Stimulation of Nitric Oxide-Cyclic GMP Pathway by L-Arginine Increases the Release of Hepatic Lipase from Cultured Rat Hepatocytes

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The nitric oxide-cyclic GMP pathway is still undefined regarding regulation of the release of hepatic lipase (HTGL). It was found that L-arginine (Arg) stimulated the release of HTGL activity from rat hepatocytes in a time- and dose-dependent manner. L-Arg-stimulated release of HTGL activity was inhibited by N-nitrosodimethyl-L-Arg, which is a nitric oxide synthase inhibitor. L-Arg markedly increased the cyclic GMP content of hepatocytes in the presence of a cyclic GMP phosphodiesterase inhibitor, zaprinast. The release of the enzyme activity was also suppressed by methylene blue (a guanylyl cyclase inhibitor) and KT5823 (a cyclic GMP-dependent protein kinase inhibitor). These results suggest that the stimulation of nitric oxide synthesis by L-Arg increases the release of HTGL activity due to processes associated with the elevation of cyclic GMP level, probably through an activation of protein kinase.

Key words  hepatic lipase; arginine; nitric oxide; cyclic GMP; hepatocyte

Hepatic lipase (HTGL; hepatic triacylglyceride lipase, EC 3.1.1.3), which may hydrolyze phospholipid and triacylglyceride in high and intermediate density lipoprotein, is thought to play an important role in lipid metabolism. 1-3) The enzyme is synthesized and secreted by hepatocytes and is bound to the surface of endothelial cells. 2) It has been reported that the release of the enzyme is accelerated by heparin, insulin, sodium orthovanadate and epidermal growth factor, and reduced by adrenaline, vasopressin and calcium ionophores. 1, 3-7) But, the role and regulation of HTGL, including its secretion and synthesis in the lipid metabolism of hepatocytes, is still largely unknown.

Recently, nitric oxide (NO) has been suggested to mediate the cell function as a messenger molecule. 8-10) NO is synthesized from L-arginine (Arg) by NO synthase (NOS), and the enzyme has been identified in a wide variety of cell types, such as vascular endothelial cells, macrophages and hepatocytes. 11-15) It is also reported that NO is an endothelium-derived relaxing factor and is able to increase cyclic GMP formation in smooth muscle cells by stimulating the activity of guanyl cyclase. 10, 16-17) However, there have been few reports describing the mechanism controlling the enzyme release of HTGL and the effect of NO production. In this study, we show that the release of HTGL activity from hepatocytes is stimulated via a process involving the activation of NO synthesis by L-Arg.

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 h before the experiments. Hepatocytes were isolated by a modification 16) of the method of Berry and Friend using collagenase. 17) Cell viability was determined by trypan blue exclusion and ranged from 85 to 95%. The hepatocytes were cultured for 24 h in monolayer in a plastic dish (1 ×10^6 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, monolayers of hepatocytes in the dish were further incubated 0-20 min in Williams’ medium E containing 2% bovine serum albumin with or without the addition of L-Arg. The hepatocytes were harvested and centrifuged at 50 × g for 5 min to remove cellular debris. The obtained supernatant served as a preparation for determining released enzyme activity.

Determination of HTGL Activity HTGL activity was determined by a method using glycerol tri-[1-14C]oleate (1.2 μM; 2.5 kBq/ml) as a substrate. 20) The HTGL activity was expressed as pmol of free fatty acids (FFA) produced/min/10^6 cells.

Determination of Cyclic GMP Content Cyclic GMP content in hepatocytes which had been incubated with or without L-Arg was measured. The incubated hepatocytes (1 ×10^8 cells) were homogenized in ice-cold 5% trichloroacetic acid (TCA), then centrifuged at 16000 × 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 cyclic GMP content using an enzymeimmunoassay by a commercially available cyclic GMP assay system from Amersham.

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

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MATERIALS AND METHODS

Materials Glycerol tri[1-14C]oleate (2.3 GBq/mmol) and a cyclic GMP enzimeimmunoassay system (RPN 226) were obtained from Amersham (Tokyo, Japan). L-Arg, N-nitrosodimethyl-L-Arg (NMMA), N-1-iminoethyl-L-ornithine (NIO), trifluoperazine, W-7, mephylene blue, KT5823, and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Williams’ medium E (L-Arg content; 50 mg/l) and zaprinast were from Gibco (N.Y.) and Sigma (MO, U.S.A.), respectively. All other chemicals used were of analytical grade.
RESULTS

The release of HTGL activity from the hepatocytes into the medium was observed with the addition of 1 mM L-Arg over a 20 min period (Fig. 1a). L-Arg linearly stimulated the release of HTGL activity in a time-dependent manner. Next, the hepatocytes were incubated with the addition of L-Arg at various concentrations for 15 min. The release of HTGL activity was increased in a dose-dependent manner up to 1 mM L-Arg addition (Fig. 1b). When HTGL was stimulated to release from the hepatocytes into the medium by L-Arg addition, the enzyme activity in the hepatocytes decreased. The possibility of the stimulation of NOS activity being involved in the action of L-Arg was examined. The hepatocytes were incubated with L-Arg in the presence of NMMA, which is an L-Arg analog inhibitor of NOS. An appreciable decrease in the L-Arg-stimulated release was observed with NMMA at concentrations of 0.1 and 0.2 mM. However, the stimulatory action by L-Arg was not suppressed by NIO, which is a different type of NOS inhibitor than NMMA (Fig. 2). To determine whether the L-Arg action is involved in the cyclic GMP content in hepatocytes, the hepatocytes were incubated with the addition of 1 mM L-Arg in the presence of a cyclic GMP phosphodiesterase inhibitor, zaprinast. A marked increase in cyclic GMP content was recognized (Fig. 3). Table 1 shows the effects of various modulators of NO formation and cyclic GMP synthesis on the L-Arg-stimulated release of HTGL activity. The action by L-Arg was suppressed by Ca²⁺/calmodulin inhibitors, such as trifluoperazine and W-7. The L-Arg-stimulated release of the enzyme activity was also suppressed by inhibitors of guanyl cyclase and cyclic GMP-dependent protein kinase, which were methylene blue and KT5823, respectively. However, the action by L-Arg was not reduced by a cyclic AMP-dependent protein kinase inhibitor, KT5720.

