Involvement of Leukotriene Production in Release of Hepatic Lipase Activity Produced by Heparin from Rat Hepatocytes

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Heparin is known to stimulate the release of hepatic lipase (HTGL) activity from hepatocytes, but the action mechanisms have not been fully confirmed. Here, we investigated the involvement of the arachidonate pathway in the heparin-stimulated release of HGTL activity from rat hepatocytes. Heparin increased phospholipase (PL) A_2 activity in the hepatocytes in a time– and dose–dependent manner. The stimulatory effect of heparin on PLA_2 activity was markedly decreased by incubation with protein tyrosine kinase (TK) inhibitors. It was also observed that heparin rapidly increased leukotriene (LT) B_4 and $LTC_4/D_4/E_4$ contents in the hepatocytes. In addition, the stimulatory release of HTGL activity by heparin was suppressed by cytosolic PLA_2, 5-lipoxygenase and LTA_4 hydrolase inhibitors, but not by cyclooxygenase and thromboxane (TX) synthetase inhibitors or a TXA_2 receptor antagonist. These findings suggest that the heparin-stimulated release of HTGL activity from hepatocytes is partly due to an action involving increases in cytosolic PLA_2 activity and LTs production with associated of TK activity.

Key words —— hepatic lipase, heparin, hepatocyte, phospholipase A_2, leukotriene

INTRODUCTION

Hepatic lipase (hepatic triacylglyceride lipase; HTGL, EC 3.1.1.3), which is involved in different steps in the metabolism of lipoprotein triacylglycerides as a lipase of the vascular compartment, is a lipolytic enzyme primarily synthesized by, and released from, hepatocytes.[1–3] This enzyme is found to be localized at the surface of endothelial cells, and is anchored to the plasma membrane of endothelial cells by electrostatic interactions with heparan sulfate proteoglycans.[4–7] The release of HTGL activity from hepatocytes is well-known for its acceleration by heparin, which is an acidic mucopolysaccharide consisting of *N*-, *N*-acetyl, *O*-sulfonic substituents for D-glucosamine, D-glucuronic acid and iduronic acid that is synthesized in the liver, lungs and most other tissues.[6–9] Heparin releases HTGL by direct interaction with the enzyme or by competing for the binding sites on the cell surface.[5,6] The mechanism of this action is thought to be due to an interaction with the negative charge of each molecule, but the detailed mechanism has not been confirmed.

Previously, Leslie *et al*. reported that heparin stimulated phospholipase (PL) A_2, which is an enzyme that hydrolyses the *sn*-2 acyl ester bond of phospholipids, generating free fatty acids and lysophospholipids, in a macrophage cytosolic fraction.[10] The release of arachidonic acid from membrane phospholipids by PLA_2 provides the substrate for the biosynthesis of leukotrienes (LTs) and prostaglandins, which are potent mediators of functions such as vasoactivity and inflammation.[11–13] Indeed, Shalit *et al*. have reported that heparin and heparan sulfate increased the production of LTB_4 and LTC_4 in cultured mouse E-mast cells.[14] Recently, we have also shown that heparin rapidly increased the LTB_4 content in cultured rat hepatocytes.[15] However, the involvement of PLA_2 and LTs in the heparin-stimulated release of HTGL activity is still unknown.

In this study, we investigated whether the release of HTGL activity from rat cultured hepatocytes by heparin is associated with increases in PLA_2 activity and the production of LTs.

