Rapid Increase in the Leukotriene B_4 Concentration of Cultured Rat Hepatocytes by Heparin

Hisashi Tagashira, Rie Kerakawati, Toshio Motoyashiki, and Tetsuo Morita*

Department of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985, Higashimura, Fukuyama, Hiroshima 729–0292, Japan

(Received April 22, 2005; Accepted April 27, 2005)

Heparin has various actions in animal tissues, but the action mechanisms have not been fully elucidated. Here, we have investigated the stimulatory increase in the leukotriene (LT) B_4 content of rat hepatocytes induced by heparin. Heparin increased the LTB_4 content of the hepatocytes in a time–dependent manner up to 5 min; its maximal effect was three-fold higher than the basal level of LTB_4. When tyrosine kinase (TK) activity in the membrane-containing preparation of the hepatocytes was suppressed, the stimulatory increase in LTB_4 by heparin was markedly reduced. It was also observed that the heparin-stimulated increase in LTB_4 was reduced by various cytosolic phospholipase A_2 (PLA_2) inhibitors. These findings suggest that heparin stimulates a rapid increase in LTB_4 in rat hepatocytes, and that this increase is partly due to an action involving the membrane TK- and the cytosolic PLA_2-sensitive pathways.

Key words —— leukotriene B_4, heparin, hepatocyte

INTRODUCTION

Heparin, which is one of the polysaccharide species, has various biological actions, such as an anticoagulant with antilipaemic effects, an inhibitor of tumor growth, a regulator of angiogenesis, as well as having antibacterial and antiviral actions.[1–4] It is ubiquitous in animal tissues, particularly in the liver, lung and intestine.[2,4] The physiological roles and action mechanisms of heparin in these tissues are still unclear.

Leukotriene (LT) B_4 is a well-known endogenous mediator active in hypersensitivity reactions and inflammation.[5,6] In addition, LTB_4 may have an additional activity as an endogenous ligand for the transcription factor peroxisome proliferator-activated receptor alpha.[7] Moreover, Striggow and Ehrlich have shown that LTB_4 activated the ryanodine receptor, but not the 1,4,5-triphosphate-gated channel, and increased the intracellular Ca^{2+} content.[8] It has been reported that LTB_4 is synthesized in leukocytes and Kupffer cells, but also that hepatocytes are capable of LTB_4 synthesis.[5,6,9–11] Therefore, here we investigated the production of LTB_4 by heparin in cultured rat hepatocytes.

MATERIALS AND METHODS

Materials —— [γ-^{32}P]Adenosine triphosphate (ATP, 111 TBq/mmol) and the LTB_4 enzymeimmunoassay system (RPN223) were obtained from Amersham (Tokyo, Japan). Heparin, quinacrine, manoalide, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Biochanin A and poly (glutamate : tyrosine, 4 : 1) were 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.[12,13] Contamination with Kupffer cells in the obtained preparation of hepatocytes was confirmed to be less than 2% by peroxidase staining.[14] Cell viability was determined by trypan blue exclusion and ranged from

*To whom correspondence should be addressed: Department of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985, Higashimura, Fukuyama, Hiroshima 729–0292, Japan. Tel.: +81-84-936-2111 (ext. 5221); Fax: +81-84-936-2024; E-mail: morita@fupharm.fukuyama-u.ac.jp

85 to 95%. The hepatocytes were cultured for 24 hr in monolayers in a plastic dish (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 in a 5% CO_2 atmosphere. After removal of the medium by aspiration, monolayers of hepatocytes in the dish were further incubated for 0–20 min in Williams' medium E containing 2% bovine serum albumin with or without the addition of heparin.

Determination of LTB_4 Content —— The LTB_4 content of hepatocytes incubated with or without heparin was measured. The incubated 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 obtained supernatant was extracted with H_2O-saturated diethyl ether to remove TCA. The TCA-soluble fraction was subjected to quantitative analysis of the LTB_4 content by enzymeimmunoassay using commercially available LTB_4 and an assay system from Amersham.

