively. It was suggested that there are at least two distinct sites at which clofibrate can inhibit respiration. One site exists between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the electron transport chain. Another, less sensitive site, exists between the interaction of succinate with succinate dehydrogenase and cytochrome *c*.

RECENTLY, Katyal *et al.*[1] showed that administration of clofibrate *in vitro* can inhibit mitochondrial oxygen consumption and oxidative phosphorylation, and increase adenosinetriphosphatase (ATPase) activity. These effects were discussed with regard to a possible mechanism for clofibrate-induced reduction of blood lipid concentration.

This present report describes a more complete study of the effects of clofibrate on mitochondrial respiratory processes, in which the primary sites of action have been identified.

METHODS

Animals. Male Sprague–Dawley rats (body wt 150–200 g) were used for all experiments. These rats were fed a commercial pelleted diet [Rockland Mouse/Rat Diet (Complete)], *ad lib.*

Isolation of intact rat liver mitochondria. Mitochondria were freshly isolated by the method of Johnson and Lardy[2] and suspended in 0·25 M sucrose at a concentration of 27–28 mg of protein/ml. Mitochondrial protein was determined by the biuret procedure of Szarkowska and Klingenberg.[3]

Preparation of mitochondrial fragments. A 5-ml aliquot of mitochondrial suspension was sonicated for 1 min at 50 W. The Sonifier (Branson Sonic Power Co.) was equipped with a 0·5-in. disruptor horn.

Clofibrate solution. Clofibrate is rather insoluble in H_2O and buffer solutions, and therefore had to be dissolved in an organic solvent. Ethanol was chosen because it was found that concentrations of up to 1% (v/v) ethanol in the mitochondrial incubation medium did not alter respiratory rate or oxidative phosphorylation.

Oxygen consumption of intact mitochondria. Oxygen consumption was determined polarographically at 30° in a Gilson oxygraph (Gilson Medical Electronics Inc., Middleton, Wis.) equipped with a vibrating platinum electrode. The basic incubation medium contained 65 mM Tris buffer (pH 7·4), 75 mM KCl, 5 mM $MgCl_2$, 12 mM phosphate buffer (pH 7·4), 1 mM EDTA and 8·6 mM substrate. The substrates were 3-hydroxybutyrate, succinate and ascorbate. When ascorbate was used, 0·2 μmole of *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylenediamine-2 HCl (TMPD)/mg of mitochondrial protein were included in the medium to facilitate transfer of electrons from ascorbate to the mitochondrial electron transport chain.[4] In the course of each experiment, incubation medium (2·6 ml), mitochondrial suspension (0·1–0·2 ml) and ethanol or clofibrate in ethanol (0–0·02 ml) were added to the oxygraph chamber followed by sequential additions (0·1 ml) of 6 mM ADP in 0·15 M KCl. ADP:O ratios (i.e. P:O ratios) and the rates of oxygen consumption in states 3 and 4 were determined by the methods of Chance and Williams.[5,6] In a second series of experiments, 2,4-dinitrophenol (DNP; 0·22 μmole/mg protein) was added before or after ADP to determine clofibrate effects on uncontrolled respiration. It was assumed that the incubation medium and ADP solution had an O_2 concentration of 0·24 mM and that the mitochondrial suspension was anaerobic.

Oxygen consumption of mitochondrial fragments. Oxygen consumption of mitochondrial fragments was measured polarographically at 30° in a Gilson oxygraph equipped with a Clark oxygen electrode. The incubation medium was the same as described above except that substrate concentration was 11 mM. However, since the sonicated mitochondria respired maximally in the absence of ADP, reactions were started by addition of substrate and ADP was not used. Reagents were added to the reaction vessel in the following order: (1) incubation medium (1·7 ml, not including substrate), (2) sonicated mitochondrial suspension (0·1–0·2 ml), (3) ethanol or clofibrate in ethanol (0–0·02 ml) and (4) substrate (0·25 ml).

Chemicals. Clofibrate (*p*-ethyl-clorophenoxyisobutyrate) was obtained from Ayerst Laboratories, N. Y. Organic reagents were from Sigma Chemical Company, St. Louis, and inorganic salts from Mallinckrodt Chemical Company, St. Louis.

RESULTS

Effects of clofibrate on oxidation of 3-hydroxybutyrate by intact rat liver mitochondria. Figure 1 shows the effects of clofibrate on oxygen consumption of states 3 and 4, and on P:O ratio. State 3 oxidation was inhibited by approximately 50 per cent at a dose of 0·12 μmole of clofibrate/mg of protein. At concentrations above 0·12 μmole/mg of protein, the state 3 and state 4 respiratory rates were approximately the same and it was not possible to determine P:O ratios via the indirect method of Chance and Williams.[5,6] Nevertheless, decreased P:O ratio was clearly evident at the lower clofibrate concentrations.

