EFFECT OF MALOTILATE (DIISOPROPYL 1,3-DITHIOL-2-YLIDENEMALONATE) ON THE PROTEIN SYNTHESIS IN RAT LIVER

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Abstract—The effect of malotilate (diisopropyl 1,3-dithiol-2-ylidene malonate) on the protein synthesis in rat liver was studied in vivo and in vitro. Oral administration of malotilate to rats caused an increase in the protein and RNA contents of the liver and led to an acceleration of 14C-leucine incorporation into microsomal and cytosol proteins. In a cell-free system, the protein synthesis was enhanced by treatment with malotilate, and an unknown factor(s) which participates in the protein synthesis was found in the cytosol fraction prepared from malotilate treated livers. These results suggest that malotilate is a new type of inducer for protein synthesis. On the basis of the observations obtained in the present study, a hypothesis can be formulated that malotilate enhances liver protein synthesis by accelerating RNA synthesis and/or increasing the transport of RNA from nuclei to cytosol in rat liver.

Malotilate (diisopropyl 1,3-dithiol-2-ylidene malonate) has been developed as a drug for chronic hepatitis and cirrhosis. In the previous paper, we have reported that this compound exerts a curative effect on CCl4-induced fatty liver in rats by improving the impaired protein synthesis in the liver (1). Malotilate is known to induce the microsomal electron transport system in rat liver, especially cytochrome b5 and NADPH-cytochrome c reductase (2, 3). These enzymes are also induced by the administration of other xenobiotics (4–6). Shuster and Jick suggested that the induction of microsomal enzymes is reflected in the increased rate of protein synthesis in the liver (7).

In the present work, the effect of malotilate on the protein synthesis in rat liver was studied in vivo and in vitro. The differences between malotilate and other chemicals with respect to their effect on liver protein synthesis are discussed.

MATERIALS AND METHODS

Chemicals: Malotilate was synthesized and obtained with greater than 99.5% purity by repeated crystallization at the Chemical Research Center of Nihon Nohyaku Co., Ltd. Radioactive L-[U-14C] leucine (351 mCi/mmol) was purchased from the Radiochemical Centre, Amersham (England). Other chemicals used here were of the reagent grade.

Treatment of animals: Male Sprague-Dawley rats (5 week-old, 110±5 g, body weight) were used in this study. They were given laboratory chow (CE-2, Nihon Clea Co.) and tap water ad libitum. Malotilate
dissolved in olive oil was given orally to them at a volume of 5 ml/kg. Control rats received an equivalent amount of olive oil. \(^{14}\)C-Leucine (50 \(\mu\)Ci/kg) was injected intraperitoneally 40 min before sacrifice. Animals were sacrificed after fasting for 15 hr.

Isolation of subcellular components: The liver was removed, rinsed, perfused with cold 0.25 M sucrose and homogenized in 4 vol. of 0.25 M sucrose containing 10 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA. The homogenate was filtered through three layers of gauze and was used for subcellular fractionations. Nuclear, mitochondrial, microsomal and cytosol fractions were prepared by the method of Ch'ih et al. (8). Free and membrane-bound polyribosomes were isolated from the post-mitochondrial supernatant by the method of Cardelli et al. (9). Ribosomes obtained by the above method exhibited an \(A_{260}/A_{280}\) ratio of 1.50–1.72. The cytosol fraction was further centrifuged at 150,000 \(\times\) g for 120 min to obtain the supernatant.

\(^{14}\)C-Leucine incorporation into the protein fraction in a cell-free system: \(^{14}\)C-Leucine incorporation in a cell-free system was measured according to the procedure described by Arimasa (10). The prepared polyribosomes were suspended in 0.25 M sucrose containing 40 mM KCl, 20 mM Tris-HCl buffer (pH 7.6), 6 mM mercaptoethanol, 5 mM Mg\((\text{CH}_3\text{COO})_2\) and 0.1 mM EDTA.