Determination of Tyrosine Kinase (TK) Activity —— The preparation of TK activity, containing zero phosphotyrosine phosphatase activity, was partially purified from the membrane fraction of the hepatocytes by the method reported previously.[15,16] TK activity was determined by the method of Braun *et al*. using poly (glutamate : tyrosine, 4 : 1) and [γ-^{32}P]ATP as substrates.[17] The TK activity was expressed as fmol of ATP used/min/mg protein.

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

RESULTS

Figure 1 shows the time dependency of the LTB_4 content of hepatocytes incubated with 2 U/ml heparin over a 20-min period. A time–dependent increase in LTB_4 in hepatocytes was observed up to 5 min. The maximal increase in LTB_4, which was three-fold higher than the basal level, was observed with a 5-min incubation. A significant increase in the LTB_4 content of hepatocytes incubated without heparin was not observed under these experimental conditions.

Recently, we reported that the activity of membrane TK from hepatocytes is elevated by heparin.[16] Then, to determine whether the membrane TK activity is involved in the heparin-stimulated increase in LTB_4 in hepatocytes, the hepatocytes were incubated with heparin in the presence or absence of TK inhibitors, such as ST-638 and biochanin A (Table 1).[18,19] When the increase in the membrane TK activity from hepatocytes incubated in the presence of heparin was largely suppressed by ST-638 and biochanin A, the heparin-stimulated increase in LTB_4 was also markedly reduced in the presence of these TK inhibitors.

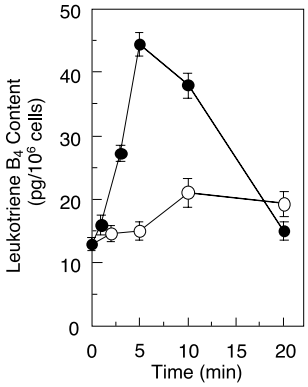

Fig. 1. Increase in the LTB_4 Content of Hepatocytes by Heparin
The hepatocytes were incubated with (2 U/ml, ●) or without heparin (○) over a 20-min period. The LTB_4 content of the hepatocytes was determined as described in MATERIALS AND METHODS.

Table 2 shows the involvement of phospholipase A_2 (PLA_2), which is a key enzyme in the arachidonate cascade to synthesize LTB_4, in the stimulatory increase of the LTB_4 content of hepatocytes by heparin. When the hepatocytes were incubated with various PLA_2 inhibitors, such as quinacrine, manoalide and $AACOCF_3$, in the presence or absence of heparin,[20–22] these PLA_2 inhibitors markedly suppressed the heparin-stimulated increase in LTB_4.

DISCUSSION

Heparin, which is an acidic mucopolysaccharide, has various important biological effects. Previously, heparin stimulated the activity of an arachidonoyl-hydrolyzing phospholipase from human neutrophils.[23] In addition, Leslie *et al*. reported that heparin increased the PLA_2 activity from the macrophage cytosolic fraction.[24] However, the effects of heparin on the arachidonate cascade in hepatocytes are still largely unknown.

In this report, we observed a rapid and transient increase in the LTB_4 content of cultured rat hepato-

Table 1. Effects of Various TK Inhibitors on Increases in TK Activity and LTB_4 Content by Heparin

Chemicals	TK Activity (fmol ATP/min/mg protein)		LTB_4 Content (pg/10^6 cells)	
	−Heparin	+Heparin	−Heparin	+Heparin
None	0.14 ± 0.01	0.57 ± 0.02	13.6 ± 0.5	39.1 ± 0.9
ST-638 (0.1 mM)	0.13 ± 0.01	0.14 ± 0.01	13.8 ± 0.6	16.5 ± 0.4
Biochanin A (1.0 mg/ml)	0.14 ± 0.02	0.16 ± 0.01	13.7 ± 0.3	19.4 ± 0.4

The hepatocytes were incubated for 5 min with or without heparin (2 U/ml) in the presence of various agents, as described in MATERIALS AND METHODS. No significant changes in the basal TK activity and LTB_4 content were observed with any single inhibitor.

