Epoxyeicosatrienoic acid stimulates ADP-ribosylation of a 52 kDa protein in rat liver cytosol

Kouichi SEKI,* Aizan HIRAI,* Masatoshi NODA,† Yasushi TAMURA,*‡ Iwao KATO† and Sho YOSHIDA*

*The 2nd Department of Internal Medicine and †The 2nd Department of Microbiology, Chiba University School of Medicine, 1-8-1 Inohana, Chiba 280, Japan

In rat liver cytosol, rapid ADP-ribosylation of a 52 kDa protein by endogenous ADP-ribosyltransferase(s) was observed. This ADP-ribosylation was stimulated dose-dependently by 14,15-epoxyeicosatrienoic acid (14,15-EET), one of the metabolites of arachidonic acid by NADPH-dependent cytochrome *P*-450 mono-oxygenase. This stimulatory effect required the presence of GTP or its non-hydrolysable analogues, guanosine 5'-[β -imido]triphosphate or guanosine 5'-[γ -thio]triphosphate. Of four regioisomeric EETs, 14,15-EET was the most potent. No stimulatory effect was observed with addition of 14,15-dihydroxyeicosatrienoic acid, a stable metabolite of 14,15-EET. The 52 kDa protein was not ADP-ribosylated by cholera toxin A subunit and pertussis toxin, and was not recognized by anti- $G_s\alpha$ and anti- $G_i\alpha$ antibodies. However, the 52 kDa protein could be photoaffinity-labelled with 8-azidoguanosine 5'-[α - 32 P]triphosphate. These results suggest that the 52 kDa protein is neither G_s nor G_i , though it may have a GTP-binding site. These results contribute to the understanding of the role of mono-oxygenase metabolites of arachidonic acid in intracellular signal transduction.

INTRODUCTION

The oxidative metabolism of arachidonic acid is known to yield a variety of biologically active substances that are thought to serve as mediators of intracellular events. In addition to being metabolized via the well-characterized cyclo-oxygenase and lipoxygenase pathways, arachidonic acid is also metabolized via the cytochrome *P*-450-dependent mono-oxygenase, an oxidative system strictly dependent on molecular oxygen and NADPH [1]. This enzyme system function as active arachidonic acid 'epoxygenase' affording four novel regioisomeric epoxyeicosatrienoic acids (EETs), i.e. 5,6-, 8,9-, 11,12- and 14,15-EET [2–4]. EETs are potent mediators for the release of certain hormones, such as luteinizing hormone, prolactin and somatostatin from brain tissue [5–7], insulin from pancreatic islet cells [8,9], and cortisol from adrenal cortex [10]. EETs also have the following biological properties: (a) inhibition of chloride transport in renal tubules [11]; (b) inhibition of Na^+ - K^+ -ATPase [12,13]; (c) relaxation of blood vessels [14]; (d) inhibition of platelet aggregation and Ca^{2+} -ionophore-induced neutrophil aggregation [15]. Recently we demonstrated that EETs dose-dependently increased both phosphorylase *a* activity and intracellular Ca^{2+} concentration in rat liver cells, and found that 14,15-EET was the most potent of four regioisomeric EETs [16]. However, little information is available on the mechanism of biological action of EETs. The EETs are produced in microsomes and released to the cytosol; they are reported to be unstable, and are rapidly hydrated to form the corresponding dihydroxyeicosatrienoic acids (DHTs) by epoxide hydratases in cytosol or microsomes [17]. EETs have been shown to mobilize microsomal Ca^{2+} ions [18], but there has been no report on the action of EETs in cytosol.