The cell-free system for protein synthesis consisted of Tris-HCl (27 mM, pH 7.6), KCl (78 mM), Mg\((\text{CH}_3\text{COO})_2\) (5.8 mM), 2-mercaptoethanol (4.6 mM), sucrose (130 mM), ATP (1 mM), GTP (0.2 mM), creatine phosphate (20 mM), creatine phosphokinase (2 units), amino acid mixture (40 mM), \(^{14}\)C-leucine (4 mM, 1.3 \(\mu\)Ci/ml), ribosomes (0–320 \(\mu\)g of RNA) and the 150,000 \(\times\) g supernatant (1.2 mg of protein) in a final volume of 0.5 ml. The mixture was incubated at 37°C for 15 min and then the reaction was stopped by the addition of 4 ml of 5% HClO\(_4\). After heating at 90°C for 15 min, the reaction mixture was kept at 0°C for 30 min and centrifuged at 3,000 rpm for 15 min. The acid insoluble fraction was washed three times with 4 ml of 5% HClO\(_4\), dissolved in 0.1 N NaOH and measured for radioactivity.

Analytical methods: Protein, RNA and DNA fractions in the liver were separated by the procedure devised by Schmidt and Thannhauser (11) and modified by Fleck and Munro (12). RNA was determined by the orcinol reaction (13) in which yeast RNA was used as the standard. DNA was quantified by the diphenylamine reaction according to the procedure of Burton (14). The protein fraction was dissolved in 0.1 N NaOH and the protein content was determined by the method of Lowry et al. (15).

Determination of radioactivity: The protein fraction dissolved in 0.1 N NaOH was neutralized with 1 N HCl and suspended in Bray's scintillation fluid. The radioactivity was determined by a scintillation spectrometer equipped with an external standard. The data were corrected for background (15 to 30 cpm) and counting efficiency (70 to 80%).

Statistics: The significance of the difference between two mean values was assessed by the Student's \(t\)-test.

RESULTS

Effect of malotilate on the liver weight and the protein, RNA and DNA contents in the liver: The liver weight and the protein, RNA and DNA content in the liver from malotilate-treated and control rats are shown in Table 1. The protein and RNA contents in the malotilate-treated group were significantly greater than those in the control group. It was also clear that those increases depended upon the dose of malotilate. However, the DNA content was not changed
Table 1. Liver weight and protein, RNA and DNA contents in livers from malotilate-treated and control rats

| Malotilate dosage (mg/kg) | 0 (control) | 100 | 250 | 500 |
|--------------------------|-------------|-----|-----|-----|
| Weight (g/100 g B.W.)    | 3.9±0.1     | 4.4±0.1 | 4.7±0.1** | 5.7±0.2** |
| Protein (mg/100 g B.W.)  | 918±32      | 1,024±33** | 1,100±38** | 1,322±75*** |
| (mg/mg DNA)              | 66.7±0.9    | 74.7±2.8* | 77.6±2.2** | 85.0±2.1** |
| RNA (mg/100 g B.W.)      | 57.0±2.9    | 62.1±2.9  | 66.3±1.7** | 76.3±3.8** |
| DNA (mg/100 g B.W.)      | 13.0±0.3    | 13.4±0.6  | 13.9±0.4  | 13.5±0.3  |

Each value is a mean±S.E. of determinations in 5 rats. Malotilate (100, 250, and 500 mg/kg) was administered orally for 3 days. Rats were sacrificed 24 hr after termination of malotilate administration. Significant difference from a control value: *(P<0.05), **(P<0.01), ****(P<0.001). For further details, see the Materials and Methods section.

by malotilate treatment. All the malotilate-treated groups had ratios of protein to DNA that were larger than those in the control group. These results suggest that malotilate enhances the protein synthesis in the liver.

Effect of malotilate on the $^{14}$C-leucine incorporation into liver protein: Figure 1 presents the time-course changes of $^{14}$C-leucine incorporation into rat liver proteins after administration of malotilate. In the malotilate-treated groups, $^{14}$C-leucine incorporation into the total and microsomal protein reached the maximum level 15 hr after administration of malotilate and was thereafter decreased, returning to a pretreatment level eventually. The maximum levels of $^{14}$C-leucine incorporation into the total and microsomal protein were 117% and 122% of the control level, respectively.

