Rapid Increase in the Leukotriene B₄ Concentration of Cultured Rat Hepatocytes by Heparin

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(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) B₄ content of rat hepatocytes induced by heparin. Heparin increased the LT B₄ 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 LT B₄. When tyrosine kinase (TK) activity in the membrane-containing preparation of the hepatocytes was suppressed, the stimulatory increase in LT B₄ by heparin was markedly reduced. It was also observed that the heparin-stimulated increase in LT B₄ was reduced by various cytosolic phospholipase A₂ (PLA₂) inhibitors. These findings suggest that heparin stimulates a rapid increase in LT B₄ in rat hepatocytes, and that this increase is partly due to an action involving the membrane TK- and the cytosolic PLA₂-sensitive pathways.

Key words — leukotriene B₄, 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) B₄ is a well-known endogenous mediator active in hypersensitivity reactions and inflammation⁵⁻⁶ In addition, LT B₄ 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 LT B₄ activated the ryanodine receptor, but not the 1,4,5-triphosphate-gated channel, and increased the intracellular Ca²⁺ content.⁸ It has been reported that LT B₄ is synthesized in leukocytes and Kupffer cells, but also that hepatocytes are capable of LT B₄ synthesis.⁹⁻¹¹ Therefore, here we investigated the production of LT B₄ by heparin in cultured rat hepatocytes.

MATERIALS AND METHODS

Materials —— [³²P]Adenosine triphosphate (ATP, 111 TBq/mmol) and the LT B₄ enzymeimmunoassay system (RPN223) were obtained from Amersham (Tokyo, Japan). Heparin, quinacrine, manosalide, arachidonyl trifluoromethyl ketone (AACOCF₃) 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
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85 to 95%. The hepatocytes were cultured for 24 hr in monolayers in a plastic dish ($1 \times 10^5$ 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 ($0.9 \times 10^6$ 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$_2$O-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 [$\gamma$-32P]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.

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, manolalide 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 hepatoc-
Table 2. Effects of Various PLA2 Inhibitors on Increases in the LTB4 Content by Heparin

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Relative LTB4 Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Quinacrine (10 µM)</td>
<td>58.2 ± 1.2</td>
</tr>
<tr>
<td>(50 µM)</td>
<td>18.3 ± 1.7</td>
</tr>
<tr>
<td>Manoalide (10 µM)</td>
<td>84.4 ± 1.9</td>
</tr>
<tr>
<td>(50 µM)</td>
<td>63.0 ± 3.0</td>
</tr>
<tr>
<td>AACOCF3 (10 µM)</td>
<td>54.1 ± 2.9</td>
</tr>
<tr>
<td>(50 µM)</td>
<td>14.2 ± 2.5</td>
</tr>
</tbody>
</table>

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 LTB4 content were observed with any single inhibitor.

Table 1. Effects of Various TK Inhibitors on Increases in TK Activity and LTB4 Content by Heparin

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>TK Activity (fmol ATP/min/mg protein)</th>
<th>LTB4 Content (pg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>– Heparin</td>
<td>+ Heparin</td>
</tr>
<tr>
<td>None</td>
<td>0.14 ± 0.01</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>ST-638 (0.1 mM)</td>
<td>0.13 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Biochanin A (1.0 mg/ml)</td>
<td>0.14 ± 0.02</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

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 LTB4 content were observed with any single inhibitor.

REFERENCES


