Suppressive Effects of Uracil, Tyrosine, and Phenylalanine Contained in Human-Placenta Extract on Acute Ethanol-Induced Liver Injury in Mice

Shin-ichi Togashi, Noriko Takahashi, Satoshi Watanabe, Akiko Ishiguro and Tetsuya Fukui*

Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa-ku, Tokyo 142–8501, Japan

(Received October 19, 1999; Accepted November 18, 1999)

Human-placenta extract (PLx) possesses various physiologically important activities, such as antioxidant activity and anti-inflammatory activity. Our previous study elucidated that uracil, tyrosine, and phenylalanine function as the main antioxidative substances in PLx. In order to confirm whether these compounds have similar function as PLx, we examined the effects of the administration on ethanol-induced liver injury in mice. As a result, PLx suppressed ethanol-induced decrease in hepatic glutathione level, increase in thiobarbituric acid reaction substance (TBARS), increase in the activities of glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, and superoxide dismutase, and decrease in the activity of catalase. A mixture of uracil, tyrosine, and phenylalanine showed similar antioxidant activity to PLx, except for failure to suppress the increase in TBARS. Although these results suggest that PLx has some other unknown components to alleviate acute alcoholic liver injury, the mixture of uracil, tyrosine, and phenylalanine appeared to have almost the same ability to suppress acute ethanol-induced liver injury in mice as that of PLx.

Key words —— ethanol, liver, placenta, antioxidant, uracil, tyrosine, phenylalanine

INTRODUCTION

Placental tissue is known to suppress lipid peroxidation in blood and protect the fetus from the toxicity of reactive oxygen by stimulating antioxidant defense mechanisms in the late gestational stage.[1] Accordingly, human-placenta extract (PLx) has been shown to possess a wide spectrum of actions in several pathological and physiological disorders.[1–4] Girotto G. *et al.*[1] demonstrated that therapeutic use of PLx was effective in improving symptoms of myopic and senile chorioretinal dystrophies. Banerjee K. *et al.*[2] reported that PLx significantly suppressed inflammation of carrageenin-induced edema in rats, and suggested that the active component of PLx is possibly a small molecule. Further, Itoh *et al.*[3] indicated that sialic acid is an important active ingredient for the anti-inflammatory effect of PLx. Very recently, we reported that the main active components showing antioxidant activity in PLx are uracil, tyrosine, and phenylalanine.[5]

In this study, we examined the effect of an antioxidant mixture consisting of uracil, tyrosine, and phenylalanine, contained in PLx, on ethanol-induced liver injury in mice. This is because ethanol ingestion is known to produce a variety of pathologic alterations in the liver, featuring a decrease in the concentration of glutathione (GSH) and an increase in lipoperoxidation,[6] which seems to result from the generation of oxygen free radicals produced in the course of metabolism of acetaldehyde derived from ethanol.[6]

*To whom correspondance should be addressed: Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 4–41, Ebara 2-Chome, Shinagawa-ku, Tokyo 142–8501, Japan. Tel.: +81-3-5498-5771; Fax: +81-3-5498-5771; E-mail: fukui@hoshi.ac.jp.

MATERIALS AND METHODS

Materials —— Lyophilized PLx was kindly provided by Snowden Co. Ltd. (Tokyo, Japan). 2-Deoxy-D-

ribose, trichloroacetic acid, and 4,6-dihydroxy-2-mercaptopyrimidine (thiobarbituric acid), GSH, glutamate oxaloacetate transaminase (GOT) assay kit and glutamate pyruvate transaminase (GPT) assay kit were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade or of the highest purity available.

Administration of Antioxidant Mixture to Mouse — Male ddy mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Japan) at 5 weeks of age and housed in plastic cages under a 12-h light/dark cycle for 1 week before experimental use. They were divided in 5 groups of 10 mice each and allowed free access to standard laboratory food and water. Antioxidant mixture was prepared by dissolving uracil, tyrosine, and phenylalanine at 1.3 mM, 4.1 mM, and 11 mM, respectively in distilled water. Concentrations of these three compounds in the mixture were adjusted so as to give the same concentrations as observed in 10% crude PLx.

