CHAPTER IV

DISCUSSION AND CONCLUSION

The results presented here show that bezafibrate can influence the important functions of mitochondria namely, oxidative phosphorylation and calcium transport. This drug inhibits both states 3 and 3u respiration (figure 14) and stimulates calcium release (figure 19) by isolated rat liver mitochondria. On the other hand, the ATPase activity is slightly affected (table 8) and no effect on MAO activity was observed (figure 22). The discussion below concerns mainly with the plausible mechanism of bezafibrate action on mitochondrial bioenergetics and whether these effects might participate in the pharmacological and/or toxicological action of bezafibrate.

Effects of Bezafibrate on Mitochondrial Oxidative Phosphorylation and ATPase Activity.

Bezafibrate was found to inhibit oxidative phosphorylation when mitochondria were respiring with glutamate plus malate. From the dose-response curves in figure 15, the rates of state 3 and state 3u respiration decreased as the concentration of bezafibrate increased. This result indicates that bezafibrate may depress oxidative

phosphorylation through effect on the respiratory chain since the inhibition pattern, the blockade of both states 3 and 3u respiration, resembles that of the respiratory chain inhibitors. When succinate was respiratory substrate, the states 3 and 3u respiratory rates were insignificantly affected by bezafibrate as shown by the dose-response curves in figure 16. In this respect, the inhibition of both states 3 and 3u respiration with NAD^+-linked substrates but not with succinate, the effect of bezafibrate is similar to that of rotenone, a well known and potent site I respiratory chain inhibitor [30,43]. Thus bezafibrate appears to exert inhibitory effect at the early part of mitochondrial respiratory chain, i.e., the segment from NADH to CoQ or site I. Further experiments with isolated complex I are needed to prove that bezafibrate acts directly on this complex to inhibit electron flow from NADH to ubiquinone. Interestingly, state 4 respiration with either glutamate plus malate or succinate was unaffected by bezafibrate. In contrast, gemfibrozil, another fibric acid derivative, has uncoupler-like activity since it has been shown to stimulate state 4 respiration [22]. Apparently, bezafibrate does not possess the uncoupling effect on mitochondrial oxidative phosphorylation.

The inhibitory effect of bezafibrate on state 3 and state 3u respiration suggests the respiratory chain but does not exclude the ATP synthase complex as the site of

bezafibrate action. The possibility that bezafibrate might also depress oxidative phosphorylation by interfering with the ATP synthase complex can be assessed by studying the effect of bezafibrate on the uncoupler-activated mitochondrial ATPase activity. The ATPase reaction induced by the DNP-type uncouplers is generally believed to represent the reversed process of the respiratory chain-linked ADP phosphorylation [44]. As reported in table 8, bezafibrate slightly inhibited the DNP-stimulated ATPase activity whereas oligomycin, a well known and powerful inhibitor of mitochondrial oxidative phosphorylation [44, 45], severely depressed the enzyme. Thus it seems unlikely for bezafibrate to retard oxidative phosphorylation by acting mainly on the mitochondrial ATP synthase system. However, the small inhibitory effect on the uncoupler-activated ATPase activity may account for the observation that bezafibrate inhibits state 3 more than state 3u respiration. At present, it is not clear why oligomycin, in the absence of DNP, further augmented the small bezafibrate-induced ATPase activity.

Factors Modifying Bezafibrate Action on Oxidative Phosphorylation.

From the study of various factors affecting bezafibrate action, it was found that different pH can influence the inhibitory effect on mitochondrial respiration.

Bezafibrate produced the strongest inhibition at acidic pH and the inhibition declined when the pH was increased (table 4). As bezafibrate is a weak acid [7] possessing ionizable carboxylic group, lowering the medium pH will increase the concentration of the unionized form. This form presumably penetrates the mitochondrial inner membrane more readily than the ionized form due to its higher lipophilic property and acts on the respiratory chain enzymes located in the inner membrane. Thus this observation suggests that the active form of bezafibrate is the unionized structure and the site of action is somewhere inside the inner membrane.

The sulfhydryl group (-SH) in the mitochondrial inner membrane is known to play an important role in several inner membrane functions, for example, the inner membrane permeability, the coupling of oxidative phosphorylation, the function of ATP synthase and several ions transport [46,47,48]. The present study shows that the mechanism of bezafibrate action on oxidative phosphorylation does not involve the mitochondrial sulfhydryl group since DTT, a sulfhydryl group-protecting substance [49], failed to attenuate or altered the inhibitory effect of bezafibrate (table 5).

