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Toxicological Aspects of Carboxylesterases -A Sensitive Biomarker of Organophosphate Toxicity-

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Abstract:

Egasyn is accessory protein of β-glucuronidase(β-G) in the liver microsomes. Liver microsomal β-G is stabilized within the luminal site of the microsomal vesicles by complexation with egasyn which is one of carboxylesterase isozymes. We investigated the effects of organophosphorus compounds(OPs) such as insecticides on the dissociation of egasyn-β-glucuronidase(EG) complex. The EG complex was easily dissociated by administration of OPs, i.e., Fenitrothion, EPN, Phenthionate, and bis-p-nitrophenyl phosphate(BNPP), and resulting β-G dissociated was released into blood, leading to the rapid and transient increase of plasma β-G level with a concomitant decrease of liver microsomal β-G level. In a case of phenthionate treatment, less increase in plasma β-G level was observed, as compared with those of other OPs. This may be explained by a fact that phenthionate was easily hydrolyzed by carboxylesterase. Similarly, carbamate insecticides such as Carbaryl caused rapid increase of plasma β-G level. In contrast, no significant increase of plasma β-G level was observed when pyrethroid insecticides were administered to rats. This is due to a fact that pyrethroids such as Phenthrin and Allethrin were easily hydrolyzed by A-esterase as well as carboxylesterase. On the other hand, addition of OPs to the incubation mixture containing liver microsomes caused the release of β-G from microsomes to the medium. From these *in vivo* and *in vitro* data, it is concluded that increase of the plasma β-G level after OPs administration is much more sensitive biomarker than cholinesterase inhibition to acute intoxication of OPs and carbamates.

Keywords: Carboxylesterase, Egasyn, β-Glucuronidase, Organophosphorus compounds Accessory protein

Abbreviations: β-G, β-glucuronidase; EG complex, egasyn-β-glucuronidase complex,; ER, endoplasmic reticulum; OPs, organophosphorus compounds

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Introduction

In 1973, Swank and Paigen(1) reported the existence of egasyn-β-glucuronidase(EG) complex in mouse liver and lung. Liver microsomal β-glucuronidase is stabilized within microsomal vesicles by complexation with the accessory protein, named egasyn. Egasyn was first purified from mouse liver microsomes by Lusis *et al.*(2). Medda *et al.*(3) reported that egasyn has esterase activity and it was one of carboxylesterase isozymes. Later, we purified three isozymes of carboxylesterases, named RL1, RL2 and RH1, from rat liver microsomes(4, 5), and egasyn was identified as RL2 isozyme. In 1989, we reported that inbred strain of rats having inherent hyperbilirubinuria, named EHBR rat, had the C phenotype of esterase-3(egasyn) judging from the absence of liver microsomal β-glucuronidase(6). β-Glucuronidase of rat liver shows a unique subcellular distribution with significant activity being associated with both microsomal and lysosomal subcellular compartments. A wide variety of biochemical and genetic evidence indicates that the mechanism of stabilization of β-glucurionidase in microsomes of the rat is explained, at least in part, by the formation of noncovalent binding complexes with the accessory protein, egasyn, and easily dissociated by heating and treatment with chemicals such as urea and deoxycholate(7) .

Recently, Satoh and Hosokawa(8) reviewed the molecular aspects of carboxylesterases, indicating that mammalian carboxylesterases represent a multigene family. Further, a comparison of the nucleotide and amino acid sequence of the mammalian carboxylesterases shows that all forms expressed in the rat can be assigned to one of three gene subfamilies with structural identities of more than 70% within each family.

Regarding the molecular basis of egasyn, Robbi *et al.*(9, 10) reported cDNA cloning of rat liver microsomal pI 6.1 esterase(ES-10) and pI 5.5 esterase(ES-3, egasyn). This was the first report to show that cDNA of liver carboxylesterase has the consensus sequence of the ER retention tetrapeptide HVEL-COOH. Ovnic *et al.*(11, 12) conducted genetic mapping and confirmed that the location of an egasyn cDNA fragment is in cluster 1 of the esterase region on chromosome 8. Two different forms of mouse liver microsomal carboxylesterase were cloned from a mouse liver cDNA library.

The present paper represents the *in vitro* and *in vivo* evidences showing that EG complex bound to the ER membrane in the liver was easily cleaved by OPs and β-glucuronidase dissociated in the liver microsomes was released to plasma, leading to the rapid and massive increase of β-glucuronidase level in the blood.

Materials and Methods

Chemicals Fenitrothion, Phenothrin and Allethrin were generously supplied by Sumitomo Chemical Co., Ltd., Osaka, Japan. Phenthioate was a gift from Nissan Chemical Industries, Ltd., Tokyo, Japan. EPN, NAC(Carbaryl) and bis-p-nitrophenylphosphate(BNPP) were purchased from WAKO Pure Chemicals, Inc., Ltd., Tokyo, Japan. All other chemicals were of reagent grade and were purchased from commercial source.