Group 1 and Group 4 were given distilled water (0.3 ml, *p.o.*). Group 2 and Group 5 were given a 10% solution of PLx (0.3 ml, *p.o.*) which was verified to contain uracil, tyrosine, and phenylalanine at 1.3 mM, 4.1 mM, and 11 mM, respectively. Group 3 and Group 6 were given the antioxidant mixture (0.3 ml, *p.o.*). Administrations were executed once a day for three days. At 15 minutes after the last administration, Group 4, Group 5, and Group 6 were given 50% ethanol (0.3 ml, i.p.), while Group 1 and Group 3 were given distilled water (0.3 ml, i.p.). At 24 h after the ethanol administration, the mice were decapitated, and blood and liver samples were collected.

Preparation of Blood and Liver Samples — The excised livers were stored in 0.1% NaCl solution. All subsequent operations were carried out at 0—4℃. Livers were wiped to remove the moisture by paper filters, weighed, and 0.8 g portions of each liver were taken into 7.2 ml of 0.1% NaCl solution. They were homogenized in a Teflon glass homogenizer for 1 min to make 10% homogenates. Homogenized livers were used for the determination of GSH. The homogenate was centrifuged at 105000 × *g* for 1 h, and the supernatant was used for the measurement of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) activities and protein content.

The collected blood was kept at room temperature for 30 min, and centrifuged at 3000 rpm for 10 min, and the supernatant was used to measure GOT and GPT activity.

Measurement of Protein — Protein was determined by the method of Lowry *et al.* using bovine serum albumin as a standard.[7]

Measurement of TBARS, GSH, SOD, Catalase and GSH-Px — The TBARS[8] and GSH contents,[9] and SOD,[10] catalase[11] and GSH-Px[12] activities were determined as described elsewhere.

Measurement of GOT and GPT Activity — GOT and GPT activities in blood serum were measured using the Wako assay kit according to the manufacturers instructions.

Measurement of Antioxidant Activity — Antioxidant activity was measured by the deoxyribose method[13] as described previously.[5]

RESULTS

Antioxidant Activity of Antioxidants in PLx

Di Luzio[14] has observed that acute ethanol-induced fatty liver formation can be prevented in rats by administration of certain antioxidants. Therefore, we examined the effect of antioxidants which had been purified from PLx by us and identified to be uracil, tyrosine, and phenylalanine.[5]

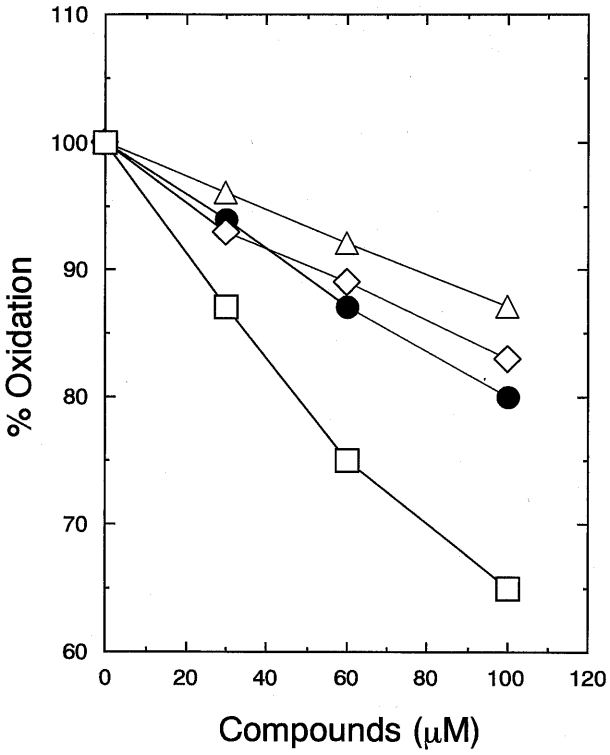

Fig. 1. Comparison of Antioxidative Activities of Uracil, Tyrosine, Phenylalanine and Mannitol by the Deoxyribose Method

Assay conditions are described in Materials and Methods. □: uracil, ◇: tyrosine, ●: phenylalanine, △: mannitol.

