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EVALUATION OF INHIBITORS OF FATTY ACID OXIDATION IN RAT MYOCYTES

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Abstract—The effects of 4-bromocrotonic acid, 2-bromopalmitic acid, 3-mercaptopropionic acid, 4-pentenoic acid, and 2-tetradecylglycidic acid on the oxidations of palmitate, octanoate, and pyruvate in adult rat myocytes were studied. Since all of these compounds inhibit the oxidation of palmitate but not of pyruvate, they are specific inhibitors of fatty acid oxidation. Fifty percent inhibition of palmitate oxidation was obtained when myocytes were preincubated for 10 min with one of the following: 0.1 μM 2-tetradecylglycidic acid, 60 μM 4-bromocrotonic acid, 60 μM 2-bromopalmitic acid, 100 μM 3-mercaptopropionic acid, or 100 μM 4-pentenoic acid. Removal of the inhibitors from the medium after preincubation relieved the inhibition caused by 3-mercaptopropionic acid or 4-pentenoic acid but did not reverse the effects of the other inhibitors. This study leads to the conclusion that 2-tetradecylglycidic acid is the compound of choice for inhibiting the mitochondrial uptake of fatty acids and thereby their oxidation, whereas 4-bromocrotonic acid is the best irreversible inhibitor of the mictochondrial β-oxidation cycle.

The importance of fatty acids as a source of energy in heart muscle is well established [1]. Studies of fatty acid oxidation, especially of its regulation, have been greatly aided by the availability of specific inhibitors which have also been investigated for their potential use as oral hypoglycemic agents.

The first inhibitor of fatty acid oxidation to be studied was hypoglycin, the toxic principle of the unripe arillus of the ackee fruit, which is believed to cause hypoglycemia as a consequence of inhibiting β-oxidation via an inhibition of butyryl-CoA dehydrogenase (EC 1.3.99.2) [2, 3]. The search for structurally simpler analogs of hypoglycin led to the identification of 4-pentenoic acid which, however, causes the inhibition of β-oxidation by inactivating mitochondrial 3-ketoacyl-CoA thiolase (EC 2.3.1.16) and acetoacetyl-CoA thiolase (EC 2.3.1.9) [4]. Other known inhibitors of thiolases and thereby of fatty acid oxidation are 2-bromooctanoic acid [5] and 4-bromocrotonic acid [6]. Most recently, 3-mercaptopropionic acid, a known convulsant agent, has been found to inhibit β-oxidation by reversibly inhibiting acyl-CoA dehydrogenase (EC 1.3.99.3) [7]. All of the above-mentioned compounds inhibit either the first or the last reaction of the β-oxidation cycle after having been taken up by mitochondria, converted to CoA thioesters, and in most cases further metabolized.

Another group of inhibitors of fatty acid oxidation inactivates carnitine palmitoyltransferase I (EC 2.3.1.21) by binding either irreversibly or reversibly, but very tightly, to the enzyme. Representatives of this group of compounds are 2-tetradecylglycidic acid [8] and 2-bromopalmitic acid [9] which, after their conversions to CoA thioesters, inhibit the mitochondrial uptake and thereby the oxidation of fatty acids. In contrast, aminocarnitine and its acyl derivatives are competitive inhibitors of carnitine palmitoyltransferase I [10].

Although the number of available inhibitors of fatty acid oxidation has increased significantly over the last few years, choosing the best one for a given study is complicated by the fact that they were evaluated by use of different biological systems. This study is aimed at establishing the effectiveness of most of the known inhibitors of fatty acid oxidation in adult rat myocytes. In addition, we attempted to answer some unresolved questions about the mechanisms of action of these inhibitors.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 220–250 g were obtained from Taconic Farms, Inc., Germantown, NY, and maintained on a standard laboratory diet.

Materials. [1-^{14}C]Palmitic acid, [1-^{14}C]octanoic acid and [2-^{14}C]pyruvate were purchased from Amersham. Collagenase (type II) was obtained from Worthington, whereas Sigma was the source of hyaluronidase (type I), bovine serum albumin (essentially free of fatty acids), 3-mercaptopropionic acid, and most standard biochemicals. 2-Bromopalmitic acid and 4-pentenoic acid were purchased from Fluka A.G., Switzerland. Fisher Scientific was the source of Scinti Verse II. 4-Bromocrotonic acid was synthesized as described [6]. 2-Tetradecylglycidic acid and 2-bromooctanoic acid were provided by Dr. John Lowenstein, Brandeis University.

