Increased expression of endothelial lipase in rat models of hypertension

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Received 12 March 2004; received in revised form 3 January 2005; accepted 12 January 2005
Available online 12 February 2005
Time for primary review 22 days

Abstract

Objective: To gain a better understanding of the involvement of endothelial lipase (EL) in vascular disease, we examined whether the EL expression is regulated in animal models of hypertension.

Methods: The rat cDNA homologue of EL was identified using reverse transcription-polymerase chain reaction. Cultured rat aortic smooth muscle cells were stimulated with angiotensin II (Ang II) and phorbol 12-myristate 13-acetate (PMA), and EL mRNA expression was analyzed by Northern blotting. EL mRNA levels in tissues from stroke-prone spontaneously hypertensive rats (SHR-SP) and Ang II-induced hypertensive rats were evaluated using RNase protection assays.

Results: Rat EL cDNA encoded a protein containing 493 amino acid residues including a signal peptide, and shares 91.9% and 80.9% sequence homology with murine and human EL, respectively. Northern blotting revealed that EL was expressed in a wide range of rat tissues. In cultured rat aortic smooth muscle cells, Ang II and PMA increased EL mRNA levels by 2.9- and 3.3-fold, respectively. In Ang II-induced hypertensive rats, EL expression was upregulated in the aorta, heart, and lung. In SHR-SP, EL expression was upregulated in the aorta and heart.

Conclusion: EL expression is increased in rat models of hypertension. Thus, EL might have a role in the local pathophysiology of vascular diseases.

Keywords: Lipid; Lipoprotein; Lipase; Cholesterol; Hypertension; Remodeling; Phospholipase

1. Introduction

The lipase gene family has a crucial role in lipoprotein metabolism and energy generation, and is associated with the development of cardiovascular diseases [1–4]. Established members of this gene family include lipoprotein lipase (LPL) and hepatic lipase (HL), which are both synthesized by non-endothelial cells. LPL is mainly synthesized in heart, muscle and adipose tissue, whereas HL is synthesized in the liver. Both lipases are secreted and then bind to the endothelial cell surface where they hydrolyze triglycerides and/or phospholipids of lipoproteins. In addition to their lipolytic enzyme properties in lipoprotein metabolism, there is accumulating evidence that lipases serve additional functions in the vascular wall. Both LPL and HL interact with cell- and matrix-associated proteoglycans as well as lipoproteins [5–7]. Lipases can form a molecular bridge between proteoglycans and circulating lipoproteins and blood cells [8]. The non-enzymatic bridging function of lipases can increase cellular lipoprotein uptake or monocyte recruitment [9,10]. These findings support the involvement of the lipase family in vascular remodeling and atherosclerotic vascular diseases [8,11,12].

The lipase gene family was extended by the characterization of endothelial lipase (EL) (gene nomenclature,
LIPG, NCBI Locuslink #9388) [13,14]. EL is expressed by endothelial cells as well as by a wide range of non-endothelial cells in a variety of tissues [13,14]. EL has phospholipase activity and relatively little triglyceride lipase activity [13–15]. Gene targeting and transgenic experiments have indicated that EL is a determinant of high density lipoprotein (HDL) cholesterol levels in the mouse [16,17]. In contrast to other members of the lipase family, EL is upregulated in cultured endothelial cells by inflammatory cytokines and biophysical forces [18–20]. Moreover, we have recently reported that EL expression is increased in an in vivo model of inflammation [21]. These findings suggest that EL has a local role in the pathogenesis of vascular diseases.

Although hypertension is an established risk factor for the development of atherosclerosis, the underlying mechanisms are not fully understood. Stroke-prone spontaneously hypertensive rats (SHR-SP) are an established model of malignant hypertension in which the animals undergo vascular remodeling and organ damage in the development of hypertension [22,23]. SHR-SP is known to have severe cardiovascular organ damage such as ventricular hypertrophy and vascular hyperplasia. To examine the functional roles of EL in the pathogenesis of vascular diseases due to hypertension, in the present study, we have identified rat EL cDNA and explored EL mRNA expression in animal models of hypertension such as SHR-SP and angiotensin II (Ang II)-induced hypertension.

