J. Biochem. 119, 1196-1201 (1996)

A New Inhibitor of Mitochondrial Fatty Acid Oxidation[1]

Takashi Hashimoto,*,[2] Yasuko Shindo,* Masayoshi Souri,* and Graham S. Baldwin†

*Department of Biochemistry, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390; and †Department of Surgery, Austin Hospital, Heidelberg, Victoria 3084, Australia

Received for publication, March 5, 1996

The mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) plays a major role in mitochondrial fatty acid oxidation. The enzyme complex consists of four molecules of α-subunit containing both hydratase and dehydrogenase domains and four molecules of β-subunit containing the thiolase domain. The primary structure of a gastrin-binding protein (GBP) was highly homologous to that of the α-subunit of the trifunctional protein. Here, we report that gastrin inhibits the hydratase, dehydrogenase, and thiolase activities of the trifunctional protein. The gastrin/cholecystokinin receptor antagonist benzotript, which inhibited binding of gastrin to the GBP, also inhibited all three activities of the trifunctional protein. In addition, benzotript inhibits the activities of multifunctional enzymes having similar structures, such as the peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein and the *Pseudomonas fragi* fatty acid oxidation enzyme complex. This reagent, however, hardly inhibited various monofunctional enzymes involved in fatty acid oxidation.

Key words: benzotript, fatty acid oxidation, gastrin, inhibition, mitochondrial trifunctional protein.

Two newly discovered fatty acid oxidation enzymes, very-long-chain acyl-CoA dehydrogenase (*1*) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) (*2*), are associated with the mitochondrial inner membrane and play a major role in fatty acid β-oxidation. A schematic hypothesis is that long-chain acyl-CoAs are first oxidized by these enzymes, and the carbon-chain-shortened acyl-CoAs are then processed by classical β-oxidation enzymes.

The trifunctional protein is a multienzyme complex composed of four molecules of α-subunit with both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase domains and 4 mol of β-subunit with the 3-ketoacyl-CoA thiolase domain (*3, 4*). The primary structure of a porcine gastrin-binding protein (GBP) (*5*) is highly homologous to the structure of the α-subunits of rat and human trifunctional protein (*3, 4*), and identical with the structure of the porcine trifunctional protein (*6*). The trifunctional protein and the GBP appear to differ in subcellular distribution: at least some of the GBP is located on the cell membrane, and the trifunctional protein is in mitochondria (*7*). The functions of these two proteins may also be different, but we supposed that gastrin and reagents which inhibited the binding of gastrin to the GBP might interact with the α-subunit of the trifunctional protein. Firstly, we examined the effect of gastrin and two inhibitors of binding of gastrin to the GBP, benzotript [*N*-(4-chlorobenzoyl)-L-tryptophan] and proglumide (*N*-benzoyl-*N'*,*N'*-dipropyl-D,L-isoglutamine) (*8*), on the enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase activities of the trifunctional protein, and found that gastrin and benzotript inhibited all of the activities of the trifunctional protein reversibly.

Since benzotript was more potent than gastrin, we chose it for further study. Benzotript inhibited not only the trifunctional protein but also multifunctional enzymes involved in fatty acid oxidation, such as the peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (bifunctional protein) and the *Pseudomonas fragi* fatty acid oxidation enzyme complex. In contrast, various monofunctional fatty acid oxidation enzymes were hardly inhibited. Benzotript was also shown to inhibit overall palmitate oxidation of mitochondria. Therefore, we conclude that benzotript is a new inhibitor of mitochondrial fatty acid oxidation.

MATERIALS AND METHODS

Materials—Human [Met[15]]-gastrin$_{2-17}$ was obtained from Research Plus (Bayonne, NJ). Benzotript was obtained from Research Biochemicals International (Natick, MA), and proglumide was from Sigma (St. Louis, MO). CoA was obtained from Kyowa Hakko Kogyo (Tokyo). NAD, NADH, and ATP were from Boehringer (Mannheim, Germany). *l*-Carnitine was a gift from Otsuka Pharmaceutical (Osaka). [1-^{14}C]Palmitic acid was purchased from

[1] This work was supported in part by Grant-in-Aid (06454175) for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and a Research Grant for the Intractable Diseases from the Ministry of Health and Welfare of Japan.

[2] To whom correspondence should be addressed: Tel: +81-263-35-4600 (Ext. 5180), Fax: +81-263-33-6458

Abbreviations: GBP, gastrin-binding protein; trifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein; bifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein.

American Radiolabeled Chemicals (St. Louis, MO). Acyl-CoAs were synthesized and purified by DEAE-cellulose column chromatography as described previously (*9*).