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Hypoglycin A was a gift of Dr. E. A. Kean, University of the West Indies.

Isolation of myocytes. Adult rat myocytes were isolated by the method of Frangakis *et al.* [11] except that the hearts were perfused with a Ca^{2+}-free Krebs–Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$ and 0.93 mM KH_2PO_4. The viability of myocytes isolated by this procedure was 80–90% as judged by trypan blue exclusion. The cells oxidized palmitic acid linearly for up to 80 min.

Metabolic studies with myocytes. Myocytes (1.8 mg protein) suspended in 0.9 ml of Krebs–Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 0.93 mM KH_2PO_4 and 0.5 mM $CaCl_2$ were placed in a 25-ml Erlenmeyer flask. To this cell suspension was added 20 μl of an inhibitor solution to give the desired concentration of the inhibitor. Stock solutions of inhibitors were prepared by dissolving compounds with short or medium hydrocarbon chains like 4-bromocrotonic acid, 3-mercaptopropionic acid, 4-pentenoic acid, 2-bromooctanoic acid, and hypoglycin in the cell suspension buffer. The pH was kept at 7.4 by the addition of KOH. 2-Bromopalmitic acid and 2-tetradecylglycidic acid were dissolved in a solution of defatted bovine serum albumin in the cell suspension buffer. The pH was maintained at 7.4 by the addition of KOH. The molar ratio of inhibitor to bovine serum albumin was 4:1. After preincubating the myocytes with one of the inhibitors for 10 min at 37° under constant shaking, 0.1 ml of a metabolic substrate was added to cell suspension to give a final concentration of either 0.2 mM [1-^{14}C]palmitic acid (2.5×10^5 dpm), or 0.2 mM [1-^{14}C]octanoate (3.2×10^5 dpm) or 2 mM [2-^{14}C]pyruvate (1.8×10^5 dpm).

Stock solutions of the substrates were prepared by dissolving sodium pyruvate or octanoic acid in the cell suspension buffer and by dissolving palmitic acid in a solution of defatted bovine serum albumin in the cell suspension buffer. The molar ratio of palmitate to albumin was 2:1. The Erlenmeyer flask was then closed with a rubber septum to which a plastic center well was attached. The incubation was continued under shaking at 37° for 30 min or as indicated. After that time, 0.4 ml of 1 M hyamine hydroxide was injected through the septum into the center well, and the reaction was terminated by injecting 0.3 ml of 70% perchloric acid through the septum into the incubation medium. The flask was shaken continuously for 2 hr at 37° at which time the plastic center well was removed, placed into a scintillation vial containing 4 ml of Scinti Verse II, and counted in a liquid scintillation counter. Control experiments with $NaH^{14}CO_3$ added to the cell suspension proved that the release of $^{14}CO_2$ was complete 1 hr after the addition of perchloric acid.

RESULTS AND DISCUSSION

Seven known inhibitors of fatty acid oxidation, for which mechanisms of inhibition are known or reasonably well established, were tested for their effectiveness in inhibiting palmitate oxidation in adult rat myocytes. As shown in Table 1, the most effective of all inhibitors was 2-tetradecylglycidic acid, which at a concentration of 0.1 mM caused almost complete inhibition of palmitate oxidation. Also very effective were 2-bromopalmitic acid, 3-mercaptopropionic acid, and 4-bromocrotonic acid. Less effective was 4-pentenoic acid which at a concentration of 0.5 mM reduced palmitate oxidation by almost two-thirds. Hypoglycin, which slightly stimulated fatty acid oxidation, and 2-bromooctanoic acid, which at a concentration of 0.5 mM inhibited this pathway by only 10%, were considered ineffective in rat myocytes and thus were not studied any further. Especially interesting was the ineffectiveness of 2-bromooctanoic acid because it has been shown to be an excellent inhibitor of fatty acid oxidation in perfused rat liver and in isolated rat liver mitochondria [5]. Since the inhibitor is also ineffective with coupled rat heart mitochondria [6], it is possible that in heart this compound is not metabolized to 2-bromo-3-ketooctanoyl-CoA which is an irreversible inhibitor of 3-ketoacyl-CoA thiolase. The reason for 2-bromooctanoic acid not being metabolized in heart mitochondria may be the inability of the intramitochondrial acyl-CoA synthetase to activate this

