Involvement of Ca^{2+} /Calmodulin-Dependent Protein Kinase II in Heparin-Stimulated Release of Hepatic Lipase Activity from Rat Hepatocytes

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The release of hepatic lipase (HTGL), which is responsible for the hydrolysis of lipoprotein triacylglyceride, produced by heparin from the isolated rat hepatocytes in primary culture has been examined. Tyrosine kinase (TK) inhibitors (ST-638 and biochanin A) inhibited the heparin-stimulated release of HTGL activity. The activity of partially purified TK preparation from the hepatocytes was found to be increased following incubation with heparin in a manner which was both time- and dose-dependent. An intracellular Ca^{2+} -chelator (Quin2/AM), a calmodulin inhibitor (W-7) and a Ca^{2+} /calmodulin-dependent protein kinase II (CaMK-II) inhibitor (KN-93) suppressed the release of HTGL activity by heparin. In addition, CaMK-II activity in the hepatocytes incubated with heparin was recognized to elevate in a time- and dose-dependent manner. The increase in CaMK-II activity by heparin was markedly reduced in the presence of the inhibitors of TK. These results suggest that the release of HTGL activity from the hepatocytes by heparin is, in part, caused through a pathway involving an activation of CaMK-II associated with an increase in membrane TK activity.

Key words hepatic lipase; Ca^{2+} /calmodulin-dependent protein kinase II; tyrosine kinase; heparin; hepatocyte

Hepatic lipase (HTGL; hepatic triacylglyceride lipase, EC 3.1.1.3), is synthesized primarily by hepatocytes, hydrolyzes triacylglyceride-rich lipoproteins, such as high and intermediate density lipoprotein, while on the luminal endothelial cell surface.^{1–3} The enzyme, which is found in plasma membranes, microsomes, and cytosol in hepatocytes, is thought to play important roles in the lipid metabolism including catabolic action for the clearance of circulatory triacylglycerides and the production of free fatty acids that are available for local uptake.^{1,4–6} It is suggested that HTGL is found in the endothelium by electrostatic interactions with cell surface proteoglycans. It has been shown that the intravenous injection of heparin leads to the appearance of large amounts of HTGL activity in the blood-stream.^{1,2} Inclusion of heparin in the medium decreases HTGL binding to the surface of cultured cells, and causes release of HTGL into the medium.^{1,3,4,6} Heparin releases HTGL by direct interaction with the enzyme or by competing for the binding sites on the cell surface. The mechanism of this action is thought to be due to an interaction with the negative charge of each molecule.⁴

Previously, we showed that the release of lipoprotein lipase (LPL) activity from tumor cells is stimulated *via* a process involving the activation of various protein kinases by dextran sulfate, which is a type of heparinoid.^{7,8}

However, the involvement of the protein kinases in the heparin-stimulated release of HTGL is still unknown.

In this report, we show that the release of HTGL produced by heparin from cultured rat hepatocytes is, in part, associated with an increase in the activities of tyrosine kinase (TK) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMK-II).

MATERIALS AND METHODS

Materials Glycerol tri[1-¹⁴C]oleate (2.3 GBq/mmol) and [γ -³²P]Adenosine triphosphate (ATP, 111 TBq/mmol) were obtained from Amersham (Tokyo, Japan). Heparin,

Quin2/AM and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). KN-93, KN-92, W-7 and W-5 were from Seikagaku Industries (Tokyo, Japan). Biochanin A and poly (glutamate:tyrosine, 4:1) were purchased from Sigma (MO, U.S.A.). ST-638 was provided by Dr. Tadayoshi Shiraishi (Kanegafuchi Chemical Industry, Osaka). Williams' medium E was from Gibco (N.Y., U.S.A.). All other chemicals used were of analytical grade.

Preparation and Incubation of Hepatocytes Male Wistar rats, weighing 200–250 g, were fed a commercial laboratory chow *ad libitum* and fasted for 24 h before the experiments. Hepatocytes were isolated by *in vitro* collagenase perfusion and low speed centrifugation with modifications.^{9–11} Cell viability was determined by trypan blue exclusion and ranged from 85 to 95%. The hepatocytes were cultured for 24 h in monolayers in a plastic dish (1×10^5 cells/cm²) in Williams' medium E containing 10% fetal calf serum, 10 nM insulin, 10 nM dexamethasone and 5 KIU/ml aprotinin under 5% CO₂ atmosphere. After removal of the medium by aspiration, monolayers of hepatocytes in the dish were further incubated for 0–90 min in Williams' medium E containing 2% bovine serum albumin with or without the addition of heparin. The hepatocytes were harvested and centrifuged at $50 \times g$ for 5 min to remove cellular debris. The obtained supernatant served as the preparation for the released HTGL activity.