Clofibrate also inhibited DNP-stimulated respiration at the concentrations which inhibited state 3 oxidation and, in addition, DNP did not relieve the clofibrate-produced inhibitions of state 3 oxidation.

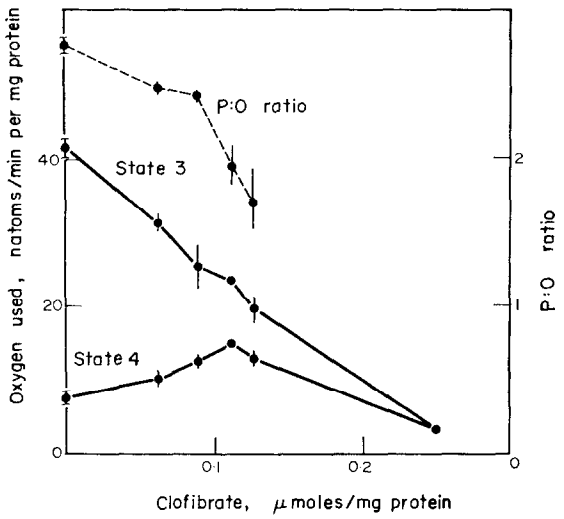

FIG. 1. Effects of clofibrate on oxygen utilization and P:O ratio of intact mitochondria with 3-hydroxybutyrate as substrate. The experimental procedure is described under Methods. Each data point indicates the mean value. The S.E.M. is shown for data points representing three or more replicate determinations.

Effects of clofibrate on oxidation of succinate by intact rat liver mitochondria. Figure 2 shows the effects of clofibrate on oxygen consumption of states 3 and 4, and on P:O ratio. In general, the effects of clofibrate on succinate oxidation were qualitatively similar to those found when 3-hydroxybutyrate was the substrate; state 3 oxidation

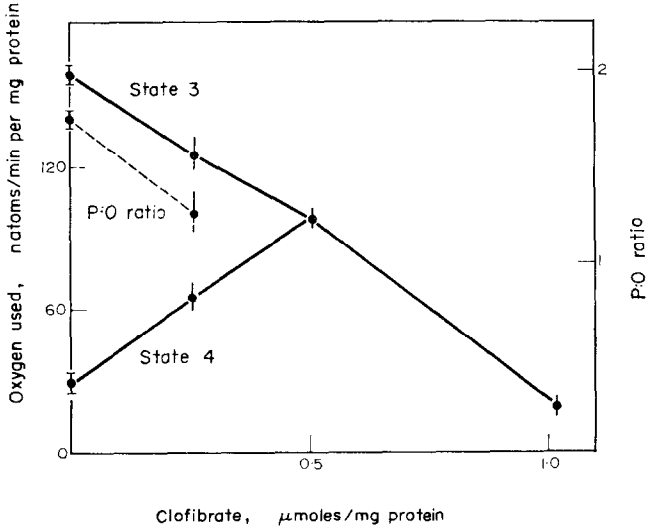

FIG. 2. Effects of clofibrate on oxygen utilization and P:O ratio of intact mitochondria with succinate as substrate. The experimental procedure is described under Methods. Each data point indicates the mean value. The S. E. M. is shown for data points representing three or more replicate determinations.

was inhibited, state 4 oxidation stimulated and P:O ratio lowered. State 3 oxidation was inhibited by approximately 50 per cent at a dose of 0·62 μmole of clofibrate/mg of protein. At concentrations above 0·5 μmole/mg of protein, the state 3 and 4 respiratory rates were approximately the same and, therefore, P:O ratios could not be determined.

Clofibrate also inhibited DNP-stimulated respiration at the same concentrations which inhibited state 3 oxidation and, in addition, DNP did not relieve the clofibrate-produced inhibitions of state 3 oxidation.

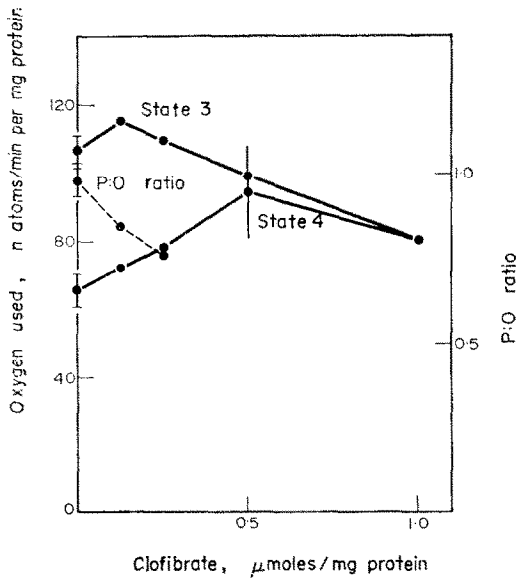

FIG. 3. Effects of clofibrate on oxygen utilization and P:O ratio of intact mitochondria with ascorbate plus TMPD as substrate. The experimental procedure is described under Methods. Each data point indicates the mean value. The S.E.M. is shown for data points representing three or more replicate determinations.