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MATERIALS AND METHODS

Materials —— Glycerol tri[1-^{14}C]oleate (2.3 GBq/mmol), L-α-1-palmitoyl-2-arachidonyl, [arachidonyl-1-^{14}C]-phosphatidylcholine (1776 MBq/mmol), the LTB_4 enzymeimmunoassay system (RPN223) and the $LTC_4/D_4/E_4$ enzymeimmunoassay system (RPN224) were obtained from Amersham (Tokyo, Japan). Heparin, manoalide, AA-861, indomethacin, aspirin, bestatin, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nordihydroguaiaretic acid (NDGA) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). ONO-3708 and OKY-046 were provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Biochanin A was obtained from Sigma (MO, U.S.A.). ST-638 was provided by Dr. Tadayoshi Shiraishi (Kanegafuchi Chemical Industry, Osaka, Japan). Williams' medium E was from Gibco (NY, 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 on a commercial laboratory chow *ad libitum* and fasted for 24 hr before the experiments. Hepatocytes were isolated by *in vitro* collagenase perfusion and low speed centrifugation with modifications.[16,17] Contamination by Kupffer cells of the hepatocyte preparation was confirmed to be less than 2% by peroxidase staining.[18] Cell viability was determined by trypan blue exclusion and ranged from 85 to 95%. The hepatocytes were cultured for 24 hr in monolayers in plastic dishes (1×10^5 cells/cm^2) in Williams' medium E containing 10% fetal calf serum, 10 nM insulin, 10 nM dexamethasone and 5 kIU/ml aprotinin under a 5%CO_2 atmosphere. After removal of the medium by aspiration, the monolayers of hepatocytes were further incubated 0–60 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 supernatant obtained served as the preparation for assaying the release of HTGL activity.

Determination of HTGL Activity —— HTGL activity was determined using glycerol tri[1-^{14}C]-oleate (1.2 μM; 2.5 kBq/ml) as a substrate.[19] The HTGL activity was expressed as pmol of free fatty acid (FFA) produced/min/10^6 cells.

Determination of PLA_2 Activity —— PLA_2 activity was determined by the method of Teramoto *et al.*[20] Briefly, the incubated hepatocytes were homogenized in 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM $CaCl_2$ by Physcotron (a microhomogenizer; NS-310E model, Niti-on Co., Tokyo, Japan), and were centrifuged at $105000 \times g$ for 60 min at 4°C. The supernatant contained the cytosolic and also the membrane fraction. The supernatant was used as the enzyme preparation of PLA_2 which hydrolyzed L-α-1-palmitoyl-2-[^{14}C] arachidonyl-phosphatidylcholine as a substrate.[10,21] The free fatty acids released were extracted and quantified. The PLA_2 activity was expressed as pmol/min/10^6 cells.

Determination of LT Contents —— Contents of LTs in hepatocytes which had been incubated with or without heparin were measured. The hepatocytes (0.9×10^6 cells) were homogenized in ice-cold 5% trichloroacetic acid (TCA), then centrifuged at 6000 $\times g$ for 10 min. The supernatant was extracted with H_2O-saturated diethyl ether to remove TCA. The TCA-soluble fraction was subjected to a quantitative analysis of LT contents using an enzymeimmunoassay with a commercially available LTs-assay system from Amersham.[22,23]

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

RESULTS

Figure 1a shows the change in PLA_2 activity in hepatocytes incubated with heparin (2 U/ml) over a 7-min period. A time-dependent increase in PLA_2 activity was observed up to 5 min. Next, the hepatocytes were incubated with heparin at different concentrations for 5 min. PLA_2 activity increased in a dose-dependent manner up to 3 U/ml (Fig. 1b).

Recently, we have reported that the release of HTGL activity by heparin from primary cultured rat hepatocytes is associated with the activation of membrane tyrosine kinase (TK).[24] Therefore, to determine whether TK is involved in the stimulatory effect on PLA_2 activity by heparin, the hepatocytes were incubated with heparin in the presence of two TK inhibitors, biochanin A and ST-638.[25,26] The heparin-stimulated increase in PLA_2 activity was markedly suppressed by both inhibitors (Fig. 2).

Whether the stimulatory release of HTGL activity by heparin is involved in the increase in PLA_2 activity was examined. The effects of PLA_2 inhibi-

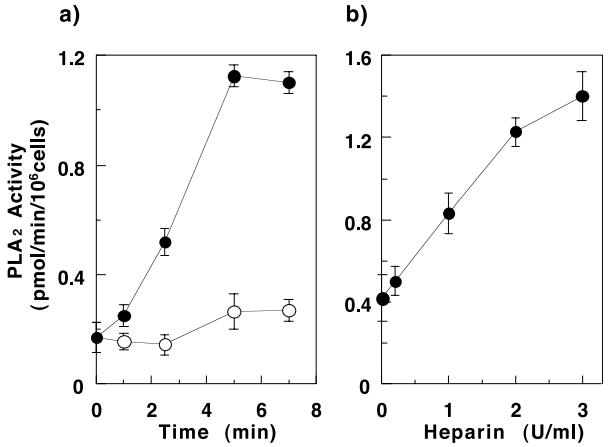

Fig. 1. Effect of Heparin on PLA_2 Activity in Hepatocytes

(a) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) over a 7-min period. (b) The hepatocytes were incubated for 5 min with various concentrations (0–3 U/ml) of heparin. PLA_2 activity was measured as described in MATERIALS AND METHODS.