Table 1. Effects of inhibitors of fatty acid oxidation on the rate of palmitate oxidation in adult rat myocytes

Inhibitor	Rate of palmitate oxidation* (% of control)	
	0.1 mM Inhibitor	0.5 mM Inhibitor
Hypoglycin	114.3 ± 0.4 (3)	115.8 ± 10.4 (4)
4-Pentenoic acid	50.3 ± 2.9 (3)	37.2 ± 0.4 (3)
2-Bromooctanoic acid	98.0 ± 7.1 (4)	89.4 ± 3.9 (4)
4-Bromocrotonic acid	35.3 ± 1.3 (3)	14.7 ± 1.2 (3)
3-Mercaptopropionic acid	48.1 ± 6.1 (4)	13.2 ± 0.9 (3)
2-Bromopalmitic acid	38.7 ± 4.7 (3)	1.7 ± 0.1 (3)
2-Tetradecylglycidic acid	4 ± 0.3 (3)	

* Myocytes were preincubated without (control) or with inhibitor (I) for 10 min at 37° and then allowed to react with 0.2 mM palmitic acid for 30 min at 37°. Values are means ± SD. Numbers in parentheses give the number of measurements on which the values are based. The control value was 1.39 ± 0.11 nmol palmitate oxidized/mg protein/30 min.

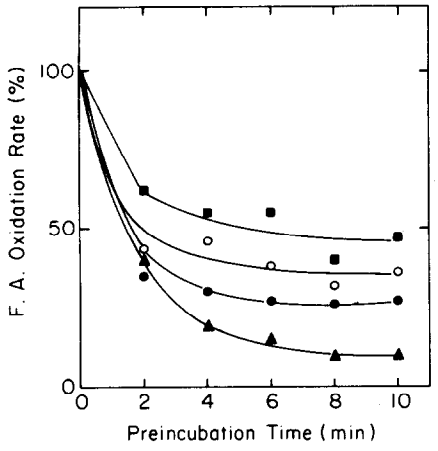

Fig. 1. Inhibition of fatty acid oxidation by several inhibitors as a function of the preincubation time. Myocytes were preincubated for the indicated periods of time with 120 μM 2-bromopalmitic acid (▲), or 125 μM 4-bromocrotonic acid (○), 300 μM 3-mercaptopropionic acid (●), or 300 μM 4-pentenoic acid (■) before fatty acid oxidation was initiated by the addition of [1-^{14}C]palmitate, as detailed under Materials and Methods.

substituted fatty acid. This explanation is supported by published data which indicate that the medium-chain acyl-CoA synthetase of heart mitochondria has a much narrower substrate specificity than the liver enzyme [12, 13].

The extent to which fatty acid oxidation was inhibited by 2-bromopalmitic acid, 4-bromocrotonic acid, 3-mercaptopropionic acid, or 4-pentenoic acid was a function of the preincubation time (Fig. 1). With all compounds optimal inhibitions were obtained after 8 min of preincubation, although some of the short-chain inhibitors seemed to cause maximal effects within shorter time periods.

The effects of the inhibitors on the oxidation rates of palmitate, octanoate, and pyruvate in rat myocytes were studied as a function of the inhibitor concentrations. 2-Tetradecylglycidic acid was the most effective of the five inhibitors. At a concentration of 0.5 μM it caused a decrease in palmitate oxidation of 80%, while pyruvate oxidation remained unaffected (Fig. 2A). However, in contrast to published reports [14, 15], the inhibitor at concentrations of up to 5 μM was found to stimulate octanoate oxidation by up to 50%, while at an inhibitor concentration of 10 μM the rate of oxidation was back to control levels. Since myocytes are expected to also oxidize endogenous and thus unlabeled fatty acids derived from intracellular triglycerides, an inhibition of their mitochondrial uptake could result in a stimulation of octanoate oxidation. The return of octanoate oxidation to control levels at a concentration of 10 μM 2-tetradecylglycidic acid may be the consequence of the inhibitor directly affecting the oxidation of medium-chain fatty acids.