While investigating the role of EETs in rat liver cytosol, we observed that ADP-ribosylation of a 52 kDa protein was stimulated dose-dependently by 14,15-EET in the presence of GTP. It is well known that ADP-ribosylation of cellular proteins is an important post-translational modification mechanism of proteins [19,20]. This reaction is catalysed by ADP-ribosyltransferase,

which transfers the ADP-ribosyl moiety from NAD to acceptor proteins. It has been clearly demonstrated that bacterial toxins such as cholera toxin and pertussis toxin catalyse ADP-ribosylation of the stimulatory or inhibitory GTP-binding proteins (G_s or G_i) of the adenylate cyclase system, thereby eliciting their biological effects [15,21,22]. Recently, enzymes with actions similar to those of bacterial toxins have been identified and purified in a large number of animal tissues and organs [23–28]. Although the physiological functions of ADP-ribosylation induced by these enzymes have not been well defined, it has been reported that this enzymic reaction may be involved in the regulation of adenylate cyclase [29–32], Ca^{2+} release from mitochondria [33], phosphorylation of nuclear proteins [34,35], and phosphate transport by renal brush-border membranes [36]. Moreover, it has been reported that agonists such as α -thrombin and phorbol 12,13-dibutyrate stimulate ADP-ribosylation of a cytosolic protein of human platelets [37] and that thyroid-stimulating hormone stimulates ADP-ribosylation of proteins in thyroid cells [29]. Thus ADP-ribosylation seems to play an important role in intracellular messenger systems. In the present study, we focused on the effect of EETs on ADP-ribosylation in rat liver cytosol.

MATERIALS AND METHODS

Materials

[adenylate- 32 P]NAD $^+$ di(triethylammonium) salt was obtained from Dupont NEN Research Products (Boston, MA, U.S.A.). 125 I-labelled anti-rabbit IgG F(ab') $_2$ fragment from donkey, nitrocellulose hybridization transfer membranes (0.45 μ m pore size) and dried milk membrane-blocking agent were purchased from Amersham International (Amersham, Bucks., U.K.). Arachidonic acid was obtained from Kurita Water Industries (Sagamihara, Japan). Thymidine, NAD $^+$, GTP, NaF, Na $_2$ EDTA, Na $_3$, dithiothreitol and BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine 5'-[γ -thio]triphosphate (GTP[S]), guanosine 5'-[β -imido]triphosphate

Abbreviations used: EET, epoxyeicosatrienoic acid; DHT, dihydroxyeicosatrienoic acid; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; 8N $_3$ -GTP, 8-azidoguanosine 5'-triphosphate; $G_{i\alpha}$, α -subunit of GTP-binding protein that inhibits adenylate cyclase; $G_{s\alpha}$, α -subunit of GTP-binding protein that stimulates adenylate cyclase.

‡ To whom correspondence and reprint requests should be addressed.

(p[NH]ppG) and guanosine 5'-[β -thio]diphosphate (GDP[S]) were purchased from Boehringer Mannheim (Mannheim, Germany). Anti-G α antiserum RM/1 [38] and anti-G β antiserum AS/7 [39] were purchased from Dupont NEN Research Products. 8-Azidoguanosine 5'-[α - 32 P]triphosphate (8N $_3$ -[α - 32 P]GTP) was purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.). Other chemicals were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan).

Preparation of cytosolic and plasma-membrane fractions of rat liver

Male Wistar rats (Charles River Japan, Kanagawa, Japan; 200–250 g body wt.) fed *ad libitum* were killed by cervical dislocation and their livers were perfused with ice-cold buffer containing 20 mM-Tris/HCl (pH 7.5), 1 mM-EDTA, 1 mM-NaN $_3$ and 250 mM-sucrose. The livers were removed quickly and placed into the same buffer. After this treatment, all subsequent procedures were performed at 0–4 °C. The livers were minced with scissors and homogenized by three passes in a tight-fitting Teflon/glass Potter–Elvehjem-type homogenizer. This homogenate was centrifuged at 700 *g* for 10 min. The resultant supernatant was centrifuged at 7000 *g* for 20 min, and then twice at 20000 *g* for 30 min. The 20000 *g* supernatant was centrifuged at 105000 *g* for 60 min. The resultant supernatant fraction was used as cytosolic fraction. Rat liver plasma-membrane fraction was prepared by the method of Prpić *et al.* [40]. These samples were stored in liquid N $_2$ until use. Then, just before the experiment, cytosol was diluted to 2 mg/ml with the ice-cold buffer described above.