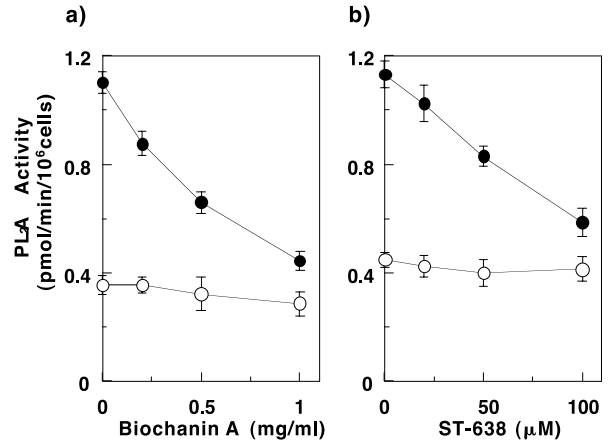

Fig. 2. Effects of TK Inhibitors on the Increase in PLA_2 Activity Caused by Heparin

(a) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of biochanin A. (b) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of ST-638. PLA_2 activity was measured as described in MATERIALS AND METHODS.

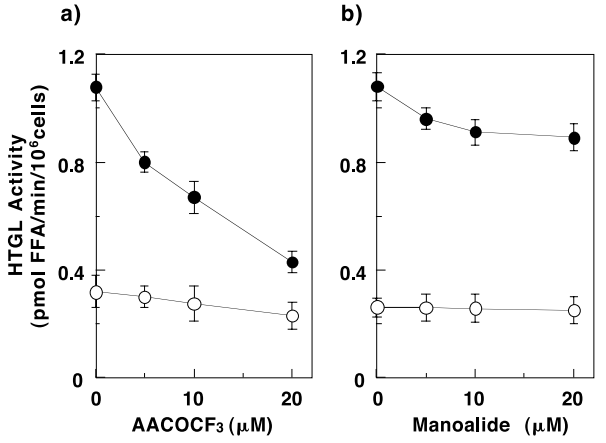

Fig. 3. Effects of PLA_2 Inhibitors on the Release of HTGL Activity by Heparin

(a) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of $AACOCF_3$. (b) The hepatocytes were incubated with heparin (2 U/ml, ●) or without (○) in the presence of manoalide. The activity of HTGL released into the medium was measured as described in MATERIALS AND METHODS.

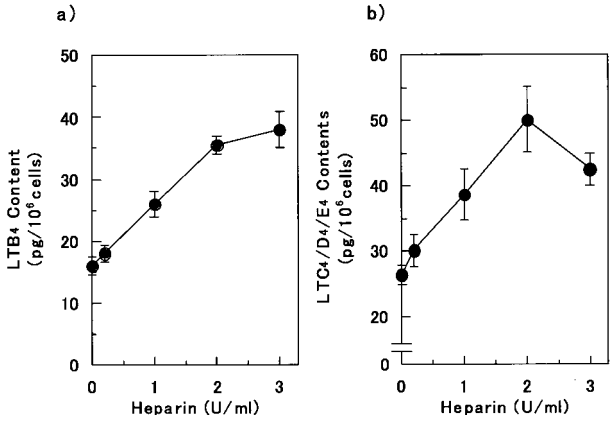

Fig. 4. Increase in LTs Contents in Hepatocytes Caused by Heparin

The hepatocytes were incubated with various concentrations (0–3 U/ml) of heparin for 5 and 15 min to measure the LTB_4 (a) and LTC_4/D_4/E_4 contents (b), respectively. The LTs contents in the hepatocytes were determined as described in MATERIALS AND METHODS.

tors, such as $AACOCF_3$ (a specific cytosolic PLA_2 inhibitor) and manoalide (a weakly specific cytosolic and/or secretory PLA_2 inhibitor), on the release of HTGL activity by heparin are shown in Fig. 3.[27,28] The stimulatory effect of heparin was reduced more by $AACOCF_3$ than manoalide.