Bovine serum albumin (BSA) is the other factor which can influence bezafibrate action on oxidative phosphorylation

(table 7). The presence of BSA in reaction mixture reduced, in a dose-related fashion, the inhibition produced by bezafibrate on state 3 respiration. Since BSA is not expected to cross mitochondrial membrane, the antagonistic effect of BSA presumably exerted extramitochondrially. Thus by complexing with extramitochondrial bezafibrate, BSA reduces the free drug concentration around mitochondrial vicinity thereby promoting bezafibrate to dissociate from mitochondrial inner membrane. Obviously, this proposal implies that BSA should have relatively higher affinity for bezafibrate than the inner membrane. In agreement with the above notion, bezafibrate is known to be highly bound to plasma albumin [1,7].

Effect of Bezafibrate on Mitochondrial Calcium Transport.

Mitochondria possess an elaborate system for transporting calcium ions across their inner membrane. They could take up large amounts of calcium ions by a uniporter, a mechanism that facilitates the diffusion of an ion down its electrochemical gradient and does not couple the transport to that of any other ion or molecule [50]. Calcium ions penetrate across the inner membrane without charge compensation in response to the negative membrane potential established inside the inner membrane by the activity of the respiratory chain or by ATP hydrolysis [51].

Preliminary study has revealed the depressive action of bezafibrate on calcium-stimulated respiration by isolated rat liver mitochondria in a dose-related manner (figures 17 and 18), suggesting that this drug has effect on mitochondrial calcium transport. Further experiments to elaborate the effect of bezafibrate on calcium transport by mitochondria were then performed with calcium-selective electrode. The results of glutamate plus malate-supported calcium transport (figure 19) show that bezafibrate slightly inhibited calcium uptake while the main effect was to stimulate calcium efflux. The calcium-releasing effect can be observed with both substrate-and ATP-supported calcium transport (figures 19 and 20). The effect of bezafibrate on mitochondrial calcium transport (influx and efflux) was found to possess a distinct pattern compared with those mediated by DNP-an uncoupler, rotenone-a site I respiratory chain inhibitor and ruthenium red-a relative specific inhibitor of mitochondrial calcium uniporter [52, 53] (figures 19 and 21). These results suggest that the mechanism of action of bezafibrate on mitochondrial calcium transport, particularly the efflux process, should be different from those of DNP, rotenone and ruthenium red.

At present, the mechanism by which bezafibrate stimulates calcium release from the mitochondria is a matter of conjecture. It is generally known that mitochondria contain an electrophoretic uniporter which is used almost

exclusively for the uptake of calcium and at least two or three calcium efflux mechanisms. The Na^+-dependent Ca^{2+} efflux [54] is mediated by a Ca^{2+}/nNa^+ exchanger which is most prominent in brain and heart mitochondria [56]. The second is Na^+-independent Ca^{2+} efflux [55] which may be mediated by a $Ca^{2+}/2H^+$ exchanger or a cotransporter of Ca^{2+} and Pi or an active mechanism in which energy for Ca^{2+} efflux comes from electron transport chain or ATP hydrolysis. This mechanism is most prominent in liver and kidney mitochondria [56]. Another attractive mechanism which, in recent years,has been investigated as a potential calcium release mechanism is the mitochondrial membrane permeability transition [50,57]. It is induced by Ca^{2+} in conjunction with another agent referred to as an inducing or a Ca^{2+}-releasing agent. The order of addition is not important, but Ca^{2+} must be accumulated into the matrix space for the transition to occur. The high permeability state eliminates energy-linked functions such as membrane potential preservation, Ca^{2+} retention, or coupling of the oxidative phosphorylation. Thus Ca^{2+} or solute movements occur by facilitated diffusion driven by their concentration gradients. This mechanism can release large Ca^{2+} loads rapidly in an energy-efficient manner [50,57]. There are many agents that can induce mitochondrial membrane transition. The mechanism of the transition is still controversial. Two candidate mechanisms have been considered. One postulated that solutes cross through a

proteinaceous pore, of which inhibitors and activators of the transition bind to specific sites affecting the tendency of the pore to open or close in an allosteric fashion [58]. Another proposed that phospholipase A_2 activity may also create the transition, particularly under pathological conditions, by causing permeability defects in the membrane lipid phase [59].