All of the fatty acid oxidation enzymes used in these experiments were purified from rat liver. Long-chain acyl-CoA synthetase was purified as described previously (*10*). The outer membrane of rat liver mitochondria was prepared (*11*) for assay of carnitine palmitoyltransferase I. Carnitine palmitoyltransferase II (*12*), short-chain, medium-chain, long-chain (*13*), and very-long-chain (*1*) acyl-CoA dehydrogenases were purified by the cited procedures. Mitochondrial short-chain enoyl-CoA hydratase (*14*), 3-hydroxyacyl-CoA dehydrogenase (*15*), 3-ketoacyl-CoA thiolase (*16*), and trifunctional protein (*2*), and the peroxisomal bifunctional protein (*14*) and peroxisomal 3-ketoacyl-CoA thiolase (*16*) were purified as described in the indicated references. *P. fragi* fatty acid oxidation multienzyme complex (*17*) was donated by Asahi Chemical Industry (Shizuoka) through Nippon Shoji Kaisha (Osaka).

Enzyme Assay—Long-chain acyl-CoA synthetase was assayed with [^{14}C]palmitate (*10*). For assay of carnitine palmitoyltransferase I, [^{14}C]palmitoyl-CoA was synthesized by preincubation with long-chain acyl-CoA synthetase, then the transferase reaction was initiated by addition of the mitochondrial outer membrane fraction, because the synthetase activity was not high enough to assay the transferase activity, although the membrane fraction contained long-chain acyl-CoA synthetase. Assay conditions and separation of the radioactive palmitoylcarnitine were the same as the procedures of Bhuiyan and Pande (*11*). The activity of carnitine palmitoyltransferase II was assayed by liberation of CoA from palmitoyl-CoA (*12*). Activities of short- and medium-chain acyl-CoA dehydrogenases were measured with butyryl-CoA and octanoyl-CoA, respectively, and those of long- and very-long-chain acyl-CoA dehydrogenases were measured with palmitoyl-CoA (*1, 13*).

The enoyl-CoA hydratase activity in the forward reaction was assayed by increase in absorbance at 303 nm in the presence of L-3-hydroxyacyl-CoA dehydrogenase. The reaction mixture contained 0.1 M Tris-Cl, pH 8.3, 50 mM KCl, 25 mM $MgCl_2$, 50 μM octenoyl-CoA, 0.2 mM NAD^+, and 5 μg/ml 3-hydroxyacyl-CoA dehydrogenase. The L-3-hydroxyacyl-CoA dehydrogenase activity was assayed by the reverse reaction. The reaction mixture contained 0.1 M Tris-Cl, pH 8.3, 50 mM KCl, 20 μM 3-ketooctanoyl-CoA, and 0.1 mM NADH. The 3-ketoacyl-CoA thiolase activity was assayed by decrease in absorbance at 303 nm in a reaction mixture containing 0.1 M Tris-Cl, pH 8.3, 50 mM KCl, 25 mM $MgCl_2$, 15 μM 3-ketooctanoyl-CoA, and 50 μM CoA.

Gastrin was dissolved in 10 mM potassium phosphate, pH 7.5 to give a 2 mM stock solution. Benzotript and proglumide were dissolved in dimethyl sulfoxide at a concentration of 100 mM or less, and 20 μl of the solution was added to the reaction mixture in a total volume of 1 ml. For the control, 20 μl of dimethyl sulfoxide was added.

All enzyme activities were measured at 30°C. One unit of enzyme was defined as the amount which utilized substrate or formed product at a rate of 1 μmol per min.

Palmitate Oxidation—Palmitate oxidation by cultured human skin fibroblasts was initiated by addition of [1-^{14}C]-palmitate to the culture medium. The reaction was stopped by addition of $HClO_4$, and the acid-soluble radioactivity was determined as described previously (*18*). Palmitate oxidation by fibroblast homogenates was assayed by production of acid-soluble radioactivity (*19*).