The hepatocytes were homogenized in 10 mM Tris-HCl buffer (pH 7.4, 4 °C) containing 0.25 M sucrose, 4 mM EDTA and 15 mM β -mercaptoethanol and centrifuged at $105000 \times g$ for 60 min at 4 °C.¹² The obtained precipitate was dissolved in 0.1% Triton X-100 containing the same buffer, and served as the particulate preparation containing membrane TK activity. The preparation was chromatographed on a DEAE-Cellulofine (Seikagaku Industries) column (0.8 $\times$ 7 cm). The active fractions were pooled and served as the preparation for the TK activity. No phosphotyrosine phosphatase activity was found in the preparation used for purified TK activity

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Fig. 1. Effects of TK Inhibitors on the Stimulatory Release of HTGL Activity by Heparin

(a) The hepatocytes were incubated for 0–90 min with heparin (2 U/ml, ●) or without (○). (b) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of ST-638. (c) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of biochanin A. The activity of HTGL released into the medium was measured as described in Materials and Methods.

(data not shown).

Determination of HTGL Activity HTGL activity was determined by a method using glycerol tri[1-¹⁴C]oleate (1.2 μ M; 2.5 kBq/ml) as a substrate.¹³⁾ The HTGL activity was expressed as pmol of free fatty acids (FFA) produced/min/10⁶ cells.

Determination of TK Activity TK activity was determined by the method of Braun *et al.* using poly (glutamate : tyrosine, 4 : 1) and [γ -³²P]ATP as substrates.¹⁴⁾ The TK activity was expressed as fmol of ATP used/min/mg protein.

Determination of CaMK-II Activity The hepatocytes incubated under the conditions described above were homogenized in Krebs-Ringer bicarbonate buffer (pH 7.4, 4 °C) containing 119 mM NaCl, 25 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ and 0.5 mM CaCl₂ and centrifuged at 105000 $\times g$ for 60 min at 4 °C. The supernatant obtained served as the preparation for CaMK-II activity, which was determined by the CaMK-II assay kit (No.17135) from Cosmo Bio (Tokyo, Japan).¹⁵⁾ The CaMK-II activity was expressed as nmol of ATP used/min/mg protein.

Data Analysis In each experiment, results are the mean $\pm$ S.E. of three or four observations for separate experiments using different hepatocyte preparations.

RESULTS

Effects of TK Inhibitors on the Stimulatory Release of HTGL Activity by Heparin Figure 1a shows the stimulatory release of HTGL activity from the hepatocytes into the medium in the presence of 2 U/ml heparin over a 90 min period. It can be seen that heparin stimulated the release in a time-dependent manner up to 60 min under our experimental conditions. Although the hepatocytes were incubated with heparin in the presence of cycloheximide, an inhibitor of protein synthesis, heparin-stimulated release of HTGL activity was still well preserved (data not shown). To determine whether TK activity is involved in the release of HTGL activity by heparin, the hepatocytes were incubated with heparin in the presence of TK inhibitors, such as ST-638 and biochanin A.^{16,17)} The stimulatory release of HTGL activity by heparin was markedly suppressed by both inhibitors (Figs. 1b, c).

Effect of Heparin on TK Activity This involvement of TK activity by heparin was examined. The TK preparation

Fig. 2. Increasing Effect of Heparin on TK Activity in Hepatocytes

(a) The partially purified TK preparation from the hepatocytes was incubated with heparin (2 U/ml, ●) or without (○) over a 10-min period. (b) The partially purified TK preparation from the hepatocytes was incubated for 10 min with various concentrations (0–5 U/ml) of heparin. TK activity was measured as described in Materials and Methods.

was partially purified from the hepatocytes by DEAE-Celulofine column chromatography as described in Materials and Methods, and no protein phosphotyrosine phosphatase activity was found in the preparation used for purified TK activity (data not shown). When the TK preparation was incubated with heparin at a concentration of 2 U/ml for up to 10 min, the TK activity was markedly increased in a time-dependent manner over this period (Fig. 2a). Next, the TK preparation was incubated with heparin at different concentrations for 10 min, and the TK activity increased in a dose-dependent manner up to 5 U/ml (Fig. 2b).