Preparation of EETs and DHTs

EETs were prepared by non-selective epoxidation of arachidonic acid as described by Chung & Scott [41]. The epoxide mixture was separated by straight-phase (SP-) h.p.l.c. using a h.p.l.c. pump 880-PU [Japan Spectroscopic Co. (JASCO), Tokyo, Japan] equipped with a silica column (Develosil 5 μ m; 20 mm $\times$ 500 mm; Nomura Chemical Co., Aichi, Japan) with a solvent system of *n*-hexane/propan-2-ol/acetic acid (100:0.6:0.05, by vol.). The u.v. absorption of the column effluent was monitored at 206 nm with a u.v. detector 875-UV (JASCO). The corresponding DHTs were obtained by acidic aqueous hydrolysis, followed by purification by reverse-phase (RP-) h.p.l.c. as previously described [4,10].

[α - 32 P]ADP-ribosylation

ADP-ribosylation was carried out basically as described previously [42]. The reaction was performed in a final volume of 300 μ l. A 100 μ l portion of cytosol/assay was preincubated with 500 μ M-GTP or its non-hydrolysable analogues, GTP[S] or p[NH]ppG, at 0–4 °C for 20 min. Then the reaction was started by addition of 150 μ l of reaction mixture containing 200 mM-Tris/HCl (pH 7.5), 20 mM-thymidine, 2 mM-EDTA, 10 mM-MgCl $_2$, 2 μ M-NAD $^{+}$ and 10 μ Ci of [32 P]NAD $^{+}$ and various concentrations of 14,15-EET. The reaction was carried out for 1 min at 30 °C and stopped by addition of 2 ml of 7.5% (w/v) trichloroacetic acid and 10 μ l of 0.1% BSA. After being kept at 0–4 °C for 30 min, samples were centrifuged at 2000 *g* for 30 min and the supernatants were removed. The precipitates were dissolved in 1% SDS/5% 2-mercaptoethanol, and were heated at 60 °C for 10 min. Proteins were separated by SDS/PAGE by the method of Laemmli [43], in 12%-polyacrylamide gels. Gels were stained with 0.2% (w/v) Coomassie Blue and 10% trichloroacetic acid in 50% (v/v) methanol, and then were destained with 0.7% acetic acid/0.5% ethanol. The gels were autoradiographed on Kodak Omat film. For the quantitative analysis of ADP-ribosylated proteins, radioactive bands corresponding to

ADP-ribosylated protein (molecular mass 52 kDa) were sliced off. The pieces of the gels containing radioactive bands were placed into glass scintillation vials, and 0.5 ml of water and 0.5 ml of Solvable (DuPont) were added. After incubation at 50 °C for 3 h, 10 ml of Atomlight (DuPont) was added and the samples were kept at room temperature overnight to gain maximal recovery of radioactivity before counting.

Immunoblot analysis

The samples were treated with 62.5 mM-Tris/HCl (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 7% glycerol and 0.01% Bromophenol Blue for 10 min at 60 °C. After electrophoresis, the proteins were electrophoretically transferred from the gel to the nitrocellulose sheet (0.45 μ m pore size) by the method of semi-dry electroblotting [44]. Non-specific protein-binding sites on the sheet were blocked with 5% (w/v) dry milk in 20 mM-Tris/HCl (pH 7.6) by incubation at 4 °C overnight [45]. The sheet was subjected to a series of treatment and wash cycles performed at room temperature as follows: treated for 120 min with a 1:1000 dilution of either rabbit anti-G α or -G β antiserum in phosphate-buffered saline containing 5% BSA [38,39] and washed three times for 15 min each with phosphate-buffered saline containing 0.1% Tween 20 (buffer A); treated for 120 min with 20 ml (3.37 kBq/ml) of 125 I-labelled anti-rabbit IgG F(ab') $_2$ fragment from donkey in buffer A and washed as described above, then washed with distilled water and dried [46]. Blot autoradiographs were obtained with X-ray film Rxo-H (Fuji Photo Film Co., Kanagawa, Japan) at –80 °C with an intensifying screen after 48 h exposure.