RESULTS AND DISCUSSION

Inhibition of the Trifunctional Protein by Gastrin and Inhibitors of the GBP—Gastrin inhibited all of the enzyme activities of the trifunctional protein (Fig. 1). The inhibition pattern of the hydratase activity was not linear. The reason for the deviation from linearity is unknown, but may be related to the observation that the N- and C-terminal halves of gastrin are capable of independent binding to both hydratase and dehydrogenase domains (*20*; Murphy, V.J. *et al.*, unpublished data). Short-chain enoyl-CoA hydratase was not inhibited even in the presence of 12 μM gastrin (data not shown). The hydratase activity of the bifunctional protein decreased linearly, and the remaining activity was about 70% in the presence of 12 μM gastrin. The dehydrogenase activity of the trifunctional protein was linearly inhibited by increasing gastrin concentration with an apparent K_i of 9 μM. The difference between this value and the IC_{50} value of 0.2 μM determined for the inhibition by gastrin of the crosslinking of [^{125}I]gastrin$_{2,17}$ to the porcine gastrin-binding protein is presumably caused by the presence of 20 μM 3-ketooctanoyl-CoA in the dehydrogenase assay: 3-ketoacyl-CoAs inhibit the binding of gastrin to the GBP with affinities which increase with increasing chain length (*8*; Baldwin and Hashimoto, unpublished data). The dehydrogenase activities of the short-chain 3-hydroxyacyl-CoA dehydrogenase and the bifunctional protein were not affected even in the presence of 20 μM gastrin.

The 3-ketoacyl-CoA thiolase activity of the trifunctional protein was also inhibited by gastrin. The inhibition was less than the inhibition of hydratase or dehydrogenase activity and was not linear. Mitochondrial and peroxisomal 3-ketoacyl-CoA thiolase were not inhibited in the presence of 20 μM gastrin.

The effects of the inhibitors, benzotript and proglumide,

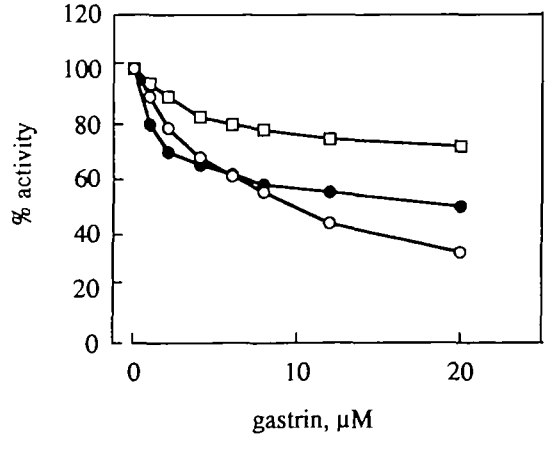

Fig. 1. **Effect of gastrin on the trifunctional protein.** The enoyl-CoA hydratase activity (●) was assayed with octenoyl-CoA, and the 3-hydroxyacyl-CoA dehydrogenase activity (○) was assayed by the reverse reaction with 3-ketooctanoyl-CoA and NADH. The 3-ketoacyl-CoA thiolase activity (□) was assayed by thiolytic cleavage of 3-ketooctanoyl-CoA in the presence of CoA.

on the dehydrogenase activities were then examined. The apparent K_i values for benzotript were 0.4 mM for the trifunctional protein and 1.8 mM for the bifunctional protein. A similar IC_{50} value of 0.2 mM was obtained for inhibition of the binding of gastrin to the porcine gastrin-binding protein by benzotript (*8*). No inhibition was found for short-chain 3-hydroxyacyl-CoA dehydrogenase. Proglumide at concentrations up to 2 mM did not inhibit these enzyme activities. Proglumide is also a much weaker inhibitor than benzotript of the binding of gastrin to the porcine gastrin-binding protein, with an IC_{50} value of 5.1 mM (*8*). Based on these results, benzotript was used for the following experiments.

Effect of Benzotript on Long-Chain Acyl-CoA Synthetase and Carnitine Palmitoyltransferases I and II—The long-chain acyl-CoA synthetase activity was increased about 1.5-fold by the addition of dimethyl sulfoxide, and this enhanced activity was further increased about 2-fold in the presence of 1 or 2 mM benzotript. The carnitine palmitoyltransferase I activity was not affected by dimethyl sulfoxide, but the activity was increased 2-fold at 1 mM benzotript, and 3-fold at 2 mM of the reagent. The activity of carnitine palmitoyltransferase II was not affected either by dimethyl sulfoxide alone or by benzotript up to 2 mM.

Effect of Benzotript on Acyl-CoA Dehydrogenases—None of the activities of short-chain, medium-chain, long-chain, and very-long-chain acyl-CoA dehydrogenases were affected either by dimethyl sulfoxide or by benzotript up to 2 mM.

Effect of Benzotript on Enzymes Catalyzing Enoyl-CoA Hydratase, 3-Hydroxyacyl-CoA Dehydrogenase, and 3-Ketoacyl-CoA Thiolase Reactions—Benzotript was concluded to be a reversible dead-end type inhibitor for all of the activities described below, because the inhibitory effects were the same with or without preincubation of the enzymes.

