Inhibition by 3-Amino-1,2,4-triazole of Fatty Acid Synthesis in Cell Free System

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In this paper, the mechanism of inhibition by aminotriazole (3-amino-1,2,4-triazole) of fatty acid synthesis was studied with cell free system of the rat liver. The concentration of this compound in the liver was 20 μM at 30 min after the injection of the compound (100 mg/100 g, i.p.). About 80% of this compound in the liver cell was recovered in the supernatant fraction (cytoplasm) by subcellular fractionation of the liver homogenate. In the experiment of cell free system, the incorporations of 14C-acetate and 14C-acetyl-CoA into fatty acid were inhibited about 50% by the addition of 20 μM aminotriazole into the system and this inhibition depended on the concentration of the compound. However, the incorporation of 14C-malonyl-CoA into fatty acid was not inhibited by aminotriazole. Aminotriazole repressed the activation and stabilization of acetyl-CoA carboxylase in the rat liver by citrate and inhibited this enzyme non-competitively.

Keywords—aminotriazole; triglyceride; fatty acid synthesis; cell free system; acetyl-CoA carboxylase

Aminotriazole (3-amino-1,2,4-triazole) has been widely used as herbicide, and it was reported recently that this compound was able to decrease the lipid contents in leaves. In addition, this compound exerted a variety of effects on mammals; catalase activities in the liver and kidney were inhibited and the activity of enzyme that metabolized drug in the liver was reduced by this compound. It was demonstrated by histological and biochemical methods that the necrosis and the accumulation of fat in hepatocyte caused by the injection of CCl₄ are prevented by pretreatment with aminotriazole. It was also reported that the lability of lysosomal membrane in the liver by the injection of CCl₄ is prevented by the injection of aminotriazole. We reported previously that aminotriazole decreases markedly triglyceride level in the rat liver and significantly represses the production of CCl₄ ethanol and ethionine-induced fatty liver. Furthermore, in vivo and in vitro experiments demonstrated that the decrease in triglyceride level by aminotriazole is due to the inhibition of fatty acid synthesis in the liver.

In order to clarify the mechanism of inhibition by aminotriazole of fatty acid synthesis in the liver, we studied the effect of aminotriazole on the synthesis in cell free system.

Materials and Methods

Materials—ATP, NADP, NAD, GSH, acetyl-CoA and malonyl-CoA were purchased from Sigma Co. Acetyl-CoA-1-14C, malonyl-CoA-2-14C and 14C-KHCO₃ were purchased from Nuclear New England. Sodium-

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2-14C-acetate was purchased from Daichii Pure Chemical Co. 3-Amino-1,2,4-triazole-5-14C was synthesized from 10 mCi sodium formate and aminoguanidine sulfate according to the method of Allen and Bell,10 and identified by comparing its mp, IR and Rf value on TLC with those of purified aminotriazole. Specific radioactivity of the product was 0.9 mCi/mmol.

**Preparation of Liver Extract**—A male Wistar rat weighing about 150 g was sacrificed by decapitation, and the liver was removed and 20% liver homogenate in 0.25 m sucrose containing 0.01 m KHCO3 was prepared in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 600 x g for 10 min and the supernatant was centrifuged at 105000 x g for 60 min. The supernatant obtained by the latter centrifugation was used as liver extract.

**Fatty Acid Synthesis from 2-14C-Acetate**—The incubation system consisted of 0.1 ml liver extract (2 mg protein), 40 #mu mol potassium phosphate buffer (pH 7.1), 15 #mu mol MgCl2, 0.3 #mu mol MnCl2, 40 #mu mol isocitrate, 5 #mu mol KHCO3, 2 #mu mol ATP, 1 #mu mol NADP, 2 #mu mol NAD, 0.05 #mu mol CoA, 4 #mu mol 1-cystein, 1.07 #mu mol 2-14C-acetate (0.3 #mu C) and various concentrations of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37°C for 45 min without shaking and the reaction was stopped by the addition of 1 ml of 20% KOH in ethanol. The system was saponified for 30 min in a boiling-water bath. After acidification with 1 ml of 5 N HCl, the system was extracted three times with 5 ml of petroleum ether. The petroleum ether extracts were pooled and evaporated to remove petroleum ether. The residue was dissolved in 5 ml of n-hexane. An aliquot of the hexane solution was transferred into vial vessel and the radioactivity of this solution was determined with liquid scintillation counter.

**Fatty Acid Synthesis from 1-14C-Acetyl-CoA**—Incorporation of 14C-acetyl-CoA into fatty acid in cell free system of the rat liver was determined according to the method of Masoro et al.11 The incubation system consisted of 0.2 ml liver extract (5 ml protein), 0.42 #mu mol 1-14C-acetyl-CoA (0.05 #mu C), 48 #mu mol KCl, 0.6 #mu mol MnCl2, 40 #mu mol p-l-isocitrate, 2 #mu mol NADP, 12 #mu mol NAD, 1.5 #mu mol ATP, 4.8 #mu mol potassium phosphate buffer (pH 8.8), 12 #mu mol KHCO3 and various concentrations of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37°C for 30 min. Saponification, extraction and determination were carried out according to the method of the fatty acid synthesis from 2-14C-acetate.