Photoaffinity labelling

The photoreaction was performed in a final volume of 150 μ l. Rat liver cytosol (100 μ l) was incubated with 2 μ M-8N $_3$ -[α - 32 P]GTP at 4 °C for 10 min. The samples were irradiated on ice at a distance of 2 cm for 10 min under gentle stirring with a u.v. lamp (254 nm, 15 W [47,48]). After photolysis, the reaction was stopped by addition of 1 ml of 15% trichloroacetic acid. These samples were prepared for and subjected to SDS/PAGE as described above. After electrophoresis, the gel was stained and used to develop an autoradiogram by using Kodak Omat film.

Protein determination

Protein concentrations were determined by the method of Bradford [49] using the Bio-Rad protein assay dye reagent (Bio-Rad, Richmond, CA, U.S.A.), with BSA as standard.

Statistical analysis

Data analysis was performed with a software package entitled 'Manual of Pharmacologic Calculations with Computer Programs' [50] on a Toshiba J 3100 SGT computer. Statistical significance was determined with procedure 39 of t-Test I: Grouped Data [50].

RESULTS

Effect of 14,15-EET on ADP-ribosylation in rat liver cytosol

In the present study, a 52 kDa protein was ADP-ribosylated by endogenous ADP-ribosyltransferase(s) in rat liver cytosol, and the reaction reached a maximum after 1 min and showed a rapid decline between 1 and 3 min (results not shown). When 10 μ M-14,15-EET was added, ADP-ribosylation of the 52 kDa protein was increased in the presence of 500 μ M-GTP (Fig. 1) and likewise reached a maximum after 1 min and showed a rapid decline between 1 and 3 min (results not shown). Similar results were observed in the presence of its non-hydrolysable analogues, GTP[S] or p[NH]ppG (Fig. 1).

Fig. 1. Effect of 14,15-EET on ADP-ribosylation in the presence of GTP or its non-hydrolysable analogues, GTP[S] or p[NH]ppG in rat liver cytosol

Rat liver cytosol (200 μ g of protein/lane) was [32 P]ADP-ribosylated with or without 10 μ M-14,15-EET in the presence of 500 μ M-GTP, -GTP[S] or p[NH]ppG and separated by SDS/PAGE as described in the Materials and methods section. The gel autoradiograph was obtained after overnight exposure. Similar results were observed in three other experiments.

Fig. 2. Time course of 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein in rat liver cytosol

Each sample was incubated with or without 10 μ M-14,15-EET in the presence of 500 μ M-GTP[S] for 0, 0.5, 1, 2, 3 or 10 min at 30 $^{\circ}$ C and separated by SDS/PAGE. Then the radioactive band of the 52 kDa protein was sliced off and counted for radioactivity as described in the Materials and methods section. The results are expressed as the difference in the radioactivity of [32 P]ADP-ribosylated 52 kDa protein in the presence and the absence of 14,15-EET. The values are means $\pm$ S.E.M. of four experiments. The degree of significance is * $P < 0.05$, ** $P < 0.01$.

Characterization of 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein

Fig. 2 shows the time course of the increase in ADP-ribosylation of the 52 kDa protein by addition of 10 μ M-14,15-EET in the presence of 500 μ M-GTP[S]. This 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein reached a maxi-

Table 1. Effect of 14,15-EET concentration on the stimulation of ADP-ribosylation of the 52 kDa protein in rat liver cytosol

Rat liver cytosol was [32 P]ADP-ribosylated with the indicated concentrations of 14,15-EET in the presence of 500 μ M-GTP[S] and separated by SDS/PAGE. The radioactive band of the 52 kDa protein was sliced off and counted for radioactivity as described in the Materials and methods section. The results are expressed as percentage stimulations of ADP-ribosylation of the 52 kDa protein by addition of the indicated concentrations of 14,15-EET. The values are means $\pm$ S.E.M. of four experiments. The degree of significance is ** $P < 0.01$ versus the result with 0 μ M-14,15-EET.