Figure 2 summarizes the effects of benzotript on various enzymes of the fatty acid β-oxidation cycle. Acyl-CoA substrates with an eight-carbon chain were used because (1) all of the enzymes examined reacted with these substrates, and (2) the medium-chain substrates did not exhibit the substrate inhibition that was observed with long-chain substrates for most of the enzymes used in this experiment.

The effects of benzotript on the enoyl-CoA hydratase activities are shown in Fig. 2A. The enoyl-CoA hydratase activity of the trifunctional protein was markedly reduced by benzotript. The apparent K_i value was 0.8 mM. In contrast, the activity of short-chain enoyl-CoA hydratase was only slightly decreased. The activity of the peroxisomal bifunctional protein was moderately decreased.

Figure 2B shows that the 3-hydroxyacyl-CoA dehydrogenase activity of the trifunctional protein was also markedly decreased in the presence of benzotript. The apparent K_i value of benzotript was about 0.7 mM. The activities of short-chain 3-hydroxyacyl-CoA dehydrogenase and the bifunctional protein were not changed by benzotript.

The 3-ketoacyl-CoA thiolase activity of the trifunctional

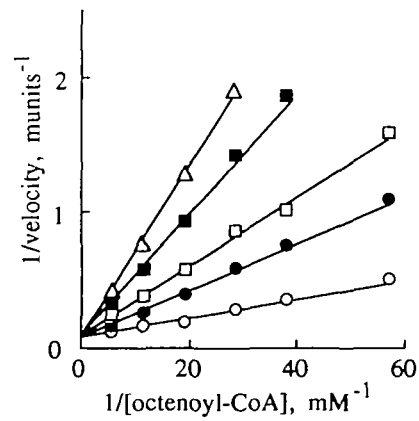

Fig. 3. **Kinetics of inhibition of the enoyl-CoA hydratase activity of the trifunctional protein by benzotript.** The amount of trifunctional protein used was 0.025 μg. Concentrations of benzotript were 0 (○), 0.5 (●), 1.0 (□), 1.5 (■), and 2.0 (△) mM.

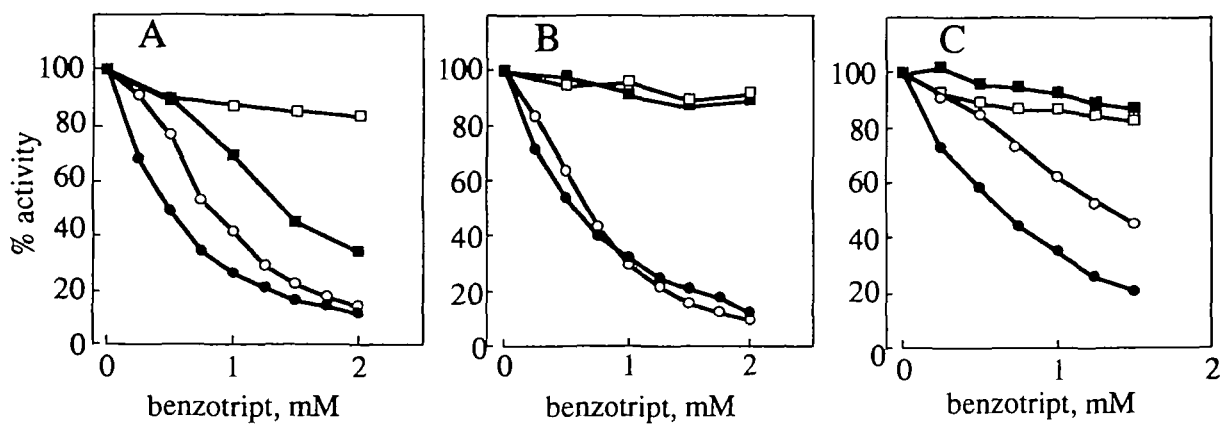

Fig. 2. **Effect of benzotript on various enzymes.** A: The octenoyl-CoA hydratase activities of short-chain enoyl-CoA hydratase (□), bifunctional protein (■), trifunctional protein (○), and *Pseudomonas fragi* fatty acid oxidation multienzyme complex (●). B: The reverse reaction of L-3-hydroxyacyl-CoA dehydrogenase with 3-ketooctanoyl-CoA:3-hydroxyacyl-CoA dehydrogenase (□), bifunctional protein (■), trifunctional protein (○), and *Pseudomonas fragi* fatty acid oxidation multienzyme complex (●). C: The 3-ketooctanoyl-CoA thiolase activities of peroxisomal (□) and mitochondrial (■) 3-ketoacyl-CoA thiolase, trifunctional protein (○), and *Pseudomonas fragi* fatty acid oxidation multienzyme complex (●).

protein was also decreased in the presence of benzotript with an apparent K_i value of 1.5 mM, but the activities of mitochondrial and peroxisomal 3-ketoacyl-CoA thiolases were only slightly reduced by this reagent (Fig. 2C).