Effect of malotilate on $^{14}$C-leucine incorporation into the subcellular protein: In each subcellular fraction, the $^{14}$C-leucine incorporation into protein was determined 15 hr after administration of various dosages of malotilate. As shown in Fig. 2, $^{14}$C-leucine was increasingly incorporated in response to the dosages into the total, microsomal, and cytosol proteins, but not into the nuclear and mitochondrial proteins. The stimulation of incorporation into microsomal protein was most remarkable; the rate

Fig. 1. Time course changes of $^{14}$C-leucine incorporation into the total and microsomal protein of rat liver. Malotilate (250 mg/kg) was administered orally to rats at zero time. $^{14}$C-Leucine (50 μCi/kg) was injected intraperitoneally 40 min before sacrifice. Animals were sacrificed at the time indicated in the figure. Each point represents a mean of determinations in 5 rats. A vertical bar shows the S.E. ○: control group; ●: malotilate-treated group. Significant difference from a control value: *(P<0.05).
of incorporation at a dose level of 500 mg/kg was 33% greater than that observed in the control group.

These findings indicate that the protein synthesis in the microsomal as well as the cytosol fraction is enhanced by malotilate.

Effect of malotilate on the $^{14}$C-leucine incorporation into free and membrane-bound polyribosomes: With a view of clarifying whether or not malotilate accelerates the synthesis of any specific protein in the liver, $^{14}$C-leucine incorporation into the protein of free and membrane-bound polyribosomes was investigated. As shown in Fig. 3, the radioactivities of the free and membrane-bound polyribosomes increased almost linearly in response to the dosages of malotilate. There was no appreciable difference in the degree of increase between the two kinds of polyribosomes. This indicates that malotilate increases the rate of protein synthesis in the intracellular and extracellular proteins.

Effect of malotilate on the $^{14}$C-leucine incorporation into acid-insoluble fraction in a cell-free system: As described above, $^{14}$C-leucine incorporation into liver proteins was

Fig. 2. $^{14}$C-Leucine incorporation into subcellular proteins of rat liver. Malotilate (250 and 500 mg/kg) was administered orally to rats, which were sacrificed after 15 hr. $^{14}$C-Leucine (50 $\mu$Ci/kg) was injected intraperitoneally 40 min before sacrifice. Each point represents a mean of determinations in 5 rats. A vertical bar shows the S.E. O: total protein, •: nuclear fraction, △: mitochondrial fraction, ▲: microsomal fraction, □: cytosol fraction. Significant difference from a control value: *($P<0.05$), **($P<0.01$).

Fig. 3. $^{14}$C-Leucine incorporation into the free and membrane-bound polyribosomal fraction. Experimental conditions were the same as described in Fig. 2. Each point represents a mean of determinations in 5 rats. A vertical bar shows the S.E. Significant differences from a control value: *($P<0.05$), **($P<0.01$).
accelerated by malotilate. In order to elucidate the effect of malotilate on the protein synthesis of the liver, the liver ribosomes and the 150,000 x g supernatant prepared from the malotilate-treated and/or control rats were incubated in the cell-free system described in Methods. Figure 4 shows the relationship between the ribosome content of the liver and the 14C-leucine incorporation into the acid-insoluble fraction in the cell-free system. The combination of the ribosomes and the 150,000 x g supernatant, both of which were obtained from the malotilate-treated rats, resulted in about 140% 14C-leucine incorporation as compared with the control combination. Similarly, the combination of ribosomes from the control rats and supernatant from the malotilate-treated rats resulted in 125% incorporation. On the other hand, when the ribosomes prepared from malotilate-treated rats and 150,000 x g supernatant prepared from control rats were incubated, the degree of incorporation was the same as that of the control. Thus, a strong possibility was raised that an unknown factor(s) which is activated by malotilate to prompt protein synthesis may be present in the 150,000 x g supernatant.

The direct effect of malotilate on the protein synthesis was also studied in the

**Table 2.** Effect of malotilate on the amino acid incorporation into the acid-insoluble fraction in a cell-free system

| Substance added to the incubation mixture | Incorporation of 14C-leucine (dpn) |
|------------------------------------------|----------------------------------|
| Dimethyl sulfoxide (%) | Malotilate (mM) | 2,539±97 | 2,716±50 | 2,630±58 | 2,787±56 | 3,114±184 |
| 0 | 0 | 2,539±97 | 2,716±50 | 2,630±58 | 2,787±56 | 3,114±184 |
| 4 | 0 | 2,716±50 | 2,630±58 | 2,787±56 | 3,114±184 |
| 4 | 0.04 | 2,630±58 | 2,787±56 | 3,114±184 |
| 4 | 0.35 | 2,787±56 | 3,114±184 |
| 4 | 3.47 | 3,114±184 |

The ribosomes and the 150,000 x g supernatant were prepared from pooled livers from 8 normal rats. The ribosomes were incubated at 37°C for 15 min in the standard cell-free system as described in the text with the 150,000 x g supernatant. Malotilate was dissolved in dimethyl sulfoxide and added to the incubation mixture. Values presented are means±S.E. of four determinations. For further details, see the Materials and Methods section.