Animals Sprague-Dauley (SD) rats(150-200g), ddY mouse(20-30g), Golden syrian Hamster(100-200g), Hertley Guinea pig(250-350g), New Zealand White rabbits(2.5-3.0kg) crab-eating monkey and human livers were used. All animals used here were males. They were freely given water and a commercial chow. Human liver specimens used for *in vitro* studies were obtained from National Disease Research Interchange (NDRI), USA through Human and Animal Bridge Discussion Group(HAB) in Japan. The animals were killed under ether anesthesia and the livers were quickly removed, and perfused with 1.15% KCl. After the ice-cold 1.15% KCl was added, 20%(v/v) homogenates were prepared in a glass homogenizer with a Teflon pestle. The microsomes were prepared as described previously(13). β-glucuronidase components of microsomal and lysosomal fractions were prepared by osmotic treatment

Determination of β-glucuronidase activity β-Glucuronidase activity was assayed using a fluorometric procedure with 4-methylumbelloferyl-β-glucuronide (Sigma)(4). One unit is the enzyme hydrolyzing 1 mmol of the substrate per hour at 37°C.

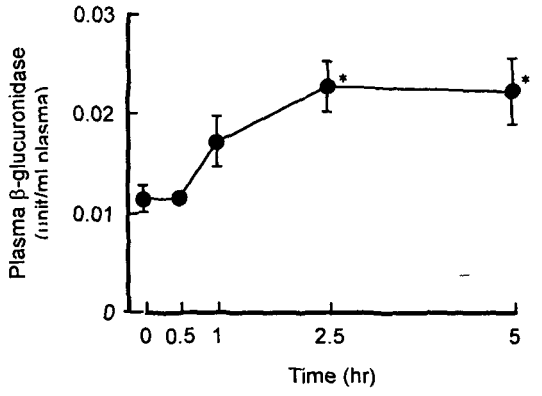

Fig. 1 Effects of BNPP treatment on plasma β-glucuronidase activity in the hamster.
* $p<0.05$ vs. 0hr.

Results and Discussion

β-Glucuronidase activities of liver microsomes of mammals.

β-Glucuronidase is located in the liver in the forms of free and bound to egasyn named EG complex. In order to know the species differences in the ratio of free to bound β-glucuronidase activities in each animal species and humans, we determined the total and microsomal β-glucuronidase activities of the animal and human livers. Microsomal β-glucuronidase activity was approximately 20-30% of total activity in the liver of animals and humans and there was no significant species difference in animals and humans.

Effects of BNPP treatment on plasma β-glucuronidase activity in the hamster.

Time course experiments of the increase of plasma β-glucuronidase activity were carried out after administration of BNPP to hamsters. Figure 1 shows the increase of plasma β-glucuronidase after oral administration of BNPP in a dose of 181 mg/kg in a time-dependent manner and reached maximum at 2.5 hr after BNPP administration.

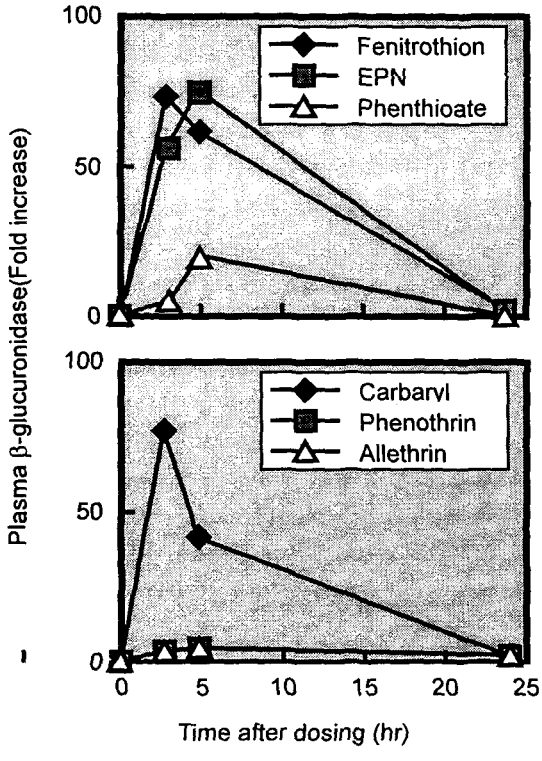

Fig. 2 Changes in the plasma β-glucuronidase activities after administration of insecticides.
Normal values of β-glucuronidase activities of rat plasma are 0.034±0.002 unit/ml.