14,15-EET concn. (μ M)	Stimulation (%)
0	0.0 $\pm$ 0.9
1	9.3 $\pm$ 5.7
3	19.6 $\pm$ 4.0**
10	37.4 $\pm$ 4.8**

Table 2. Effect of GTP[S] concentration on 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein in rat liver cytosol

Rat liver cytosol was preincubated with the indicated concentration of GTP[S] at 0–4 $^{\circ}$ C for 20 min and reactions were carried out with or without 10 μ M-14,15-EET as described in the Materials and methods section. The results are expressed as percentage stimulations of ADP-ribosylation of the 52 kDa protein by addition of 10 μ M-14,15-EET. The values are means $\pm$ S.E.M. of four experiments. The degree of significance is ** $P < 0.01$ versus the result with 0 μ M-GTP[S].

GTP[S] concn. (μ M)	Stimulation (%)
0	9.9 $\pm$ 4.4
50	16.6 $\pm$ 4.1
125	15.9 $\pm$ 8.5
250	20.2 $\pm$ 8.9
500	31.3 $\pm$ 1.3**
1000	29.6 $\pm$ 4.7**

mum after incubation for 1 min, and a rapid decline was observed for 3 min after that. As shown in Table 1, 14,15-EET dose-dependently increased ADP-ribosylation of the 52 kDa protein over the concentration range 1–10 μ M; 30–40% stimulation of ADP-ribosylation of the 52 kDa protein was obtained after addition of 10 μ M-14,15-EET. Further stimulation of ADP-ribosylation of the 52 kDa protein was not obtained by increasing the concentration of 14,15-EET over 10 μ M (results not shown).

The effect of the concentration of GTP[S] on 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein is shown in Table 2. Addition of GTP[S] increased the 14,15-EET-stimulated ADP-ribosylation in a dose-dependent manner with concentrations of 0.05–1 mM. When GTP[S] was omitted from the reaction, the stimulation by 14,15-EET was not observed. ADP-ribosylation of the 52 kDa protein was not stimulated by GTP or its non-hydrolysable analogues in the absence of 14,15-EET. The stimulatory effect of 14,15-EET was not observed in the presence of GDP[S], a non-hydrolysable analogue of GDP, or other nucleotides such as ATP, CTP or GDP, at a concentration of 500 μ M (results not shown).

Dithiothreitol at 2 μ M concentration produced 25% inhibition of ADP-ribosylation of the 52 kDa protein in the absence of 14,15-EET. On the other hand, 14,15-EET-stimulated ADP-

Table 3. Effect of four regioisomeric EETs and 14,15-DHT on the stimulation of ADP-ribosylation of the 52 kDa protein in rat liver cytosol

Reactions were carried out as in Table 1 with 10 μM -5,6-, 8,9-, 11,12- or 14,15-EET (a) or with the indicated concentrations of 14,15-DHT (b). The results are expressed as percentage stimulations of ADP-ribosylation of the 52 kDa protein by each indicated EET or by 14,15-DHT. The values are means $\pm$ S.E.M. of four experiments. The degree of significance is ** $P < 0.01$ versus control (a) or 0 μM -14,15-DHT (b).

Addition	Concn. (μM)	Stimulation (%)
(a) None (control)		0.0 $\pm$ 0.3
5,6-EET	10	1.6 $\pm$ 6.2
8,9-EET	10	5.0 $\pm$ 3.3
11,12-EET	10	6.6 $\pm$ 1.9
14,15-EET	10	32.7 $\pm$ 2.3**
(b) 14,15-DHT	0	0.0 $\pm$ 0.3
	1	4.4 $\pm$ 2.1
	3	0.7 $\pm$ 4.8
	10	4.9 $\pm$ 3.2
14,15-EET	10	31.2 $\pm$ 2.8**

Fig. 3. Photoaffinity labelling of rat liver cytosol and membrane with 8N₃-[α -³²P]GTP

Photoaffinity labelling of rat liver cytosol (200 μg of protein/lane) (lanes 1–3) and membrane (100 μg of protein/lane) (lane 5) with 2 μM -8N₃-[α -³²P]GTP were carried out as described in the Materials and methods section. Lane 1 was not irradiated with a u.v. lamp after addition of 2 μM -8N₃-[α -³²P]GTP, and to lane 3 1 mM-GTP[S] and 2 μM -8N₃-[α -³²P]GTP were added before irradiation. Lane 4 is the autoradiogram developed after ADP-ribosylation of cytosol as for Fig. 1. The gel autoradiograph was obtained after overnight exposure. Similar results were observed in three other experiments.

ribosylation of the 52 kDa protein was not affected by addition of 2 μM -dithiothreitol (results not shown).