The *P. fragi* fatty acid oxidation multienzyme complex was studied, because both the *Escherichia coli* (*21, 22*) and *P. fragi* (*23*) multienzyme complexes were very similar to the trifunctional protein (*3, 4*) in molecular structures and functions. All three activities of this enzyme complex were decreased by benzotript (Fig. 2, A, B, and C). The inhibition seemed to be more pronounced than the inhibition of rat trifunctional protein.

Kinetics of the Inhibition of the Trifunctional Protein—A detailed kinetic study was carried out to elucidate the mechanism of inhibition of the trifunctional protein. Inhibition of the enoyl-CoA hydratase activity was competitive with the substrate (Fig. 3). Inhibition of the 3-hydroxyacyl-CoA dehydrogenase activity was noncompetitive with 3-ketooctanoyl-CoA and NADH (Fig. 4, A and B). Inhibition of the 3-ketoacyl-CoA thiolase activity was noncompetitive with 3-ketooctanoyl-CoA and uncompetitive with CoA (Fig. 5, A and B).

The effects on the bifunctional protein (Fig. 2, A and B) suggest that the inhibitor binds to the site related to the hydratase activity of this multifunctional enzyme, but not to the dehydrogenase site. Competitive inhibition of the hydratase activity of the trifunctional protein was also found. This result seems to be compatible with the above idea that the inhibitor binding site is the hydratase domain of bifunctional protein. However, the results for the trifunctional protein are not explained by the simple assumptions that each of the catalytic centers are independent, and that the inhibitor binds only to the hydratase domain. Intimate interactions among the three active centers may be involved in catalysis, because possible intermediate channeling between the hydratase and the dehydrogenase sites have been suggested for the large subunit of *E. coli* fatty acid oxidation complex (*24*) and the mitochondrial trifunctional protein (*2*). One possible explanation for our

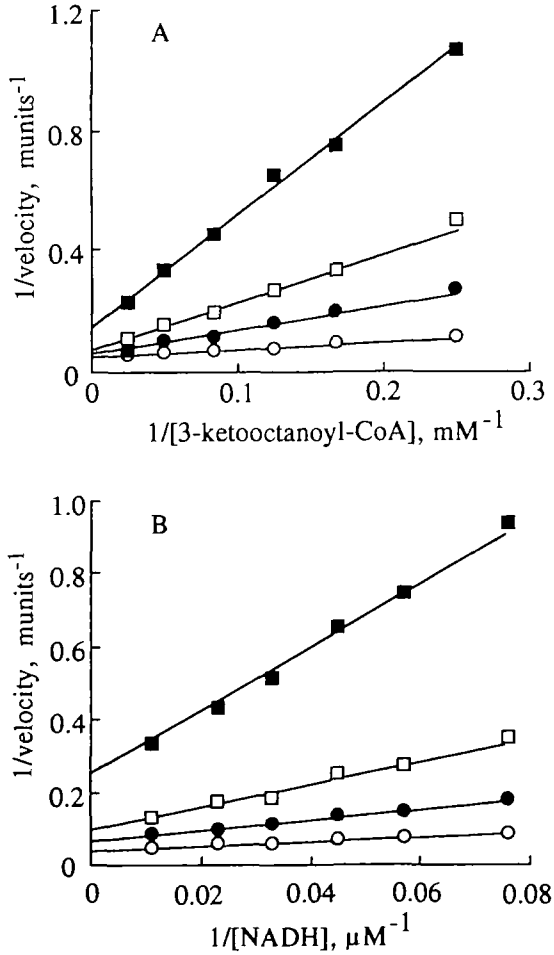

Fig. 4. **Kinetics of inhibition of the dehydrogenase activity of the trifunctional protein by benzotript.** The amount of trifunctional protein used was 1 μg. In experiment A, the concentration of 3-ketooctanoyl-CoA was varied at a fixed concentration of NADH of 50 μM. In experiment B, the concentration of 3-ketooctanoyl-CoA was fixed at 20 μM and the concentration of NADH was varied. Concentrations of benzotript were 0 (○), 0.5 (●), 1.0 (□), and 2.0 (■) mM.