Effects of Various Modulators for Intracellular Ca²⁺, Calmodulin and CaMK-II Activity Table 1 shows the effects of various chemicals on the release of HTGL activity by heparin. The heparin-stimulated release of HTGL activity was reduced by Quin2/AM (an intracellular Ca²⁺-chelator), W-7 (a calmodulin inhibitor) and KN-93 (a CaMK-II inhibitor), but was not reduced by inactive analogues of W-7 and KN-93, namely W-5 and KN-92, respectively.^{18–20)}

Effect of Heparin on CaMK-II Activity This increase in CaMK-II activity by heparin was examined. When the hepatocytes were incubated with heparin at a concentration of 2 U/ml for up to 5 min, the CaMK-II activity in the prepara-

Table 1. Effects of Various Chemicals on Release of HTGL by Heparin

Chemicals		Relative HTGL activity (%)
None		100
Quin2/AM	(5 μ M)	70.5 $\pm$ 0.8
	(20 μ M)	16.3 $\pm$ 1.4
W-5	(25 μ M)	98.2 $\pm$ 1.2
	(100 μ M)	94.0 $\pm$ 2.7
W-7	(25 μ M)	85.8 $\pm$ 0.9
	(100 μ M)	51.8 $\pm$ 0.5
KN-92	(25 μ M)	98.3 $\pm$ 1.5
	(100 μ M)	96.6 $\pm$ 0.8
KN-93	(25 μ M)	79.1 $\pm$ 1.6
	(100 μ M)	48.8 $\pm$ 2.2

The hepatocytes were incubated for 60 min with or without heparin (2 U/ml) in the presence of various agents, as described in Materials and Methods. No significant change in basal HTGL activity was found with any inhibitor alone. Percentage values were calculated from net activity.

Fig. 3. Increasing Effect of Heparin on CaMK-II Activity in Hepatocytes

(a) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) over a 10-min period. (b) The hepatocytes were incubated for 10 min with various concentrations (0–2 U/ml) of heparin. CaMK-II activity was measured as described in Materials and Methods.

tion from the incubated hepatocytes as described in Materials and Methods was increased in a time-dependent manner over this period (Fig. 3a). Next, the hepatocytes were incubated with heparin at different concentrations for 10 min when the CaMK-II activity was seen to increase in a dose-dependent manner up to 2 U/ml (Fig. 3b).

Involvement of TK Activity in the Stimulatory Increase in CaMK-II Activity by Heparin To determine whether the stimulation of TK activity is involved in an increase in CaMK-II activity, the hepatocytes were incubated with heparin in the presence of TK inhibitors. An appreciable decrease in the heparin-stimulated increase in CaMK-II activity was observed with ST-638 at 2.5 mM and biochanin A at 0.5 mg/ml, respectively (Figs. 4a, b).

DISCUSSION

The release of HTGL, which is a secretory protein, may be an important step for enzyme regulation. It has been reported that release of the enzyme is accelerated by heparin.^{4,21} In addition, Volpi and Venturelli demonstrated the level of plasma heparin in normal human.²² That is, the heparin-stimulated release of HTGL is suggested to be physiologi-

Fig. 4. Effects of TK Inhibitors on Increase of CaMK-II Activity by Heparin

(a) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of ST-638. (b) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of biochanin A. CaMK-II activity was measured as described in Materials and Methods.

cally important. However, the action by heparin is not clear except for an interaction with the negative charge of heparin.

In this report, we employed rat cultured hepatocytes, and corroborated the release of HTGL activity by heparin in a time-dependent manner. In addition, the obtained results suggest that the stimulatory release of HTGL activity by heparin is due to the TK inhibitors-sensitive pathway. When the TK preparation, which is partially purified from membrane-containing fraction in hepatocytes, is incubated with heparin, TK activity is markedly increased. The stimulatory release of HTGL activity by heparin may thus be associated with the activation of membrane TK activity in hepatocytes. It has been reported that heparin binds to specific proteins on cell surfaces of a number of cell types.²³ Loo and Kreuger reported the binding of heparin/heparan sulfate to fibroblast growth factor receptor 4.²⁴ It is possible that heparin makes an attack on the specific protein, which accompanies TK activity, of the surface of the hepatocytes. Further details, however, remain to be disclosed.