Changes in the plasma and liver microsomal β-glucuronidase activities after administration of insecticides.

The time course studies on the increase of plasma β-glucuronidase and decrease of liver microsomal β-glucuronidase activities were carried out. In order to determine the relationship between β-glucuronidase activities in plasma and liver microsomes, changes in β-glucuronidase activities in both fractions were monitored up to 24 hours after administration of Fenitrothion, NAC and Phenothrin. As shown in Fig. 2, there was a significant increase of plasma β-glucuronidase activity in rats treated with Fenitrothion and NAC up to 4 hours. This strongly suggests that increase of plasma β-glucuronidase was derived from the microsomal β-glucuronidase after dissociation of EG complex by OP and carbamate insecticides. In case of Phenothrin treatment, no significant changes in β-glucuronidase activities in plasma and liver microsomes were observed. Under same experimental conditions, no significant increase of plasma β-glucuronidase was observed in rats treated

with pyrethroids. Administration of BNPP which is a specific inhibitor of carboxylesterase, caused a rapid and transient increase of β-glucuronidase activity. It is well recognized that the differences in the susceptibility of OPs, carbamates and pyrethroids to release liver microsomal β-glucuronidase seem to be closely related to the extents of the inhibition of plasma cholinesterase and liver microsomal egasyn (carboxylesterase) activities. In fact, it is likely to speculate that pyrethroids are much more easily hydrolyzed by plasma and liver esterase(s) to form the inactive metabolites involved in the inhibition of egasyn (carboxylesterase) than OPs as well as carbamates.

Comparison of cholinesterase inhibition and increase of plasma β-glucuronidase activities in rats after administration of EPN.

In order to compare the extents of plasma ChE inhibition and increase of plasma β-glucuronidase activities, rats were treated with EPN in doses of 1, 5, 10 and 30 mg/kg intraperitoneally and sacrificed 5 hours after administration. of EPN. Figure 3 shows that increase of β-glucuronidase and cholinesterase inhibition of rat plasma were observed in a dose-dependent manner. Cholinesterase activity was decreased

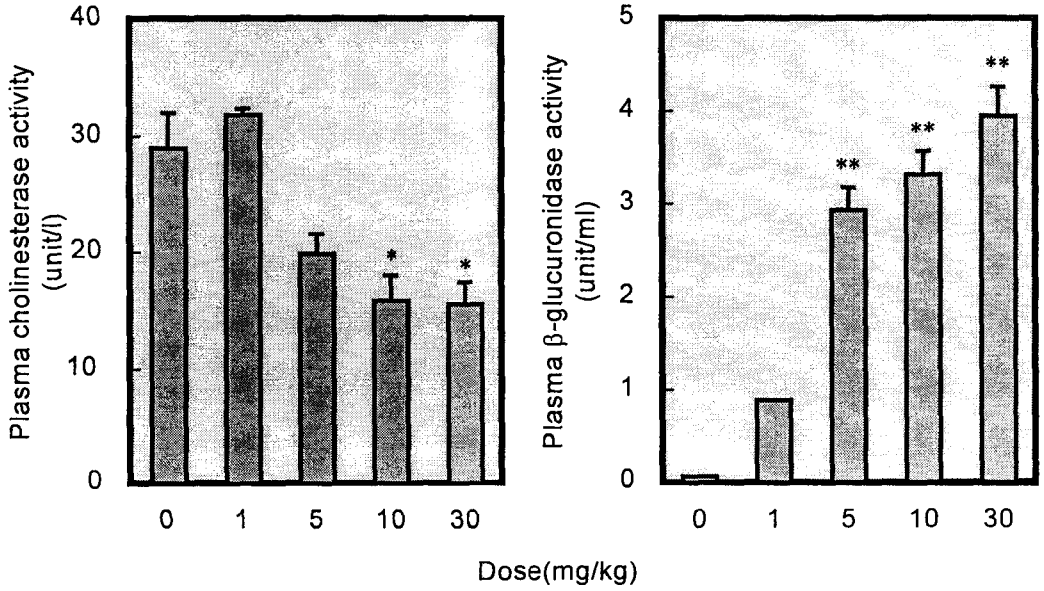

Fig. 3 Inhibition of plasma cholinesterase and increase of plasma β-glucuronidase after EPN treatment in rats.