DISCUSSION

The release of HTGL activity from the hepatocytes was stimulated by L-Arg (Fig. 1a, b). The stimulatory effect of L-Arg was suppressed by NMMA (Fig. 2), but was well reserved in the presence of NIO. In addition, the cyclic

![Figure 1](image1.png)

Fig. 1. Stimulatory Effects of L-Arg on the Release of HTGL Activity from Cultured Hepatocytes

a) The hepatocytes were incubated for 0–20 min with (●) or without (○) 1 mM L-Arg addition. HTGL activity released into the medium was measured as described in Materials and Methods. b) The hepatocytes were incubated for 15 min by the addition of various concentrations of L-Arg.

![Figure 2](image2.png)

Fig. 2. Effect of NMMA on the Release of HTGL Activity by L-Arg

The hepatocytes were incubated for 15 min with (●, ■) or without (○, □) 1 mM L-Arg addition in the presence of NMMA (○, ■) and NIO (○, □) at concentrations of 0–0.2 mM.

![Figure 3](image3.png)

Fig. 3. Increase in Cyclic GMP Content in Hepatocytes by L-Arg

The hepatocytes were incubated with 1 mM L-Arg (hatched bar) or without (white bar) for 15 min at the indicated concentrations of zaprinast. Cyclic GMP content in the hepatocytes was measured as described in Materials and Methods.

Table 1. Effects of Various Inhibitors on L-Arg-Stimulated Release of HTGL Activity

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Relative HTGL activity released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Trifluoperazine (10 μM)</td>
<td>45.9 ± 3.4</td>
</tr>
<tr>
<td>W-7 (20 μM)</td>
<td>40.1 ± 2.9</td>
</tr>
<tr>
<td>Methylene blue (5 μM)</td>
<td>37.1 ± 2.8</td>
</tr>
<tr>
<td>KT 5823 (5 μM)</td>
<td>40.1 ± 3.2</td>
</tr>
<tr>
<td>KT 5720 (5 μM)</td>
<td>92.6 ± 1.4</td>
</tr>
</tbody>
</table>

The hepatocytes were incubated for 15 min with or without 1 mM L-Arg in the presence of various agents described in Materials and Methods. No significant change in basal HTGL activity was found with any inhibitor alone. The net release of HTGL activity by L-Arg (1 mM) was 0.43 ± 0.04 pmol FFA/min/10⁶ cells. Percent values were calculated from net activity.
GMP content in the hepatocytes was clearly increased by L-Arg and zaprinast (Fig. 3). Table 1 showed that the L-Arg-stimulated release of HTGL activity was suppressed by some inhibitors of Ca^{2+}/calmodulin, guanylyl cyclase, and cyclic GMP-dependent protein kinase. However, the release of the enzyme activity was not suppressed by a cyclic AMP-dependent protein kinase inhibitor. These findings suggest that the stimulatory release of HTGL activity from primary cultured hepatocytes by L-Arg is due to a process involving the activation of cyclic GMP-dependent protein kinase caused by an elevation in NO production and a subsequent increase in cyclic GMP content in the hepatocytes. Recently, Renier and Lambert provided evidence in macrophages of a link between L-Arg metabolism and lipoprotein lipase (LPL), which is a lipoprotein-metabolic enzyme, constitutively expressed by macrophages and synthesized by parenchymal cells.\(^{28}\) According to their report, L-Arg-dependent NO production may be induced by treatment with LPL and interferon-gamma. However, no release of LPL from macrophages and/or parenchymal cells by the addition of L-Arg to the medium was shown. NO has been known to produce various biological reactions, such as the elevation of cyclic GMP production, ADP-ribosylation, and deamination from purine- and pyrimidine-base in nucleic acid.\(^{10}\) In this study, the obtained results suggest that the stimulatory release of HTGL activity from hepatocytes by the addition of L-Arg is due to the activation of guanylyl cyclase. NOS is reported to have at least three isoforms.\(^{11-15,29}\) NOS, which is involved in the release of HTGL activity under our experimental conditions, is sensitive to NMMA and Ca^{2+}/calmodulin inhibitors. It may be the constitutive type of NOS. Moreover, the L-Arg content in liver seems to be largely changed by the physiological conditions of amino acid metabolism, including ingestion.\(^{30}\) The release of HTGL activity from the hepatocytes by the addition of L-Arg to the culture medium suggests that the NOS may possess a relatively high \(K_m\) value for L-Arg. Further details, however, are being gathered.

In conclusion, we have shown that the release of HTGL activity from hepatocytes is stimulated by L-Arg, and that it may be caused by a process sensitive to the elevation of NO production and the intracellular cyclic GMP level.

REFERENCES