When the cytosol was heated at 50 °C for 5 min, ADP-ribosylation of the 52 kDa protein was not observed in the absence or the presence of 14,15-EET. The 52 kDa protein was not ADP-ribosylated by cholera toxin A subunit and pertussis toxin. When the mixture of 20 mM-NaF and 10 μM -AlCl₃ (hereafter referred to as AlF₄⁻, which is considered to be a general activator of GTP-binding proteins [51,52]) was added instead of GTP[S], the stimulatory effect by 10 μM -14,15-EET was not observed (results not shown).

Isomeric specificity

The effect of addition of chemically synthesized EETs (10 μM) on ADP-ribosylation of the 52 kDa protein is shown in Table 3(a). Of the four regioisomers, 14,15-EET was found to be the most potent. When up to 10 μM -14,15-DHT was added, little increase in ADP-ribosylation of the 52 kDa protein was observed (Table 3b).

Immunoblot analysis

The ADP-ribosylated 52 kDa protein of the rat liver cytosol was studied with antisera designed specifically for α -subunits of

G_s and G_i. The 52 kDa protein in the liver cytosol was recognized by neither the anti-G_s α antiserum RM/1 nor the anti-G_i α antiserum AS/7 (results not shown).

Photoaffinity labelling

A photolysable analogue, 8N₃-GTP, has been utilized to determine the guanine-nucleotide-binding site of various proteins [47,48]. This compound was shown to compete for the guanine-nucleotide-binding site. Fig. 3 shows the results of an experiment testing the photoincorporation of 8N₃-[α -³²P]GTP into cytosol and membrane of rat liver cells. Lanes 1, 2, 3 and 5 are autoradiograms developed after photolabelling with 8N₃-[α -³²P]GTP to cytosol (lanes 1–3) and membrane (lane 5). Lane 4 is the autoradiogram developed after ADP-ribosylation of cytosol as described above. In the absence of u.v. irradiation, there was no appreciable covalent incorporation of the radioactivity. With u.v. irradiation, ³²P was covalently incorporated into several proteins of the cytosol, including the 52 kDa protein. GTP[S] (1 mM) effectively blocked these photoincorporations.

DISCUSSION

A cytosolic protein with an apparent molecular mass of 52 kDa was ADP-ribosylated by endogenous ADP-ribosyltransferase(s). The reaction showed rapid onset; ADP-ribosylation of the 52 kDa protein already reached a maximum after 1 min. Then a rapid decline was observed for 3 min. Brüne and co-workers reported that cytosolic ADP-ribosyltransferase of rat liver caused ADP-ribosylation of several cytosolic proteins [53] and that α -thrombin-stimulated ADP-ribosylation of a 42 kDa cytosolic protein of human platelets showed a rapid onset and reached a peak at 1–3 min [37].

We first demonstrated that addition of 10 μM -14,15-EET *in vitro* stimulates ADP-ribosylation of the 52 kDa protein of rat liver cytosol in the presence of 500 μM -GTP or its non-hydrolysable analogues, GTP[S] or p[NH]ppG. Addition of 14,15-EET in the presence of GTP or its non-hydrolysable analogues had no effect on the time when ADP-ribosylation of the 52 kDa protein reached a maximum. ADP-ribosylation of the 52 kDa protein was not stimulated by GTP or its non-hydrolysable analogues in the absence of 14,15-EET. Addition of GTP[S], over the concentration range 0.05–1 mM, dose-dependently increased 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein. On the other hand, the stimulatory effect of 14,15-EET was not observed in the presence of GDP[S], a non-hydrolysable analogue of GDP, and other nucleotides. These results suggested that 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein requires the presence of GTP or its non-hydrolysable analogues. Thus, further experiments were carried out in the presence of 500 μM -GTP[S]. When the cytosol was heated at 50 °C for 5 min, the ADP-ribosylation of the 52 kDa protein was completely abolished. This result suggests that the reaction may be heat-labile. A concentration of 2 μM -dithiothreitol produced 25% inhibition of ADP-ribosylation of the 52 kDa protein in the absence of 14,15-EET. A similar result was reported by Molina y Vedia *et al.* [54]. The mechanism of the inhibition of ADP-ribosylation of the 52 kDa protein by dithiothreitol is still unclear. On the other hand, 14,15-EET-stimulated ADP-ribosylation of the 52 kDa protein was not affected by the addition of 2 μM -dithiothreitol.