2-Bromopalmitic acid, which after conversion to its CoA thioester, acts as an inhibitor of carnitine palmitoyltransferase I [9], inhibited both palmitate and octanoate oxidations (Fig. 2B). The unexpected inhibition of octanoate oxidation may be due to the inhibitor entering mitochondria and affecting octanoate activation or β-oxidation. Since pyruvate oxidation was not inhibited, depletion of the cofactors CoASH and NAD^+, necessary for the oxidation of both pyruvate and fatty acids, cannot be the reason for the observed octanoate inhibition. Also, inhibition of acetyl-CoA metabolism, which is formed from fatty acids as well as pyruvate, can be ruled out as the cause for octanoate inhibition. Although 2-bromopalmitoylcarnitine is known to enter mitochondria and to cause an inhibition of β-oxidation [9], it is not clear how the acid can be converted to its carnitine derivative and pass the inner mitochondrial membrane if it causes the irreversible inactivation of carnitine palmitoyltransferase I. Clearly, the effects of 2-bromopalmitic acid on fatty acid oxidation need to be studied further.

The observed stimulation of pyruvate oxidation by 2-bromopalmitic acid (Fig. 2B) was unexpected. In a previous study [9] with rat liver mitochondria, 2-bromopalmitoyl-CoA was found to have no effect on pyruvate oxidation, while 2-bromopalmitoylcarnitine was observed to cause an inhibition of pyruvate-supported respiration which could be partially reversed by the addition of carnitine. The stimulation of pyruvate oxidation may be the consequence of 2-bromopalmitic acid inhibiting the oxidation of endogenous fatty acids derived from myocardial triglycerides, thereby allowing more pyruvate to be degraded. However, if this hypothesis were correct, 2-tetradecylglycidic acid should also stimulate pyruvate oxidation which it does not do. Thus, we cannot explain satisfactorily the observed stimulation of pyruvate oxidation by 2-bromopalmitic acid.

4-Bromocrotonic acid, an inhibitor of both 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase [6], inhibited the oxidation of palmitate and less severely that of octanoate while hardly or not at all affecting pyruvate oxidation (Fig. 2C).

4-Pentenoic acid caused the expected inhibition of palmitate oxidation without affecting pyruvate oxidation (Fig. 2D). Surprisingly, the oxidation of octanoate was not inhibited even though the suggested irreversible inhibition of 3-ketoacyl-CoA thiolase by a metabolite of 4-pentenoate [4, 16] should repress octanoate oxidation.

Finally, 3-mercaptopropionic acid, which so far has been studied only with rat heart mitochondria [7], was found to also inhibit palmitate oxidation in rat myocytes (Fig. 2E). The virtual absence of any effect on octanoate and pyruvate oxidations agrees with the proposal that intramitochondrially formed *S*-acyl-3-mercaptopropinyl-CoA thioesters reversibly inhibit long-chain acyl-CoA dehydrogenase and thereby the β-oxidation of long-chain fatty acids [7]. Thus, the partial inhibition of octanoate oxidation observed with isolated mitochondria [7] is most likely a consequence of octanoate and 3-mercaptopropionate competing for the same intramitochondrial acyl-CoA synthetase.

In an attempt to confirm the proposed mechanisms by which these inhibitors affect fatty acid oxidation, the reversibility of the inhibition of palmitate oxidation in rat myocytes was studied. For this purpose