It is proposed that bezafibrate may act as an inducing agent causing mitochondrial membrane transition in as much as calcium release mediated by bezafibrate is complete and the calcium releasing pattern of this drug is different from the uncoupler (i.e., DNP), respiratory chain inhibitor (i.e., rotenone) and inhibitor of calcium uniporter (i.e.,ruthenium red). Alternatively, bezafibrate may act by stimulating the Na^+-independent Ca^{2+} efflux mechanism which is particularly prominent in liver mitochondria. Clearly, much more work is needed to clarify this point.

Comparison of Bezafibrate and Clofibric Acid Effects on Oxidative Phosphorylation and Calcium Transport.

Clofibrate is the prototype of the fibric acid derivatives. It also possesses the efficacious lipid-lowering property and was used widely for the treatment of hypertriglyceridemia [60]. However, its use has become

increasingly circumscribed because it has not been proven to be effective for the prevention of atherosclerosis; furthermore, awareness of latent adverse effects has grown [61]. Comparative study of bezafibrate and clofibric acid, the active form of clofibrate, on oxidative phosphorylation shows that clofibric acid produces similar effects to bezafibrate in inhibiting states 3 and 3u respiration with glutamate plus malate as substrates. This result indicates that clofibric acid also act as the respiratory chain inhibitor. However, in contrast with bezafibrate, clofibric acid was found to significantly stimulate state 4 respiration, i.e., it has uncoupling activity. In this respect clofibric acid behaves like gemfibrozil [22].

Clofibric acid, gemfibrozil and bezafibrate are in the same group of fibric acid and all are weak acidic compounds like the DNP-type uncouplers [62]. The reason why bezafibrate fails to uncouple the mitochondria may be related to its relatively large molecular size compared with other two drugs. This makes it more difficult for bezafibrate to transport H^+ into matrix by shuttling across inner membrane in the same manner as the DNP-type uncouplers [63]. The chemical structures of the three fibric acid derivatives are shown in figure 25.

The main effect of clofibric acid on mitochondrial calcium transport is to enhance calcium release. However,