Effects of clofibrate on oxidation of ascorbate plus TMPD by intact rat liver mitochondria. Figure 3 shows the effects of clofibrate on oxygen consumption in states 3 and 4, and on P:O ratio. As with both 3-hydroxybutyrate and succinate substrates, clofibrate inhibited state 3 oxidation, stimulated state 4 oxidation, and lowered the P:O ratio. However, the inhibition of state 3 respiration was not marked and 1·0 μmole of clofibrate/mg of protein produced only about a 30 per cent decrease in rate.

Effects of clofibrate on oxidation of NADH, succinate and ascorbate plus TMPD in mitochondrial fragments. In order to determine if clofibrate inhibited state 3 respiration by direct action on the respiratory chain, experiments were performed using mitochondrial fragments prepared by sonication. The sonication procedure disrupts mitochondrial membranes and exposes the inner membrane surfaces to the external medium; this procedure reduces the probability of permeability barriers, between medium and matrix, affecting the respiratory rates.[7] The fragments respired when substrate was added and showed complete loss of respiratory control.[5,6]

We found that sonicated mitochondria respired quite rapidly when ascorbate plus TMPD was added but not when 3-hydroxybutyrate was added. NAD concentration was found to be rate limiting for the 3-hydroxybutyrate oxidation. Since the fragments oxidized the NADH synthesized from free NAD added to the system, we decided to switch to NADH as substrate and, in this manner, eliminate or indirectly identify a possible clofibrate inhibition of 3-hydroxybutyrate dehydrogenase.

The mitochondrial fragments also showed a reduced rate of succinate-linked respiration. However, the rate was measurable and, therefore, succinate was a suitable substrate.

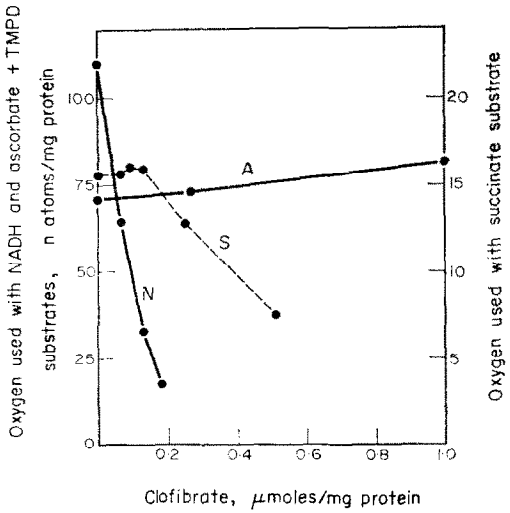

FIG. 4. Effects of clofibrate on oxygen utilization of mitochondrial fragments with NADH (N), succinate (S) and ascorbate plus TMPD (A) as substrates. The experimental procedures are described under Methods. Data points represent the mean values of two to four replicate determinations.

The results of our experiments with mitochondrial fragments are summarized in Fig. 4. It can be seen that clofibrate inhibited succinate- and NADH-linked oxidations by fragments as well as by intact mitochondria. However, ascorbate plus TMPD oxidation was not inhibited by 1·0 μmole of clofibrate/mg of protein. In the intact mitochondria, this concentration produced a 30 per cent inhibition of state 3 oxygen consumption. Concentrations above 1 μmole/mg of protein were not tested because of the low solubility of clofibrate in aqueous media even in the presence of ethanol.

DISCUSSION

In attempting to determine the primary site, or sites, of clofibrate action on mitochondrial respiration, it was convenient to confine our work to three areas of investigation: (1) effects on membrane permeability, (2) effects on the dehydrogenases and the electron transport chain and (3) effects on the phosphorylating chain. The electron transport and phosphorylating chains are shown schematically in Fig. 5. Our experiments, by process of elimination, have enabled us to show that clofibrate, *in vitro*, inhibited the electron transport chain and uncoupled oxidative phosphorylation.