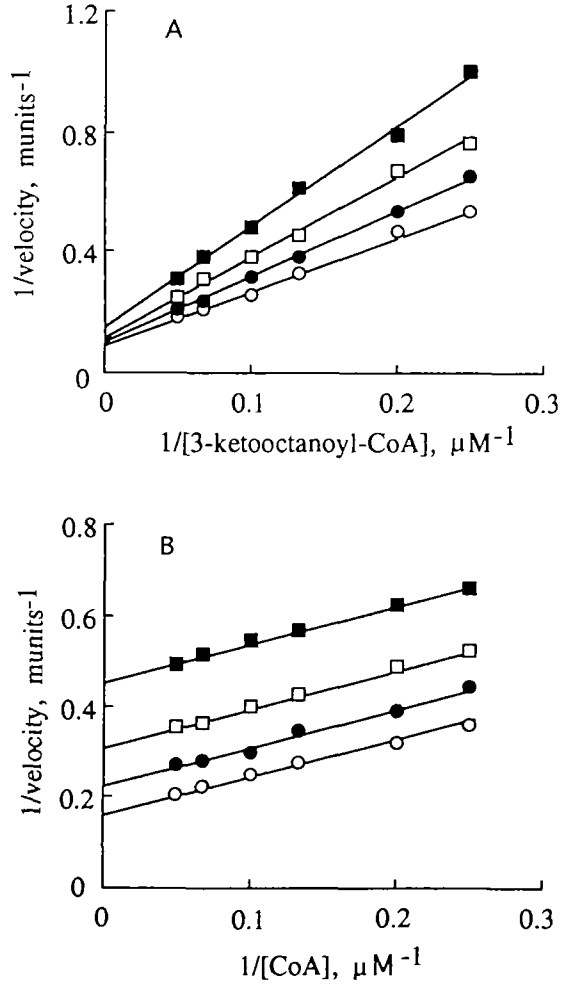

Fig. 5. **Kinetics of inhibition of the thiolase activity of the trifunctional protein by benzotript.** The amount of trifunctional protein used was 2 μg. In experiment A, the concentration of 3-ketooctanoyl-CoA was varied, and the CoA concentration was fixed at 10 μM. In experiment B, the concentration of 3-ketooctanoyl-CoA was fixed at 10 μM and the concentration of CoA was varied. Concentrations of benzotript were 0 (○), 0.5 (●), 1.0 (□), and 1.5 (■) mM.

data is that benzotript binds to the site primarily related to the hydratase domains of the multifunctional proteins and the other catalytic reactions are secondarily affected.

Inhibition of Palmitate Oxidation by Benzotript—Overall fatty acid oxidation of rat liver mitochondria was measured by production of acid-soluble radioactivity from [1-^{14}C]-palmitate. The oxidation activity was reduced to about 50% at 1 mM benzotript, and to about 10% at 2 mM of the reagent.

We used human skin fibroblasts from patients with trifunctional protein deficiency to investigate whether the reduction of the mitochondrial palmitate oxidation by benzotript was due to inhibition of the trifunctional protein. Trifunctional protein deficiency is classified into two groups (*25*). In one group, levels of both α- and β-subunits of the trifunctional protein are extremely low and all three enzyme activities are undetectable in the patients' fibroblasts. In the second, more common group, the enzyme protein is present and only the dehydrogenase activity is deficient. Palmitate oxidation by the patients' cells is 1/5-1/3 of that of the control cells. This reduction suggests that the trifunctional protein plays a major role in mitochondrial fatty acid oxidation.

We determined the rate of fatty acid oxidation of cultured human skin fibroblasts lacking the trifunctional protein by measuring the acid-soluble radioactivity produced from [1-^{14}C]palmitate added to the culture medium. When benzotript was added to the medium, the palmitate oxidation of the fibroblasts was hardly changed. The ineffectiveness of benzotript may be due to inability of the reagent to penetrate into the cells.

Palmitate oxidation by a homogenate of the mutant fibroblasts was inhibited nearly completely in the presence of 1 mM benzotript (Fig. 6), and the inhibition profiles were similar for both the control and the patients' fibroblasts. If the inhibition by benzotript were solely due to an inhibition of the trifunctional protein, the inhibition profiles of palmitate oxidation for the control and patients' homogenates would not be similar, since no inhibition of palmitate oxidation would be observed in the patients' homogenate, and incomplete inhibition of palmitate oxidation would be observed in the control homogenate. It therefore seems likely that benzotript inhibits both the trifunctional protein and some other mitochondrial function. Enzymes of the TCA cycle do not appear to be the secondary target, since malonate was included in the reaction to block the TCA cycle.

The peroxisomal palmitate oxidation rate of the fibroblasts was 5-8% of the mitochondrial activity for the control cells, and 15-20% for the patients' cells when determined using whole cells. However, the peroxisomal fatty acid oxidation activity could not be assayed under the same conditions as the mitochondrial activity because of the inhibitory effect of BSA. Therefore, it is uncertain whether the peroxisomal activity is affected by benzotript or not.