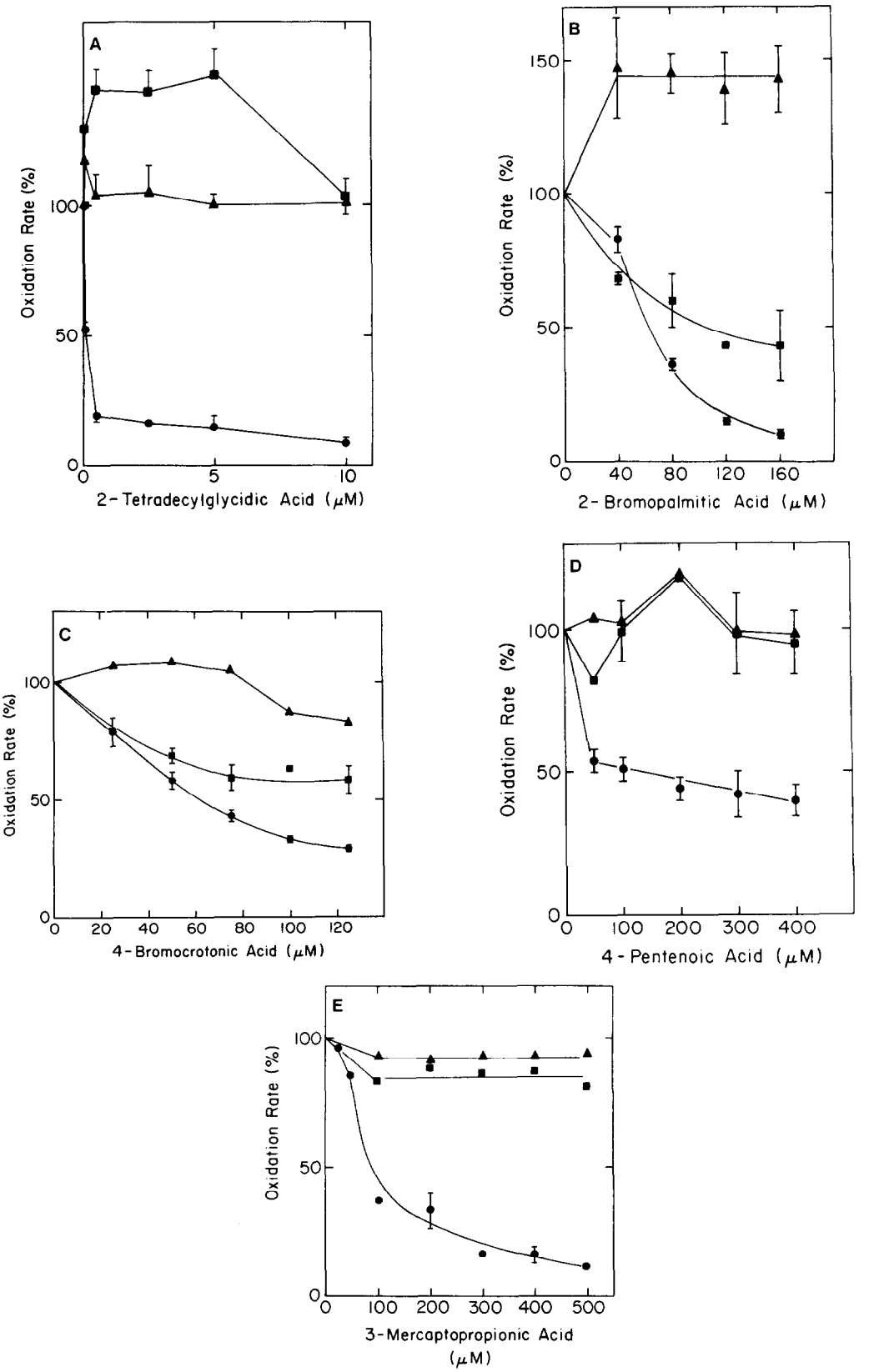

Fig. 2. Effects of inhibitors of fatty acid oxidation on the oxidations of palmitate, octanoate, and pyruvate by rat myocytes. (A) 2-Tetradecylglycidic acid; (B) 2-bromopalmitic acid; (C) 4-bromocrotonic acid; (D) 4-pentenoic acid; and (E) 3-mercaptopropionic acid. Palmitate (●), octanoate (■), and pyruvate (▲) served as metabolic substrates, as described in detail under Materials and Methods.