To determine whether the mechanism of the heparin-stimulated release of HTGL activity affects the LTs contents in hepatocytes, the hepatocytes were incubated with heparin for 5 and 15 min. When the hepatocytes were incubated with various concentrations of heparin, LTB_4 and LTC_4/D_4E_4 contents increased in a dose-dependent manner up to 3 and 2 U/ml of heparin, respectively (Fig. 4).

In addition, we investigated the effects of various modulators of eicosanoid production on the release of HTGL activity by heparin (Table 1). The heparin-stimulated release of HTGL activity was markedly suppressed by a widely specific 5-lipoxygenase (LPO) inhibitor, NDGA and a specific 5-LPO inhibitor, AA-861, and an LTA_4 hydrolase inhibitor, bestatin.[29–31] In contrast, cyclooxygenase inhibitors,

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

Chemicals		Relative HTGL Activity (%)
None		100
NDGA	(25 μM)	72.3 ± 1.8
	(50 μM)	43.5 ± 3.1
AA-861	(25 μM)	53.0 ± 2.9
	(50 μM)	15.2 ± 2.1
Bestatin	(25 μM)	71.6 ± 3.2
	(50 μM)	59.8 ± 2.2
Indomethacin	(50 μM)	106.5 ± 3.1
	(200 μM)	106.3 ± 4.3
Aspirin	(50 μM)	99.9 ± 1.5
	(200 μM)	101.5 ± 2.7
OKY-046	(25 μM)	105.8 ± 0.2
	(50 μM)	93.1 ± 3.4
ONO-3708	(25 μM)	96.0 ± 2.1
	(50 μM)	96.4 ± 2.7

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.

such as indomethacin and aspirin, a thromboxane (TX) synthetase inhibitor, OKY-046, and a TXA_2 receptor antagonist, ONO-3708 did not suppress the release of HTGL activity by heparin.[32–35]

DISCUSSION

Recently, we have reported that the release of HTGL activity from cultured rat hepatocytes by heparin is associated with the activation of membrane TK.[24] However, the action mechanisms of the heparin-stimulated release of HTGL activity are still unclear. Here, we further investigated the stimulatory effects of heparin on the release of HTGL activity. It was observed that PLA_2 activity in hepatocytes incubated with heparin increased, and that the heparin-stimulated release of HTGL activity was markedly suppressed by a specific cytosolic PLA_2 inhibitor, $AACOCF_3$. These findings suggest that the stimulatory release of HTGL activity by heparin may be closely involved with the increase in cytosolic PLA_2 activity. In addition, the stimulatory increase in PLA_2 activity by heparin was thought to be sensitive to membrane TK activity from our results. According to one report, cytosolic PLA_2 is phosphorylated at serine 505 by mitogen-activated protein kinase, and then activated.[36] Goldberg *et al*. found that PLA_2 is also stimulated by TKs of the growth factor-activated receptor.[37] In this study, it is possible that the activation of membrane TK by heparin caused an increase in PLA_2 activity. The details, however, remain to be elucidated. Also, cytosolic PLA_2 plays an important role in the separation of arachidonate from membrane phospholipids, and the arachidonate produced is converted to LTs by 5-LPO.[12] We found that the heparin-stimulated release of HTGL activity was suppressed by 5-LPO and LTA_4 hydrolase inhibitors, and that the LTB_4 and $LTC_4/D_4/E_4$ contents in hepatocytes incubated with heparin rapidly increased. These results suggest that the heparin-stimulated release of HTGL activity occurs *via* an LTs-sensitive process. It has been reported that epidermal growth factor, the receptor of which has TK activity, stimulates production of LTC_4 in P19 8-39 cells.[38] According to Yokomizo *et al*., the activation of 5-LPO caused by a receptor-mediated agonist in neutrophils is due to an elevation of the intracellular Ca^{2+} concentration and activation of TK.[39] Activation of the membrane TK by heparin in cultured rat hepatocytes was also reported to cause an increase in the intracellular LTB_4 contents.[15]

In conclusion, our results suggest that the stimulatory release of HTGL activity by heparin is, in part, caused through a pathway involving an increase in PLA_2 activity and the resulting rise in LTs contents and an association with TK activity on the cell membrane.

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