Animals were sacrificed 5hr after EPN administration. β-Glucuronidase activity of non-treated rats is 0.03±0.009 unit/ml. Values represent mean±S.E. from three to five rats. Significantly different from non-treated rats: *$p<0.05$; **$p<0.01$.

to approximately 50% of the control level after administration of EPN in a dose of 30 mg/kg, while, plasma β-glucuronidase was significantly increased approximately 1000-fold that of control level. This indicates that increase of plasma β-glucuronidase activity is much more susceptible than cholinesterase inhibition to exposure to OPs such as EPN. OPs and carbamate insecticides, but not pyrethroids, caused a rapid dissociation of the EG complex which was followed by massive and rapid secretion of microsomal β-glucuronidase into plasma. OP-induced inhibition of plasma ChE activity seems to be more rapid than increase in plasma β-glucuronidase although there are no significant data at present, because it takes much more time to release liver microsomal.β-glucuronidase into plasma after dissociation of EG complex in liver micirosomes than direct inhibition of plasma ChE by OPs. In terms of the increase in plasma β-glucuronidase activities, there were significant species differences in the release of β-glucuronidase from microsomes into plasma. At present, the reason for the species difference in the extent of the release of β-

glucuronidase from liver microsomes to plasma remain unclear.

Figure 4 summarizes the localization of EG complex, dissociation and release of β-glucuronidase from liver microsomes into plasma. OPs that covalently modify the active site of carboxylesterases including egasyn caused rapid dissociation of EG complex. This dissociation was observed when OPs were injected *in vivo* or added *in vitro* to liver microsomes containing EG complex. The specific binding of OPs to the active site of carboxylesterases suggests that the OPs interact with the complex directly rather than by causing dissociation of the complex by more indirect mechanisms, and *in vitro* dissociation is rapidly followed by massive release of β-glucuronidase into plasma. Several observations in addition to the known ability of OPs to bind covalently to the active site of carboxylesterases indicate that the target molecule for these compounds is egasyn rather than β-glucuronidase. From these results, it is concluded that release of liver microsomal β-glucuronidase into plasma is the most rapid and sensitive marker to OP and carbamate insedcticide-induced acute toxicity.

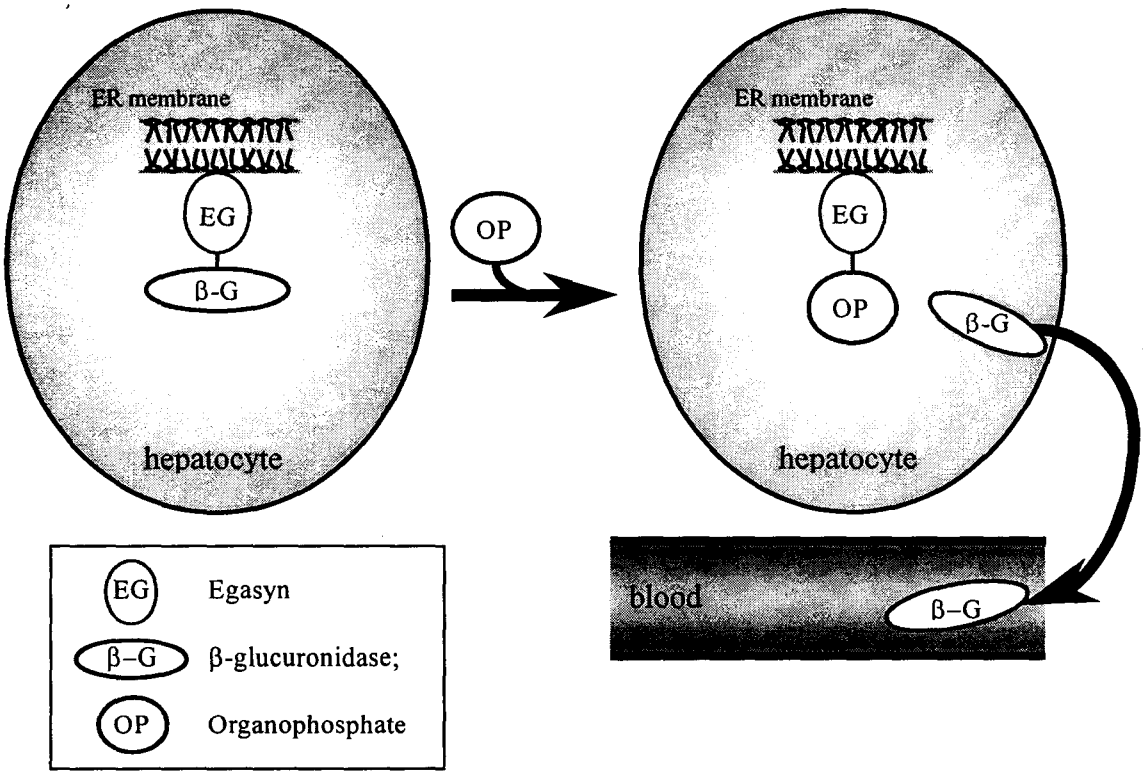

Fig. 4 Schematic picture of release of β-G from hepatocytes to blood by OP administration in animals

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