**Fatty Acid Synthesis from 2-14C-Malonyl-CoA**—The incubation system consisted of 0.1 ml liver extract (0.1 mg protein), 1.5 #mu mol 14C-malonyl-CoA (0.04 #mu C), 2 nmol acetyl-CoA, 48 #mu mol KCl, 0.6 #mu mol MnCl2, 40 #mu mol p-l-isocitrate, 1.6 #mu mol NADP, 4.8 #mu mol potassium phosphate buffer (pH 8.8) and various concentrations of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37°C for 10 min. Saponification, extraction and determination were carried out as described above.

**Partial Purification of Acetyl-CoA Carboxylase**—Acetyl-CoA carboxylase of the rat liver was partially purified according to the method of Hashimoto and Numa.16 Wistar rats were fasted for 48 hr and subsequently refed with a fat free diet for 48 hr before removing the livers. The animals were killed by decapitation and the livers were quickly removed. All phosphate buffers employed in this section were potassium phosphate buffer pH 7.5 containing 5 mm 2-mercaptoethanol and 1 mm EDTA. The livers (160 g) were homogenized in 2 volumes of 0.25 m sucrose containing 0.01 m phosphate buffer. The homogenate was centrifuged at 35000 x g for 120 min. The supernatant fluid was collected and filtered though cheesecloth. The filtrate was brought to 30% saturation by the addition of solid ammonium sulfate. After stirring for 20 min, the resulting precipitate was collected by centrifugation at 23000 x g for 20 min and dissolved in 10 mm phosphate buffer. The solution thus obtained was stirred with calcium phosphate gel suspension (17.0 mg/ml) (protein: gel=1:1.8). After stirring for 20 min, the gel was collected by centrifugation at 2600 x g for 10 min and washed 3 times with 33 mm phosphate buffer. The enzyme adsorbed on the gel was then eluted twice with 0.2 m phosphate buffer. The eluate was brought to 30% saturation by the addition of solid ammonium sulfate. After stirring for 30 min, the resulting precipitate was collected by centrifugation at 23000 x g for 20 min and dissolved in 0.1 m phosphate buffer. The solution was used as partially purified enzyme and stored at -20°C. The specific activity of this enzyme in the solution increased to 21 fold compared with that of the supernatant fluid from liver homogenate.

**Subcellular Fractionation**—Subcellular fractionation of the rat liver was carried out according to the method of de Duve et al.19. After rats were killed, the livers were perfused with ice-cold saline and 10% liver homogenate in 0.25 m sucrose was prepared in a Potter—Elvehjem type homogenizer. The homogenate was centrifuged at 600 x g for 10 min, the pellet was designated nuclear fraction (N). The post-nuclear supernatant was centrifuged at 3300 x g for 10 min, the pellet was designated heavy mitochondrial fraction (M). The supernatant of fraction M was centrifuged at 15000 x g for 20 min, the pellet was designated light mitochondrial fraction (L). The supernatant of fraction L was centrifuged at 105000 x g for 60 min, the resulting pellet was designated microsomal fraction (P). The resulting supernatant was used as supernatant fraction (S).

Assays — The activity of acetyl-CoA carboxylase was determined by H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{-} fixation assay according to the method of Hashimoto and Numai.\textsuperscript{13} The H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{-} fixation assay follows the rate of acetyl-CoA-dependent incorporation of H\textsuperscript{14}CO\textsubscript{3}\textsuperscript{-} into acid-stable material, i.e. malonyl-CoA. Acid phosphatase activity was determined by using β-glycerophosphate as substrate, and the inorganic phosphate liberated was measured according to the method of Lindberg and Ernster.\textsuperscript{14} Protein content was determined by the method of Lowry et al.,\textsuperscript{15} with bovine serum albumin as a standard.

Results and Discussion

Results of the experiments in vitro\textsuperscript{11} by liver slice and in vivo\textsuperscript{6} suggested that aminotriazole inhibits fatty acid synthesis in the liver. In order to clarify the mechanism of this inhibition by aminotriazole of fatty acid synthesis, the time-course of the content of aminotriazole in the liver was first investigated (Fig. 1). At 30 min after the injection relatively high amounts of aminotriazole were found in the liver, about 20 mmol per g. liver. Then its content decreased rapidly in the time between 30 min and 2 hr after the injection. However, after 2 hr the content decreased very slowly and even at 6 hr after the injection the value was still 5 mmoles per g. liver. Thus, it was indicated that aminotriazole is incorporated into the liver soon after the injection. Therefore, its subcellular distribution in the liver was examined. Fig. 2 shows the distribution at 30 min after the injection. A few percent of aminotriazole uptake into the liver was found in light mitochondrial fraction (L frac-

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tion), although most of this material was distributed in the supernatant fraction (S fraction, cytoplasm). Recovery of aminotriazole in the supernatant fraction was about 50% of the total material in the cell. The same result was also observed at 6 hr and 24 hr after the injection. This subcellular fractionation is justified proper since the activity of acid phosphatase was the highest in light mitochondrial fraction as indicated by de Duve et al.\textsuperscript{13} Thus, most of aminotriazole uptaken into the liver was distributed in the cytoplasm of the cell, the site of fatty acid synthesis.