This stimulatory effect by 14,15-EET was observed over the range 1–10 μM and reached a plateau at 10 μM . These concentrations are almost comparable with the concentrations of EETs that produce biological effects in liver cells [16], platelets [15] and endocrine tissues [5–10]. Of four regioisomeric EETs, 14,15-EET was the most potent in stimulating ADP-ribosylation

of the 52 kDa protein in rat liver cytosol. This result suggests that the stimulatory effect of EETs on ADP-ribosylation may exhibit isomeric specificity. An interesting finding is that 14,15-DHT, a stable metabolite of 14,15-EET, is almost inactive. The present study indicated that 14,15-EET itself exhibits the stimulatory effect on ADP-ribosylation of the 52 kDa protein, and not by its conversion into 14,15-DHT. Recently, we reported that mono-oxygenase metabolites of arachidonic acid may be involved in vasopressin-stimulated glycogenolysis in isolated rat liver cells, and that 14,15-EET is the most potent of these metabolites in stimulating glycogenolysis and intracellular Ca^{2+} mobilization in rat liver cells [16]. Moreover, Kutsky *et al.* [18] found 14,15-EET to be the most potent stimulant of Ca^{2+} mobilization from rat liver microsomes. Together with these results, the present study suggests that 14,15-EET, one of the major metabolites of arachidonic acid by the mono-oxygenase in the liver [3], might be physiologically more active than other EETs in rat liver cells.

A photolysable analogue, 8N_3 -GTP, has been utilized to determine the guanine-nucleotide-binding site of various proteins [47,48]. Our study of photoreaction showed the photoincorporation of 8N_3 -GTP into a 52 kDa protein and the blocking of this photoincorporation by addition of high concentration of GTP[S]. These results show a possibility that the 52 kDa protein may have the GTP-binding site. Cholera toxin A subunit and pertussis toxin did not catalyse ADP-ribosylation of the 52 kDa protein. When AlF_4^- , which is considered to be a general activator of GTP-binding proteins [51,52], was added instead of GTP[S], the stimulatory effect of $10\text{ }\mu\text{M}$ -14,15-EET was not observed. Moreover, the 52 kDa protein was not recognized by anti- G_α antibody and anti- G_β antibody. These results indicate that the 52 kDa protein is neither G_s nor G_i , though it may have the GTP-binding site. How GTP is involved in the stimulation of ADP-ribosylation of the 52 kDa protein is still to be determined. It is unclear whether the GTP-dependency of the stimulatory effect of 14,15-EET can be ascribed to the presence of this GTP-binding site of the 52 kDa protein.

The present results give little information on the mechanism of the stimulation of ADP-ribosylation of the 52 kDa protein by mono-oxygenase metabolites of arachidonic acid. It is unlikely that 14,15-EET itself provides the catalytic activity for ADP-ribosylation. Further investigation should be performed.

ADP-ribosylation of cytosolic proteins by endogenous ADP-ribosyltransferases is thought to play an important role in intracellular messenger systems [17,18,29,30,37,53–56]. Accordingly, in recent years much attention has been focused on the regulation of endogenous ADP-ribosyltransferase. The present investigation showed that ADP-ribosylation of the 52 kDa protein in rat liver cytosol can be modulated by 14,15-EET, one of the major metabolites of arachidonic acid in rat liver cells. Although the physiological role of ADP-ribosylation of the 52 kDa protein in rat liver cytosol has not yet been determined, the stimulatory effect on ADP-ribosylation of the 52 kDa protein might play a role in biological activities of 14,15-EET in liver cells, such as the stimulation of phosphorylase *a* activity and intracellular Ca^{2+} mobilization [16].

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