In conclusion, benzotript was shown to inhibit multifunctional enzymes of fatty acid oxidation, but not most of the monofunctional enzymes. The cDNA encoding the α-subunit of the trifunctional protein, the bifunctional protein, and the α-subunit of bacterial fatty acid oxidation multienzyme complex have regions similar to the structure of mitochondrial enoyl-CoA hydratase on the amino-terminal

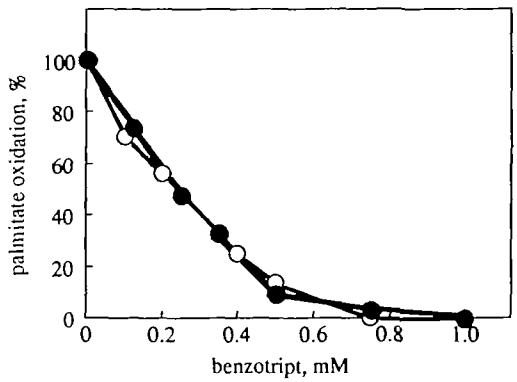

Fig. 6. **Palmitate oxidation by fibroblast homogenates.** The reaction mixture contained 150 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate, 0.1 mM EDTA, 1 mM potassium phosphate, 5 mM malonate, 1.5 mg/ml BSA, 10 mM $MgCl_2$, 5 mM ATP, 1 mM l-carnitine, 50 μM CoA, 10 nM [1-^{14}C]palmitate (18.5 kBq in 1 ml), and fibroblast homogenate (300-500 μg protein/ml). The pH was 7.2 and the total volume of reaction mixture was 0.2 ml. After incubation for 1 h at 25°C, 0.2 ml of 1 M $HClO_4$ was added, and the mixture was centrifuged. The supernatant was extracted three times with 1 ml of hexane, and the radioactivity in the aqueous phase was determined. ●, an average of the results obtained with the fibroblasts from two control persons; ○, an average of the results obtained with the fibroblasts from two patients with trifunctional protein deficiency.

side, and a domain similar to 3-hydroxyacyl-CoA dehydrogenase on the carboxyl-terminal side (*3, 4*). The binding site for gastrin and benzotript may be not related to a single region of the primary structure, but may be created by fusion of the catalytic domains.

Inhibition by benzotript of overall fatty acid oxidation of mitochondria seems to be not only due to the inhibition of the trifunctional protein but also to some other disturbance of mitochondrial function.

REFERENCES

1. Izai, K., Uchida, Y., Orii, T., Yamamoto, S., and Hashimoto, T. (1992) Novel fatty acid β-oxidation enzymes in rat liver mitochondria. I. Purification and properties of very-long-chain acyl-coenzyme A dehydrogenase. *J. Biol. Chem.* **267**, 1027-1033
2. Uchida, Y., Izai, K., Orii, T., and Hashimoto, T. (1992) Novel fatty acid β-oxidation enzymes in rat liver mitochondria. II. Purification and properties of enoyl-coenzyme A (CoA) hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein. *J. Biol. Chem.* **267**, 1034-1041
3. Kamijo, T., Aoyama, T., Miyazaki, J., and Hashimoto, T. (1993) Molecular cloning of the cDNAs for the subunits of rat mitochondrial fatty acid β-oxidation multienzyme complex. Structural and functional relationships to other mitochondrial and peroxisomal β-oxidation enzymes. *J. Biol. Chem.* **268**, 26452-26460
4. Kamijo, T., Aoyama, T., Komiyama, A., and Hashimoto, T. (1994) Structural analysis of cDNAs for subunits of human mitochondrial fatty acid β-oxidation trifunctional protein. *Biochem. Biophys. Res. Commun.* **199**, 818-825
5. Mantamadiotis, T., Sobieszczuk, P., Weinstock, J., and Baldwin, G.S. (1993) Nucleotide sequence encoding a novel member of the hydratase/dehydrogenase family. *Biochim. Biophys. Acta* **1170**, 211-215
6. Yang, S.-Y., He, X.-Y., Styles, J., Luo, M.J., Schulz, H., and Elzinga, M. (1994) Primary structure of the large subunit of trifunctional β-oxidation complex from pig heart mitochondria. *Biochem. Biophys. Res. Commun.* **198**, 431-437