The enzymes relating to fatty acid synthesis in the mammalian cell is divided into two enzymes, acetyl-CoA carboxylase and fatty acid synthetase (multi-enzyme complex), both of which are distributed in the supernatant fraction of liver cell.\textsuperscript{16,17} In order to study the mechanism of inhibition by aminotriazole of these enzymes in fatty acid synthesis, effect of aminotriazole on the synthesis of fatty acid from $^{14}$C-acetate, $^{13}$C-acetyl-CoA and $^{14}$C-malonyl-CoA was studied in a cell free system of the liver (Fig. 3). Since the concentration of aminotriazole in the liver was 20 mM at 30 min after the injection (Fig. 1) and most of aminotriazole was found in cytoplasm (Fig. 2), the experiment was carried out with the concentration of aminotriazole from 0 to 20 mM. Incorporation of $^{14}$C-acetate into fatty acid was inhibited with an increase in the concentration of aminotriazole. The degree of this inhibition depends on the concentration of aminotriazole and reached 50% of control at 20 mM of aminotriazole. These results indicated that aminotriazole inhibits the over all reaction of fatty acid synthesis in cell free system. Similarly the incorporation of $^{14}$C-acetyl-CoA into fatty acid was inhibited by aminotriazole and the degree of the inhibition depends on the concentration of this material. In this case the incorporation into fatty acid was inhibited about 40% at 20 mM of aminotriazole. However, the incorporation of $^{14}$C-malonyl-CoA into fatty acid was not inhibited by aminotriazole and was not effected even with an increase in the concentration of aminotriazole. These findings indicate that aminotriazole inhibits acetyl-CoA carboxylase but does not inhibit fatty acid synthesis (multi-enzyme complex) in the liver.

Consequently, effect of aminotriazole on partially purified acetyl-CoA carboxylase was examined. The enzyme occurs in two forms, an inactive protomer and an active polymer. Citrate, the positive allosteric modulator, is known to stimulate the enzyme activity by aggregating protomer to form polymer.\textsuperscript{18} Fig. 4 shows an influence of aminotriazole on the stimulatory effect of citrate. The enzyme was activated by preincubation for 30 min with 10 mM citrate. However, the activation of this enzyme by citrate in this system was completely inhibited by the addition of aminotriazole. On the other hand, the enzyme activity was markedly decreased by the preincubation without citrate. This repression of the enzyme activity was further enhanced by the addition of aminotriazole into the system. These findings indicate that aminotriazole inhibits the stimulatory effect of citrate. Thus the inhibition by aminotriazole of the enzyme activity may be due to the interference in the formation of the polymerized enzyme. The mode of inhibition by aminotriazole of the

\textsuperscript{17} F. Lynen, \textit{Biochem. J.}, 102, 381 (1967).
enzyme was further studied by the method of Dixon plot (Fig. 5). The straight line in each concentration of substrate \((\text{KHCO}_3)\) crossed in one point on the abscissa. This result indicates that aminotriazole inhibits non-competitively the enzyme. The \(k_i\) value of aminotriazole on the enzyme was 33 mm.

![Graph](image)

**Fig. 4.** Effect of Aminotriazole on the Effect of Citrate on Acetyl-CoA Carboxylase

The enzymes were preincubated with each mixture of final volume of 1 ml for various times, and then 0.1 ml of preincubation mixture was added to the reaction mixture containing \(^{14}C\)-\(\text{KHCO}_3\) and the reaction was carried out for 10 min at 37\(^\circ\).  
- ●: The enzyme was preincubated with 10 mm citrate.  
- ●●: The enzyme was preincubated with 10 mm citrate and 50 mm aminotriazole.  
- ○○: The enzyme was preincubated without citrate and aminotriazole.  
- ○-○: The enzyme was preincubated without citrate with 50 mm aminotriazole.

![Graph](image)

**Fig. 5.** Dixon Plot of Inhibition of Aminotriazole on Acetyl-CoA Carboxylase

Aminotriazole was added to various final concentrations in the preincubation and reaction mixture. The reaction was carried out for 10 min at 37\(^\circ\) with 2, 5 and 10 \(\mu\)mol \(^{14}C\)-\(\text{KHCO}_3\) as substrate.

It was demonstrated in this paper by the experiment of cell free system that the decrease in liver triglyceride level caused by the injection of aminotriazole is due to the inhibition of fatty acid synthesis. Furthermore, it was established that the relatively high amounts of aminotriazole are incorporated into the liver rapidly after the injection, and that most of the material is distributed in cytoplasm, the site of fatty acid synthesis. The inhibition of the synthesis by aminotriazole was proved to be due to the inhibition of acetyl-CoA carboxylase in cytoplasm, the rate-limiting enzyme in fatty acid synthesis.