Table 2. Effects of Various PLA_2 Inhibitors on Increases in the LTB_4 Content by Heparin

Chemicals	Relative LTB_4 Content (%)
None	100
Quinacrine (10 μM)	58.2 ± 1.2
(50 μM)	18.3 ± 1.7
Manoalide (10 μM)	84.4 ± 1.9
(50 μM)	63.0 ± 3.0
$AACOCF_3$ (10 μM)	54.1 ± 2.9
(50 μM)	14.2 ± 2.5

The hepatocytes were incubated for 5 min with or without heparin (2 U/ml) in the presence of various agents, as described in MATERIALS AND METHODS. No significant changes in the basal LTB_4 content were observed with any single inhibitor. Percentage values were calculated from the net content.

cytes by heparin. It has been reported that hepatocytes may not only degrade but also produce LTs.[10,11] According to Huwyler *et al*., hepatocytes contain enzymes that are capable of catalyzing the biosynthesis of LTB_4.[11] The obtained results suggest that the stimulatory increase in LTB_4 by heparin is due to a TK inhibitor sensitive process, probably associated with the activation of membrane TK activity in hepatocytes. The details, however, remain to be elucidated. In addition, the heparin-stimulated increase in the LTB_4 content of hepatocytes was suppressed by quinacrine (a widely specific PLA_2 inhibitor), manoalide (a weakly specific cytosolic and/or secretory PLA_2 inhibitor), and $AACOCF_3$ (a specific cytosolic PLA_2 inhibitor). That is, the cytosolic PLA_2 may be largely involved in the stimulatory increase in LTB_4 by heparin.

In conclusion, our results suggest that heparin stimulates a rapid increase in the LTB_4 content of cultured rat hepatocytes, and that this increase, in part, involves the cytosolic PLA_2-sensitive pathways associated with an elevation in membrane TK activity.

REFERENCES

1) Regelson, W. (1968) The antimitotic activity of polyanions (antitumor, antiviral, and antibacterial action of heparin, heparinoids, anionic dyes, and synthetic polymers). *Adv. Chemother*., **3**, 303–370.
2) Ehrlich, J. and Stivala, S. S. (1973) Chemistry and pharmacology of heparin. *J. Pharm. Sci*., **62**, 517–544.
3) Hook, M., Bjork, I., Hopwood, J. and Lindahl, U. (1976) Anticoagulant activity of heparin: separation of high-activity and low-activity heparin species by affinity chromatography on immobilized antithrombin. *FEBS Lett*., **66**, 90–93.
4) Engelberg, H. (1996) Actions of heparin in the atherosclerotic process. *Pharmacol. Rev*., **48**, 327–352.
5) Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science*, **220**, 568–575.
6) Hammarstrom, S. (1983) Leukotrienes. *Ann. Rev. Biochem*., **52**, 355–377.
7) Fiedler, J., Simon, F. R., Iwahashi, M. and Murphy, R. C. (2001) Effect of peroxisome proliferator-activated receptor alpha activation on leukotriene B_4 metabolism in isolated rat hepatocytes. *J. Pharmacol. Exp. Ther*., **299**, 691–697.
8) Striggow, F. and Ehrlich, B. E. (1997) Regulation of intracellular calcium release channel function by arachidonic acid and leukotriene B_4. *Biochem. Biophys. Res. Commun*., **237**, 413–418.
9) Sakagami, Y., Mizoguchi, Y., Seki, S., Kobayashi, K., Morisawa, S. and Yamamoto, S. (1989) Rlease of leukotriene B_4 from rat Kupffer cells. *Prostaglandins Leukot. Essent. Fatty Acids*, **36**, 125–128.
10) Perez, H. D., Roll, F. J., Bissell, D. M., Shak, S. and Goldstein, I. M. (1984) Production of chemotactic activity for polymorphonuclear leukocytes by cultured rat hepatocytes exposed to ethanol. *J. Clin. Invest*., **74**, 1350–1357.
11) Huwyler, J., Burgin, M., Zeugin, T. and Gut, J. (1991) Transformation of 5-hydroperoxyeicosatetraenoic acid into dihydroxy- and cysteinyl-leukotrienes by rat hepatocytes: effects of glu-