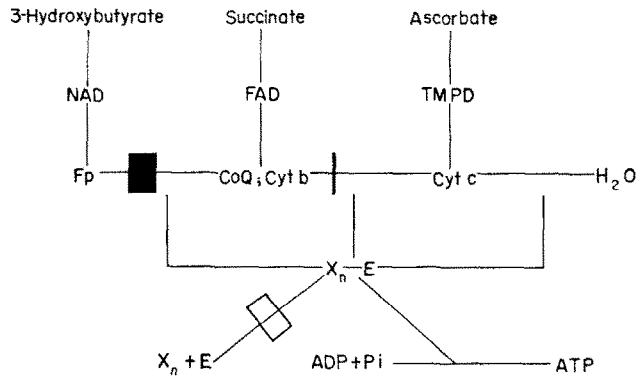

FIG. 5. Hypothetical scheme of the energy-transfer system, linked to the electron transport chain, and the sites of clofibrate action. Abbreviations: Fp, NADH dehydrogenase; Co Q, coenzyme Q; Cyt, cytochrome; TMPD, tetramethylphenylenediamine; X_n, hypothetic energy transfer carriers; E, energy. The sites of clofibrate action are at or before the sites indicated by the bars. The widths of the bars represent relative activity (see Lee and Ernster[8] and Hoch[9]).

Stepwise analysis of our results is as follows:

Mitochondrial inhibition. The experiments of Figs. 1–3 show that clofibrate inhibited mitochondrial oxygen consumption when appropriate dose levels, relative to the substrate used, were applied. Clofibrate was most potent when 3-hydroxybutyrate was the substrate, about one-fifth as potent when succinate was the substrate, and almost ineffective when ascorbate plus TMPD was the substrate. These inhibitions were not relieved by addition of DNP, the classical uncoupler of oxidative phosphorylation[10] and, in addition, DNP-stimulated respiration was inhibited in a manner similar to that shown for state 3. These findings showed that the inhibitory effects of clofibrate were not located within the phosphorylating system but occurred proximal to the DNP sensitive site ($X_n - E \rightarrow X_n + E$; Fig. 5). Inhibition exerted distal to the DNP sensitive site is reversed by addition of DNP.[11–13]

In order to examine the possibility that clofibrate reduced the state 3 respiratory rates by interfering with mitochondrial substrate uptake, we studied the effects of clofibrate on respiration of mitochondrial fragments. The results, shown in Fig. 4, indicated that clofibrate-induced inhibition of respiration did not require intact membranes when either succinate or NADH were the substrates. However, the slight inhibition of ascorbate plus TMPD oxidation in the intact mitochondria at the highest clofibrate concentration (1 μmol/mg of protein; Fig. 3) was not seen with the mitochondrial fragments (Fig. 4). It can, therefore, be suggested that this particular inhibition was, in some manner, related to a permeability factor.

Our results allow us to suggest that there are at least two distinct sites at which clofibrate can exert inhibitory effects on respiration (Fig. 5). At least one site exists between the interaction of NADH with NADH dehydrogenase and the point at which electrons from succinate oxidation enter the electron transport chain. This must be the case since inhibition of NADH-linked oxidation occurred at drug concentrations which did not affect succinate oxidation (Figs. 1, 2 and 4). At least one other, less sensitive site, exists between the interaction of succinate with succinate dehydrogenase and cytochrome *c*, the probable point at which electrons from ascorbate plus TMPD

oxidation enter the electron transport chain.[14] Lack of inhibition with ascorbate plus TMPD as substrate indicates the absence of a clofibrate inhibitory site distal to cytochrome *c*.

Mitochondrial oxidative phosphorylation. Uncoupling of oxidation from phosphorylation causes energy to be released, probably as heat, rather than conserved as ATP. This decreased efficiency results in more oxygen consumption per quantity of ADP phosphorylated. In our *in vitro* system, uncoupling was indicated by a fall in the ratio ADP phosphorylated:oxygen consumed (i.e. P:O ratio). Uncoupling is usually accompanied by increased ATPase activity and increased state 4 oxygen consumption. The effects of uncoupling on mitochondrial respiration and oxidative phosphorylation have been recently reviewed.[9]

Clofibrate produced a fall in the P:O ratio and stimulated state 4 respiration suggestive of uncoupling. In accord with these findings, Katyal *et al.*[1] showed that clofibrate, *in vitro*, increases ATPase activity. We found evidence of uncoupling in the dose range 0·06–1·0 μmole of clofibrate/mg of protein with all of the substrates and, it seems there is no evidence suggesting site specificity for the effect. The uncoupling activity always accompanied the inhibitory activity such that it did not appear that one effect would occur without the other. However, additional experiments utilizing an NAD-linked substrate and low concentrations of clofibrate (< 0·06 μmole/mg of protein) are required to demonstrate this conclusively.

Physiological considerations. Our results do not permit us to conclude that clofibrate, at physiological concentrations, produces inhibition and uncoupling of oxidative phosphorylation. However, Thorp[15] has shown that respiratory quotient and oxygen consumption are decreased by administration of clofibrate to rats, and this finding might be explained by an inhibition of mitochondrial oxidative processes.

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