Table 1. Effects of Antioxidants in PLx (Uracil, Tyrosine, and Phenylalanine) on Body Weight, Liver Weight, Liver GSH, and Liver TBARS of Mice Suffering from Acute Alcoholic Liver Injury

Group number	Preadministration (*p.o.*)	EtOH administration (i.p.)	Body weight (g)	Liver weight ratio (Liver weight/Body weight) (%)	Liver GSH (μg/g liver)	Liver TBARS (μg/g liver)
1	Distilled water	−	31.78±0.96 (100)	5.00±0.12 (100)	2183.1±215.7 (100)	4.52±0.39 (100)
2	PLx	−	30.25±0.80 (95)	5.20±0.27※ (104)	2453.2±198.3※ (112)	4.20±0.59※ (93)
3	Antioxidants	−	34.04±0.44#※ (107)	5.08±0.18※ (102)	2416.8±244.8※ (111)	4.34±0.10※
4	Distilled water	+	32.64±1.04 (103)	5.76±0.32# (115)	826.7± 72.2# (38)	7.62±0.20# (169)
5	PLx	+	30.96±0.71 (97)	5.73±0.22# (115)	1624.1±254.7※ (74)	4.15±0.50※ (92)
6	Antioxidants	+	31.58±1.09 (99)	5.66±0.22# (113)	1463.7± 83.8#※ (67)	7.70±0.56# (170)

Antioxidants contain uracil (13 μmol/kg body weight), tyrosine (41 μmol/kg body weight), and phenylalanine (110 μmol/kg body weight). Pretreatment (*p.o.*) of antioxidants was given 48 h, 24 h, and 15 min before ethanol administration (3.95 g/kg body weight, i.p.). The data are expressed as means±S.E.

#) Significantly different from Group 1 ($p<0.05$), ※) Significantly different from Group 4 ($p<0.05$).

Fig. 1 shows the antioxidant activities of the three compounds. Uracil had about 2 times stronger antioxidant activity than tyrosine and phenylalanine, which have almost the same activity.

Effects of Antioxidant Pretreatment on Body Weight and Liver Weight

There were no significant differences in body weights between Group 1 (vehicle control) and the other Groups, except for Group 3 (antioxidant control), in which body weight was significantly greater by 7% than others (Table 1). As for liver weight ratio, all of the ethanol administered Groups (Groups 4, 5, and 6) exhibited significant increases (ranging from 15% to 13%), indicating ethanol toxicity on the liver.

Effects of the Antioxidant Mixture Pretreatment on Hepatic GSH and TBARS Content

The hepatic GSH content decreased to 38% of that of control group by ethanol administration (Group 4)(Table 1). However, these decreases were partly abolished by preadministration of PLx (Group 5) or the antioxidant mixture (Group 6).

The hepatic TBARS content increased by 69% by ethanol administration (Group 4). However, pre-administration of the antioxidant mixture did not abolish the increase of hepatic lipid peroxidation (Group 6), whereas PLx inhibited it completely (Group 5), indicating that PLx possesses some unknown components other than uracil, tyrosine, and phenylalanine.

Effects of Antioxidant Pretreatment on GOT and GPT Activity in Blood Serum

As shown in Fig. 2 (A) and (B), GOT and GPT activities in blood serum by ethanol administration (Group 4), were increased 1.7 times and 3.5 times, respectively as compared with the control group. On the other hand, GOT and GPT activities in the PLx and antioxidant pre-administered groups were significantly reduced, indicating that the antioxidant mixture, as well as PLx, have potential to alleviate liver injury.

Effects of Antioxidants on SOD, Catalase, and GSH-Px Activity in Liver Cytosol

As shown in Fig. 2 (C), (D) and (E), SOD activity was enhanced by 12% by ethanol administration. Similar results were obtained by Valenzuela *et al.*,[15] who demonstrated that alcohol-induced enhancement of liver lipoperoxidation was associated with the increased activity of SOD, as an adaptive change to an elevated supply of superoxide anions. PLx and the antioxidant mixture pre-administration suppressed this en-