tathione. *Mol. Pharmacol*., **39**, 314–323.
12) Berry, M. N. and Friend, D. S. (1964) High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol*., **43**, 506–520.
13) Mori, M., Morita, T., Ikeda, F., Amaya, Y., Tatibana, M. and Cohen, P. P. (1981) Synthesis, intracellular transport, and processing of the precursors for mitochondrial ornithine transcarbamylase and carbamoyl-phosphate synthetase I in isolated hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6056–6060.
14) Wisse, E. (1974) Observations on the fine structure and peroxidase cytochemistry of normal rat liver Kupffer cells. *J. Ultrastruct. Res*., **46**, 393–426.
15) Foulkes, J. G., Erikson, E. and Erikson, R. L. (1983) Separation of multiple phosphotyrosyl- and phosphoseryl- protein phosphatases from chicken brain. *J. Biol. Chem*., **258**, 431–438.
16) Tagashira, H., Nakahigashi, S., Kerakawati, R., Motoyashiki, T. and Morita, T. (2005) Involvement of Ca^{2+}/calmodulin-dependent protein kinase II in heparin-stimulated release of hepatic lipase activity from rat hepatocytes. *Biol. Pharm. Bull*., **28**, 409–412.
17) Braun, S., Raymond, W. E. and Racker, E. (1984) Synthetic tyrosine polymers as substrates and inhibitors of tyrosine-specific protein kinases. *J. Biol. Chem*., **259**, 2051–2054.
18) Shiraishi, T., Domoto, T., Imai, N., Shimada, Y. and Watanabe, K. (1987) Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamamide derivatives. *Biochem. Biophys. Res. Commun*., **147**, 322–328.
19) Geahlen, R. L., Koonchanok, N. M., Mclaughlin, J. L. and Pratt, D. E. (1989) Inhibition of protein-tyrosine kinase activity by flavanoids and related compounds. *J. Nat. Prod*., **52**, 982–986.
20) Daniels, I., Lindsay, M. A., Keany, C. I. C., Burden, R. P., Fletcher, J. and Haynes, A. P. (1998) Role of arachidonic acid and its metabolites in the priming of NADPH oxidase in human polymorphonuclear leukocytes by peritoneal dialysis effluent. *Clin. Diagn. Lab. Immunol*., **5**, 683–689.
21) Husain, S. and Abdel-Latif, A. A. (1998) Role of protein kinase C alpha in endothelin-1 stimulation of cytosolic phospholipase A_2 and arachidonic acid release in cultured cat iris sphincter smooth muscle cells. *Biochim. Biophys. Acta*, **1392**, 127–144.
22) Bartoli, F., Lin, H. K., Ghomashchi, F., Gelb, M. H., Jain, M. K. and Apitz-Castro, R. (1994) Tight binding inhibitors of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. *J. Biol. Chem*., **269**, 15625–15630.
23) Alonso, F., Henson, P. M. and Leslie, C. C. (1986) A cytosolic phospholipase in human neutrophils that hydrolyzes arachidonoyl-containing phosphatidylcholine. *Biochim. Biophys. Acta*, **878**, 273–280.
24) Leisle, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. and Zelarney, P. T. (1988) Properties and purification of an arachidonoyl-hydrolyzing phospholipase A_2 from a macrophage cell line, RAW 264.7. *Biochim. Biophys. Acta*, **963**, 476–492.