7. Baldwin, G.S. (1993) Comparison of sequences of the 78 kDa gastrin-binding protein and some enzymes involved in fatty acid oxidation. *Comp. Biochem. Physiol.* **104B**, 55-61
8. Baldwin, G.S. (1994) Anti-proliferative gastrin/cholecystokinin receptor antagonists target the 78-kDa gastrin-binding protein. *Proc. Natl. Acad. Sci. USA* **91**, 7593-7594
9. Kobayashi, A., Jiang, L.L., and Hashimoto, T. (1996) Two mitochondrial 3-hydroxyacyl-CoA dehydrogenases in bovine liver. *J. Biochem.* **119**, 775-782
10. Miyazawa, S., Hashimoto, T., and Yokota, S. (1985) Identity of long-chain acyl-coenzyme A synthetase of microsomes, mitochondria, and peroxisomes in rat liver. *J. Biochem.* **98**, 723-733
11. Bhuiyan, A.K.M.J. and Pande, S.V. (1992) One-step synthesis of radioactive acyl-CoA and acylcarnitines using rat liver mitochondrial outer membrane as enzyme sources. *Lipids* **27**, 392-395
12. Miyazawa, S., Ozasa, H., Osumi, T., and Hashimoto, T. (1983) Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. *J. Biochem.* **94**, 529-542
13. Furuta, S., Miyazawa, S., and Hashimoto, T. (1981) Purification and properties of rat liver acyl-CoA dehydrogenases and electron transfer flavoprotein. *J. Biochem.* **90**, 1739-1750
14. Furuta, S., Miyazawa, S., Osumi, T., Hashimoto, T., and Ui, N. (1980) Properties of mitochondrial and peroxisomal enoyl-CoA hydratases from rat liver. *J. Biochem.* **88**, 1059-1070
15. Osumi, T. and Hashimoto, T. (1980) Purification and properties of mitochondrial and peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. *Arch. Biochem. Biophys.* **203**, 372-383
16. Miyazawa, S., Osumi, T., and Hashimoto, T. (1980) The presence of a new 3-oxoacyl-CoA thiolase in rat liver peroxisomes. *Eur. J. Biochem.* **103**, 589-596
17. Imamura, S., Ueda, S., Mizugaki, M., and Kawaguchi, A. (1990) Purification of the multienzyme complex for fatty acid oxidation from *Pseudomonas fragi* and constitution of the fatty acid oxidation system. *J. Biochem.* **107**, 184-189
18. Aoyama, T., Souri, M., Ushikubo, S., Kamijo, T., Yamaguchi, S., Kelley, R.I., Rhead, W.J., Uetake, K., Tanaka, K., and Hashimoto, T. (1995) Purification of human very-long-chain acyl-coenzyme A dehydrogenase and characterization of its deficiency in seven patients. *J. Clin. Invest.* **95**, 2465-2473
19. Shindo, Y., Osumi, T., and Hashimoto, T. (1978) Effect of administration of di-(2-ethylhexyl)phthalate on rat liver mitochondria. *Biochem. Pharmacol.* **27**, 2683-2688
20. Baldwin, G.S. (1995) Binding of progastrin fragments to the 78 kDa gastrin-binding protein. *FEBS Lett.* **359**, 97-100
21. Yang, X.-Y.H., Schulz, H., Elzinga, M., and Yang, S.-Y. (1991) Nucleotide sequence of the promotor and *fadB* gene of the *fadBA* operon and primary structure of the multifunctional fatty acid oxidation protein from *Escherichia coli*. *Biochemistry* **30**, 6788-6795
22. Yang, S.-Y., Yang, X.-Y.H., Healy-Louie, G., Schulz, H., and Elzinga, M. (1990) Nucleotide sequence of the *fadA* gene. Primary structure of 3-ketoacyl-coenzyme A thiolase from *Escherichia coli* and the structural organization of the *fadAB* operon. *J. Biol. Chem.* **265**, 10424-10429
23. Sato, S., Hayashi, M., Imamura, S., Ozeki, Y., and Kawaguchi, A. (1992) Primary structures of the genes, *fao* A and *fao* B, from *Pseudomonas fragi* B-0771 which encode the two subunits of the HDT multienzyme complex involved in fatty acid β-oxidation. *J. Biochem.* **111**, 8-15
24. Yang, S.-Y., Bittman, R., and Schulz, H. (1985) Channeling of a β-oxidation intermediate on the large subunit of the fatty acid oxidation complex from *Escherichia coli*. *J. Biol. Chem.* **260**, 2862-2868
25. Kamijo, T., Wanders, R.J.A., Saudubray, J.-M., Aoyama, T., Komiyama, A., and Hashimoto, T. (1994) Mitochondrial trifunctional protein deficiency. Catalytic heterogeneity of the mutant enzyme in two patients. *J. Clin. Invest.* **93**, 1740-1747