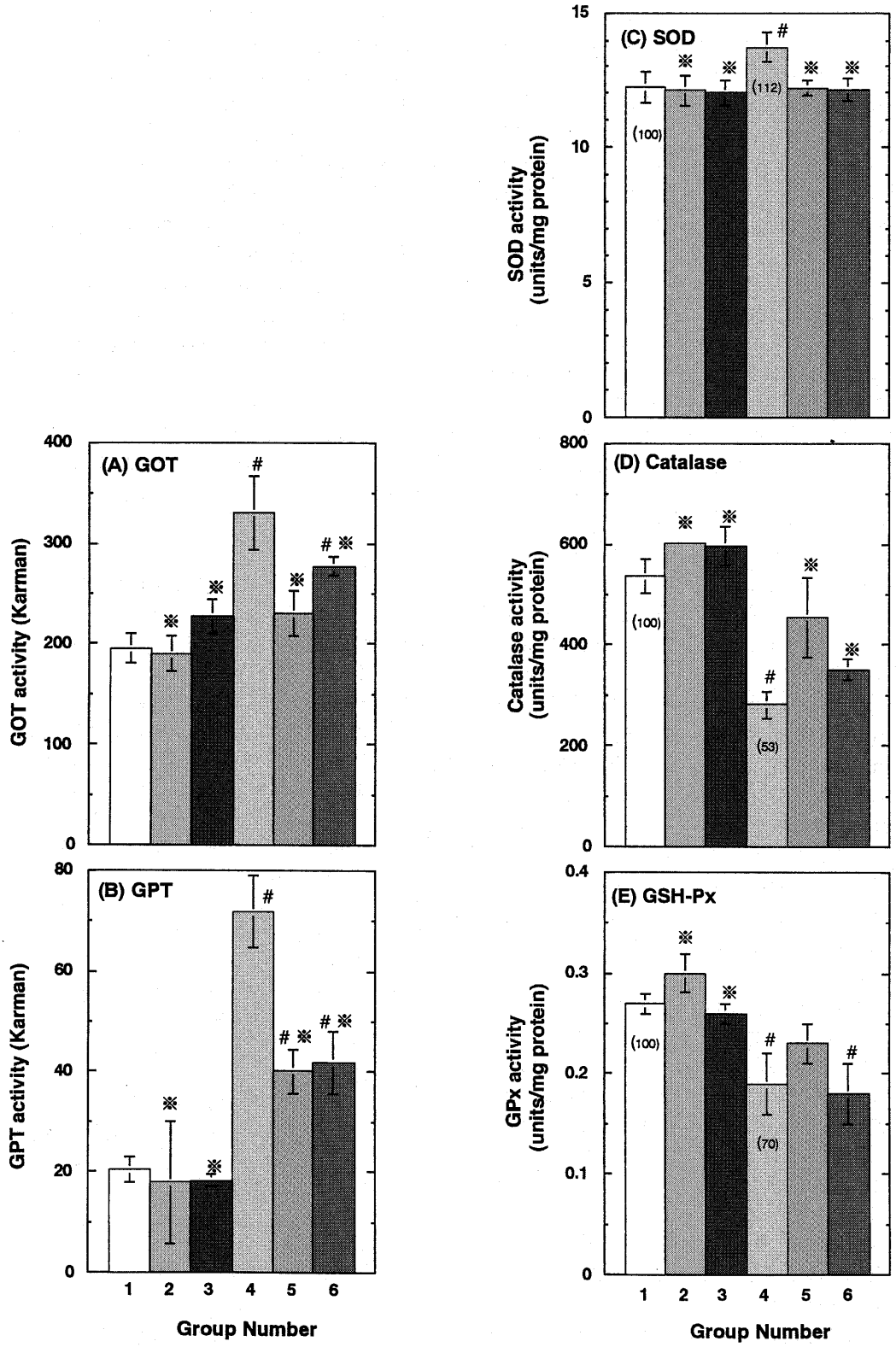

Fig. 2. Effects of Antioxidants in PLx (Tyrosine, Phenylalanine and Uracil) on GOT and GPT in Blood Serum and SOD, Catalase, and GSH-Px in Liver Cytosol of Mice Suffering from Acute Alcoholic Liver Disease

Antioxidants contain uracil (13 μmol/kg body weight), tyrosine (41 μmol/kg body weight), and phenylalanine (110 μmol/kg body weight). Pretreatment (*p.o.*) was given 48 h, 24 h, and 15 min before ethanol administration (3.95 g/kg body weight, i.p.). The data are expressed as means ± S.E.

#) Significantly different from Group 1 ($p<0.05$), ※) Significantly different from Group 4 ($p<0.05$).

□: Group 1: Water (*p.o.*), ▨: Group 2: PLx (*p.o.*), ■: Group 3: Antioxidants (*p.o.*), ▨: Group 4: EtOH (i.p.), ▨: Group 5: EtOH (i.p.)+PLx (*p.o.*), ▨: Group 6: EtOH (i.p.)+Antioxidants (*p.o.*).

hancement significantly.

Catalase and GSH-Px activities decreased to 53% and 70% of that of control group, respectively. Pre-administration of PLx or the antioxidant mixture demonstrated the ability to abolish the decrease of catalase by ethanol administration significantly. However, the decrease in GSH-Px activity was not effected.

DISCUSSION

In the present study, we have investigated the suppressive effects of a mixture of uracil, tyrosine, and phenylalanine, which were isolated as antioxidants from PLx, on ethanol-induced liver injury in mice.