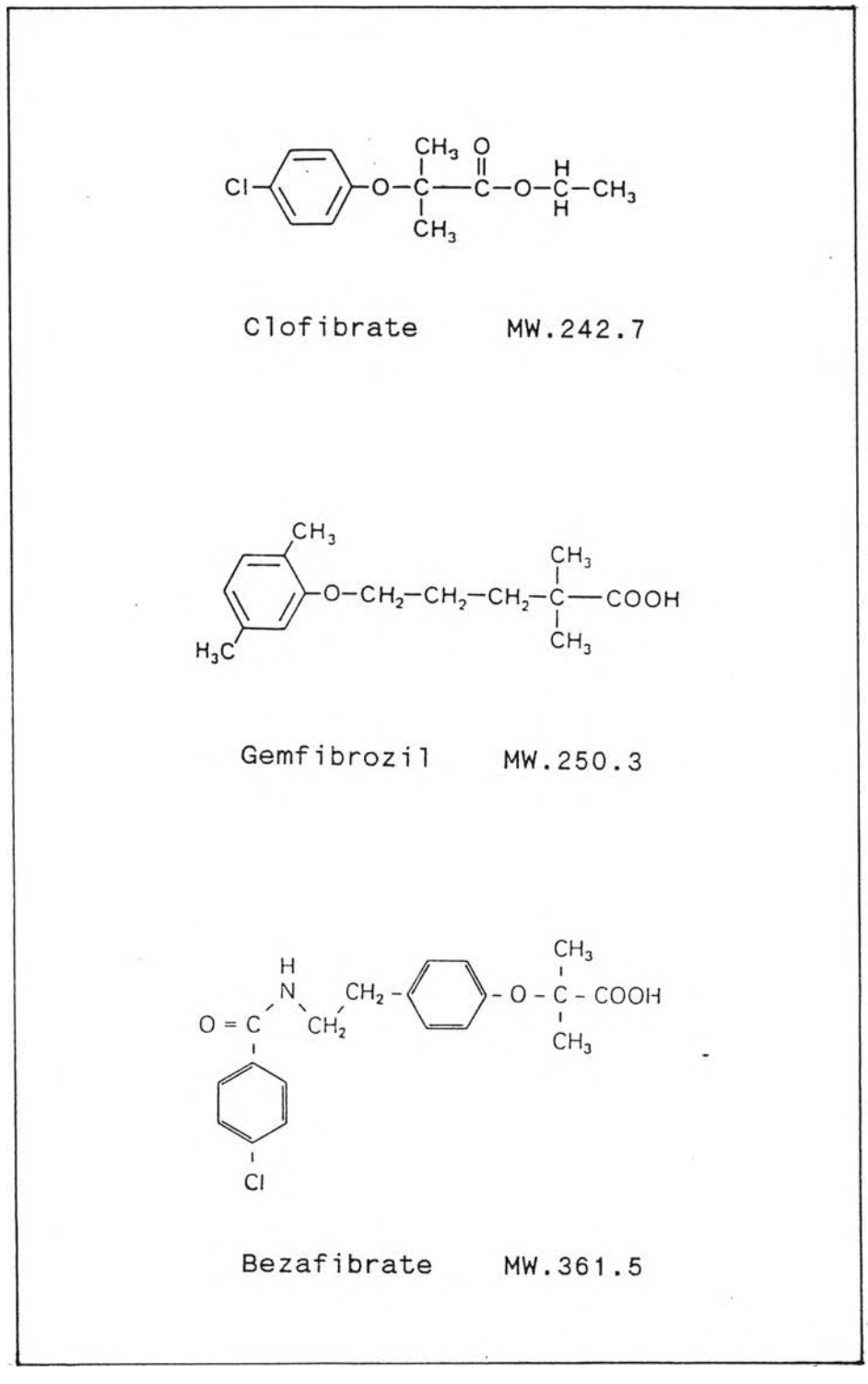

Figure 25. Chemical structure of three fibric acid derivatives.

clofibric acid appears to be considerably less potent than bezafibrate. It is not certain to what extent the uncoupling action contributes to the calcium releasing activity of clofibric acid. Nevertheless, in view of its weak uncoupling activity, this action is unlikely to play a major role in the clofibric acid-evoked calcium efflux. It should be noted in this connection that gemfibrozil has also been shown to be particularly active in stimulating calcium efflux by isolated rat liver mitochondria [22].

The possible participation of the mitochondrial effects of bezafibrate described here in the pharmacological and/or toxicological actions of this drug is at best conjectural. Because bezafibrate, clofibric acid as well as gemfibrozil [22] can affect important mitochondrial functions, it is tempting to speculate that these mitochondrial effects should play a role in their hypolipidemic activity. The depressive effect on oxidative phosphorylation could conceivably reduce intracellular ATP level and, consequently, impede biosynthetic capacity of hepatic cell. In addition, the mitochondrial calcium efflux induced by these drugs may disturb intracellular calcium homeostasis causing the intracellular Ca^{2+} concentration to rise. The increase in cytosolic Ca^{2+} level may possibly interfere with cellular enzymes activity [64] including those involved in lipid metabolism. Alternatively, the mitochondrial functional disturbances described above may

largely involve in the adverse effects of these fibric acid derivatives. In this regard, muscle myopathy, a side effect caused by bezafibrate has been found to associate with primary lesions in mitochondrial functions [26]. Much further work is needed to establish the relationship between the mitochondrial effects and biological activities of these lipid-lowering drugs. It is noteworthy that bezafibrate has been reported to cause proliferation of peroxisome [19], an organelle containing enzymes of β-oxidation [65], in the rodent liver; and this phenomenon may be an adaptive response to mitochondrial inhibition [20].

In conclusion, bezafibrate has dual actions on mitochondrial bioenergetics. Firstly, bezafibrate acts as a site I respiratory chain inhibitor to depress oxidative phosphorylation. Secondly, this drug enhances calcium efflux possibly by acting as an inner membrane transition-inducing agent. The site of action of bezafibrate is presumably on mitochondrial inner membrane where the respiratory chain enzymes are located. Whether these mitochondrial effects contribute to the hypolipidemic activity and/or adverse effect of this drug remains to be investigated.