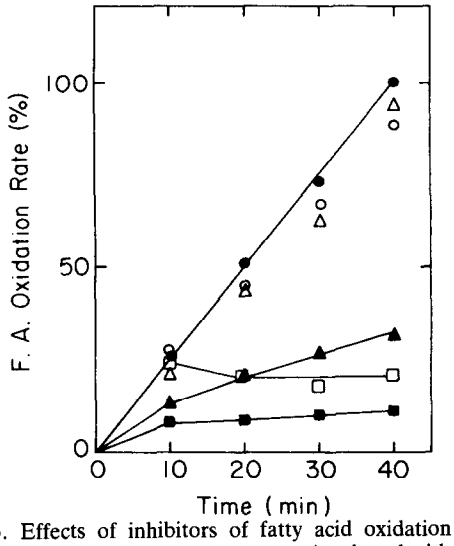

Fig. 3. Effects of inhibitors of fatty acid oxidation on palmitate oxidation in rat myocytes preincubated with the inhibitors followed by their removal from the incubation medium. Myocytes were preincubated for 10 min with 5 μM 2-tetradecylglycidic acid (□), 120 μM 2-bromopalmitic acid (■), 125 μM 4-bromocrotonic acid (▲), 300 μM 3-mercaptopropionic acid (○), 300 μM 4-pentenoic acid (△), or no inhibitor (●). After preincubation the cell suspension medium was replaced with an inhibitor-free medium. Fatty acid oxidation was initiated by the addition of 0.2 mM [1-^{14}C]palmitate and allowed to proceed for 40 min.

myocytes were preincubated with one of the inhibitors for 10 min. At the end of the preincubation period, the cell suspension medium was replaced by inhibitor-free buffer, and palmitate oxidation was initiated. As seen in Fig. 3, the inhibitions caused by 2-tetradecylglycidic acid, 2-bromopalmitic acid, and 4-bromocrotonic acid persisted after removal of the inhibitors from the incubation medium. Thus, inhibitions caused by these compounds seems to be irreversible, as suggested by previous studies [6, 8, 9]. In contrast, 3-mercaptopropionic acid, which was reported to inhibit β-oxidation reversibly [7], caused little inhibition after its removal from the preincubation medium.

A surprising finding was the almost complete reversibility of the 4-pentenoate-induced inhibition of fatty acid oxidation in myocytes. This result contradicts the reported irreversible inhibition of 3-ketoacyl-CoA thiolase and thus β-oxidation in coupled heart mitochondria [4]. However, 4-pentenoic acid is metabolized by two pathways, only one of which, the minor one, yields a metabolite that inhibits 3-ketoacyl-CoA thiolase reversibly as well as irreversibly [11]. If the intramitochondrial concentrations of 4-pentenoate in isolated mitochondria and myocytes differ significantly, the flux of the inhibitor through the minor pathway could be affected, thereby giving rise to different patterns of inhibition.

Fifty percent inhibition of palmitate oxidation in myocytes was achieved after 10 min of preincubation with one of the following: 0.1 μM 2-tetradecylglycidic acid, 60 μM bromocrotonic acid, 60 μM bromopalmitic acid, 100 μM 3-mercaptopropionic acid or 100 μM 4-pentenoic acid. Thus, it is obvious that, at low concentrations, 2-tetradecylglycidic acid is the most effective inhibitor of long-chain fatty acid oxidation. However, since it inhibits fatty acid oxidation indirectly by inhibiting carnitine palmitoyltransferase I and thus the uptake of long-chain fatty acids, it has no effect on the oxidation of medium-chain or short-chain fatty acid which enters mitochondria apart from the carnitine-dependent fatty acid uptake system. If it is necessary to inhibit the β-oxidation cycle directly and irreversibly in order to affect the oxidation of all types of fatty acids, 4-bromocrotonic acid seems to be a good choice. This inhibitor enters mitochondria directly where it is activated and metabolized to 3-keto-4-bromobutyryl-CoA, which irreversibly inactivates 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA thiolase, thereby inhibiting the β-oxidation cycle.

All of the other inhibitors have at least one property which limits their use as universal inhibitors of fatty acid oxidation. For example, 2-bromooctanoic acid is effective in liver but not in heart. However, the tissue specificity of this inhibitor may be useful in an *in vivo* study aimed at estimating the contribution of liver to the total oxidation of fatty acids. In addition, this compound could possibly be used for controlling ketogenesis by inhibiting hepatic fatty acid oxidation without affecting the same pathway in heart and perhaps skeletal muscle.

The use of hypoglycin is also limited because it is not universally effective, possibly because it is only metabolized in some tissues as, for example, liver. The effects of 4-pentenoic acid and 2-bromopalmitic acid on fatty acid oxidation are complex, thereby limiting the use of these two inhibitors. Although 3-mercaptopropionic acid is a good reversible inhibitor of fatty acid oxidation, its usefulness is limited because it induces seizures in animals.

In conclusion, 2-tetradecylglycidic acid and 4-bromocrotonic acid seem to be the best compounds for inhibiting fatty acid oxidation in myocytes and possibly in the perfused heart.

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