As a result, antioxidants could suppress the ethanol-induced increase in GOT and GPT activities, which are important markers of hepatocyte function. Remmer *et al.*[16] suggested that the primary target of the oxygen-radical attack promoted by ethanol is cellular proteins, rather than lipids, and that the increased TBARS by ethanol should not be viewed as a result of lipid peroxidation only. Our observation that PLx, but not the antioxidants, could suppress TBARS, indicates that PLx has some unknown components having the ability to suppress ethanol-induced TBARS enhancement.

Resistance of many cells to oxidative stress is associated with high intracellular levels of GSH.[17] GSH acts directly as free radical scavenger by neutralizing hydroxyl radical, restores damaged molecules by hydrogen donation, reduces peroxides, and maintains protein thiols in the reduced state.[18] Speisky[19] reported that the increased reduction of GSH from liver and inhibition of GSH synthesis constitute the mechanism of ethanol-induced decrease in GSH. Whether the antioxidant mixture suppresses enhanced hepatic permeability for GSH and/or suppresses depressed GSH synthesis requires further investigation.

SOD,[20] catalase and GSH-Px are considered to be essential enzymes for defense against the toxic effects of oxygen. Valenzuela *et al.*[15] demonstrated that alcohol-induced enhancement of liver lipoperoxidation occurred concomitantly with an increase in the activity of SOD. This result is consistent with ours. PLx and antioxidants abolished the decrease in catalase activity. Since antioxidants inhibited the increase in SOD activity and the decrease in catalase activity, the mixture can function to alleviate the toxic effects of oxygen *in vivo*.

Our data indicated that antioxidants like PLx can significantly alleviate the decrease in GSH content in the liver, enhancement of GOT and GPT activity in blood serum, enhancement of SOD activity, and decrease of catalase activity in hepatic cytosol, which are induced by ethanol.

Further studies are necessary in order to identify another unknown antioxidants present in PLx.

Acknowledgments This investigation was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1) Girotto G., Malinverni W., *Int. J. Tissue React.*, **4**, 169—172 (1982).
2) Banerjee K., Bharadwaj D., Ghosh D.P., Hati R. N., *Indian Med. Gaz.*, **124**, 243—244 (1990).
3) Itoh H., *Fragrance J.*, **6**, 67—71 (1990).
4) Takehara Y., Yoshioka T., Sasaki J., *Acta Med. Okayama*, **44**, 103—111 (1990).
5) Togashi S., Takahashi N., Kubo Y., Shigihara A., Higashiyama K., Watanabe S., Fukui T., *J. Health Science*, **46**(2), 117—125 (2000).
6) Videla L.A., Valenzuela A., *Life Sci.*, **31**, 2395—2407 (1982).
7) Taniguchi M., Hara T., *J. Nutr. Sci. Vitaminol.*, **29**, 283—292 (1983).
8) Hissin P.J., Hilf R., *Anal. Biochem.*, **74**, 214—226 (1976).
9) Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., *J. Biol. Chem.*, **193**, 265—275 (1951).
10) Beauchamp C., Fridovich I., *Anal. Biochem.*, **44**, 276—287 (1971).
11) Beers R.F.Jr., Sizer I.W., *J. Biol. Chem.*, **195**, 133—140 (1952).
12) Lawrence R.A., Burk R.F., *Biochem. Biophys. Res. Commun.*, **71**, 952—958 (1976).
13) Hashimoto S., Recknagel R.O., *Exp. Mol. Pathol.*, 8, 225—242 (1968).
14) Di Luzio N.R., *Physiologist*, **6**, 169—173 (1963).
15) Valenzuela A., Fernandez N., Fernandez V., Ugarte G., Videla L.A., *FEBS Lett.*, **111**, 11—13 (1980).
16) Remmer H., Kessler W., Einsele H., Hintze T., Diaz de Toranzo G., Gharaibeh A.M., Frank H.,

Drug Metab. Rev., **20**, 219—232 (1989).
17) Meister A., *Pharmacol. Ther.*, **51**, 155—194 (1991).
18) Sies H., *Angew. Chem. Int. Ed. Engl.*, **25**, 1058—1071 (1986).
19) Speisky H., Macdonald A., Giles G., Orrego H., Israel Y., *Biochem. J.*, **225**, 565—572 (1985).
20) Fridovich I., *Science*, **201**, 875—880 (1978).