Miralen and Templeton reported that heparin inhibited CaMK-II activation in rat mesangial cells.²⁵ However, our results in rat hepatocytes suggested that the heparin-stimulated release of HTGL activity was associated with the activation of the intracellular Ca^{2+} , calmodulin- and CaMK-II-sensitive process. Moreover, the CaMK-II activity in the hepatocytes incubated with heparin was seen to markedly increase. Both the stimulatory increase in CaMK-II activity and the stimulatory release of HTGL activity by heparin were suppressed by TK inhibitors.

In conclusion, we have shown that the release of HTGL activity from rat hepatocytes by heparin is, in part, involved in the activation of CaMK-II activity and this activation may be caused by membrane TK.

REFERENCES

- 1) Assmann G., Krauss R. M., Fredrickson D. S., Levy R. I., *J. Biol. Chem.*, **248**, 1992–1999 (1973).
- 2) LaRosa J. C., Levy R. I., Windmueller H. G., Fredrickson D. S., *J. Lipid Res.*, **13**, 356–363 (1972).

- 3) Kuusi T., Nikkila E. A., Virtanen I., Kinnunen P. K. J., *Biochem. J.*, **181**, 245—246 (1979).
- 4) Nilsson-Ehle P., Garfinkel A. S., Schotz M. C., *Ann. Rev. Biochem.*, **49**, 667—693 (1980).
- 5) Landin B., Nilsson A., Twu J. S., Schotz M. C., *J. Lipid Res.*, **25**, 559—563 (1984).
- 6) Laposata E. A., Laboda H. M., Glick J. M., Strauss J. F., *J. Biol. Chem.*, **262**, 5333—5338 (1987).
- 7) Morita T., Kanagawa A., Fujii M., Ueki H., *Biol. Pharm. Bull.*, **17**, 724—726 (1994).
- 8) Morita T., Ueki H., Kanagawa A., *Cancer Lett.*, **122**, 37—42 (1998).
- 9) Zahlten R. N., Kneer N. M., Stratman F. W., Lardy H. A., *Arch. Biochem. Biophys.*, **161**, 528—535 (1974).
- 10) Mori M., Morita T., Ikeda F., Amaya Y., Tatibana M., Cohen P. P., *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6056—6060 (1981).
- 11) Berry M. N., Friend D. S., *J. Cell Biol.*, **43**, 506—520 (1964).
- 12) Foulkes J. G., Erikson E., Erikson R. L., *J. Biol. Chem.*, **258**, 431—438 (1983).
- 13) Schotz M. C., Garfinkel A. S., Huebotter R. J., Stewart J. E., *J. Lipid Res.*, **11**, 68—69 (1970).
- 14) Braun S., Raymond W. E., Racker E., *J. Biol. Chem.*, **259**, 2051—2054 (1984).
- 15) Muthalif M. M., Benter I. F., Uddin M. R., Malik K. U., *J. Biol. Chem.*, **271**, 30149—30157 (1996).
- 16) Shiraishi T., Domoto T., Imai N., Shimada Y., Watanabe K., *Biochem. Biophys. Res. Commun.*, **147**, 322—328 (1987).
- 17) Geahlen R. L., Koonchanok N. M., McLaughlin J. L., Pratt D. E., *J. Nat. Prod.*, **52**, 982—986 (1989).
- 18) Tsien R. Y., *Biochemistry*, **19**, 2396—2404 (1980).
- 19) Hidaka H., Sasaki Y., Tanaka T., Endo T., Ohno S., Fujii Y., Nagata T., *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 4354—4357 (1981).
- 20) Sumi M., Kiuchi K., Ishikawa T., Ishii A., Hagiwara M., Nagatsu T., Hidaka H., *Biochem. Biophys. Res. Commun.*, **181**, 968—975 (1991).
- 21) Morita T., Mikami F., Kanagawa A., Sera M., Ueki H., *Chem. Pharm. Bull.*, **39**, 3287—3289 (1991).
- 22) Volpi N., Cusmano M., Venturelli T., *Biochim. Biophys. Acta*, **1243**, 49—58 (1995).
- 23) Imai K., Iida T., Takano Y., Uozumi N., *J. Chromatogr. B*, **780**, 1—12 (2002).
- 24) Loo B. M., Kreuger J., Jalkanen M., Lindahl U., Salmivirta M., *J. Biol. Chem.*, **276**, 16868—16876 (2001).
- 25) Miralem T., Templeton D. M., *Biochem. J.*, **330**, 651—657 (1998).