![Fig. 4. 14C-Leucine incorporation into the acid-insoluble fraction in a cell-free system. Malotilate (250 mg/kg) was administered orally to rats, which were sacrificed after 15 hr. The ribosomes and the 150,000 x g supernatant fraction were prepared from pooled livers from each of 5 control and 5 malotilate-treated rats. The ribosomes of control or malotilate-treated liver were incubated at 37°C for 15 min in the standard cell-free system as described in the text with the 150,000 x g supernatant fraction prepared from control or malotilate-treated livers in the following combinations: ○: control ribosomes+control supernatant; ●: control ribosomes+malotilate-treated supernatant; △: malotilate-treated ribosomes+control supernatant; ▲: malotilate-treated ribosomes+malotilate-treated supernatant.](image-url)
cell-free system with the outcome given in Table 2. The $^{14}$C-leucine incorporation into protein was not much influenced by the addition of malotilate.

**DISCUSSION**

As shown in Table 2, oral administration of malotilate to rats led to an increase in liver weight. Similar tendencies of increase were also found with respect to the RNA and protein contents of the liver. The total DNA content is known to be proportional to the number of cells (16). The protein/DNA ratio, which is used as an indicator of protein content per cell, was raised significantly by malotilate (Table 1). Therefore, the malotilate-induced increase in liver weight is considered to represent the enlargement of liver cells which is due to the increase in their protein and RNA contents.

As shown in Fig. 1, the $^{14}$C-leucine incorporation into liver protein was increased by malotilate administration. The increase was predominant in the microsomal protein and some increase was also observed in the cytosol protein (Fig. 2). Some inducers of drug metabolizing enzymes, such as phenobarbital and polychlorinated biphenyls, affect only microsomal protein synthesis. Kato et al. showed that phenobarbital promoted the incorporation of amino acids into the microsomal fraction only (17). Narbonne also showed that ingestion of polychlorinated biphenyls gives rise to an increase in the protein synthesis in the microsomal fraction of rat liver (18). The fact that malotilate enhanced both protein syntheses in the microsomal and cytosol fractions suggests that it may be a new type of inducer for protein synthesis in the liver. Rollestein has shown that the membrane-bound polyribosome participates in the synthesis of secretory proteins, while the free polyribosome is active in synthesizing intracellular proteins in rat liver (19). In this study, the incorporation of $^{14}$C-leucine into free and membrane-bound polyribosomes was equally increased by malotilate (Fig. 3). This result suggests that malotilate enhances both the synthesis of the intracellular and extracellular (secretory) proteins.

In the cell-free system, addition to an incubation mixture of the 150,000 x g supernatant of livers from malotilate-treated rats resulted in an acceleration of protein synthesis (Fig. 4). The factors which take part in protein synthesis such as t-RNA, aminoacyl-t-RNA synthetase, etc. are known to exist in the 150,000 x g supernatant. In this respect, it may be postulated that the malotilate-induced acceleration of protein synthesis was the result of stimulation of those factors in the supernatant fraction.

In general, phenobarbital and 3-methylcholanthrene have been thought to bring about gene activation and are often considered together when an explanation was sought to characterize the molecular mechanisms of their action. Kumar et al. recently reported, however, that phenobarbital acted primarily at a cytoplasmic level by enhancing the transport of RNA from nuclei to cytoplasm (20). On the other hand, 3-methylcholanthrene or its active metabolites bind directly to the chromatin and lead to specific gene activation by enhancing the synthesis of limited proteins (20).

On the basis of these observations, a hypothesis can be built that malotilate accelerates RNA synthesis and/or increases the transport of RNA from nuclei to cytosol in rat liver. Further studies are in progress to produce data that may verify this hypothesis.

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