2. Materials and methods

2.1. Animal preparations and experimental design

All animal preparations were performed in accordance with the Institutional Guidelines of Kobe University and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male Wistar–Kyoto (WKY) rats and SHR-SP (30 weeks of age) were obtained from Charles River Japan (Osaka, Japan). Male Sprague–Dawley (SD) rats (7 weeks of age) were obtained from Japan SLC (Shizuoka, Japan). The SD rats were randomly divided into two groups (Ang II-treated group, vehicle-treated group, n=7). For in vivo stimulation with Ang II, an ALZET Mini-Osmotic pump (Alza, Palo Alto, CA) was implanted in the flank region of SD rats under sodium pentobarbital. The mini-pump is designed to release Ang II (Sigma-Aldrich Chemical Co., St. Louis, MO) at a rate of 60 ng/min according to the manufacturer’s instructions. Two weeks after treatment with Ang II or vehicle, animals were anesthetized by administration of pentobarbital, and tissues were excised for RNA analysis. All rats were maintained on a standard chow with 0.9% NaCl for the entire study.

2.2. Blood pressure measurement and plasma chemical analysis

Blood pressure of the rats was measured using the tail-cuff method (model MK-1100; Muromachi Kikai, Japan). Blood was collected from tail veins, and total cholesterol and triglyceride levels were determined with an automated clinical chemical analyzer. HDL cholesterol levels were quantified by the heparin–Mn2+ precipitation method using a commercially available kit (Wako, Tokyo, Japan).

2.3. Cell culture

Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aorta of SD rats by enzymatic dissociation [24]. Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Chemical Co.) supplemented with 10% fetal bovine serum, l-glutamine, penicillin, and streptomycin. Cells were grown to 90% confluence and made quiescent by serum starvation for 24 to 48 h prior to the stimulation assay with Ang II or phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Chemical Co.). Ang II stimulates diacylglycerol formation, which activates protein kinase C (PKC). PMA, the phorbol ester, was used to investigate the involvement of PKC activation in AngII-induced EL expression.

2.4. Molecular cloning of the rat homologue of EL

Total RNAs were isolated from cultured RASMC or rat tissues from SD rats by the guanidine thiocyanate method using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNAs were synthesized by reverse transcription (RT) with 2 µg total RNA using a RETROscript First Strand Synthesis Kit (Ambion, Austin, TX). Double-stranded cDNAs were synthesized by polymerase chain reactions (PCR) with the following primers: 5’-CCGGAAATTCATGCGAAACACGGTTTTCCTGCTC-3’ and 5’-CCGCCTCGAGTCAGGCCAAGTTCACAAAGG-GACTGGTTT-3’. The primer pair was designed based on the 5’- and 3’-untranslated region of murine and human EL cDNA sequences. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and then sequenced by the dideoxy method.

2.5. Northern blot and RNase protection

For Northern blotting, 20 µg of total RNA was size-fractionated on 1% agarose–formaldehyde gels and transferred to nylon membranes. Membranes were then hybridized with the rat EL probe, a 1329-base pair cDNA fragment. The probe was labeled with [32P]dCTP by a random priming method. Hybridizations were performed at 68 °C for 16 h in the PerfectHyb solution (TOYOBO, Japan). After hybridization, membranes were washed with a final stringency of 2× saline sodium citrate (SSC) and 0.5%...
sodium dodecyl sulfate (SDS) at 65 °C for 30 min. Visualization was achieved using a bio-imaging analyzer, Fujix BAS 2000 (FUJI XEROX, Japan). Membranes were stripped and reprobed with a radiolabeled cyclophilin probe to normalize the RNA content. An RNase protection assay was used to analyze EL mRNA expression in hypertensive rats because the RNase protection assay is a more sensitive and quantitative technique for investigating mRNA expression. For RNase protection assays, an EcoRI–DraII fragment (coding from +1 to +178) of rat EL cDNA was cloned into the pBluescript vector (Stratagene, La Jolla, CA), and linearized with EcoRI. Cyclophilin cDNA was purchased from Ambion Inc. (Austin, TX). [32P]UTP-labeled antisense riboprobes were synthesized with T3 RNA polymerase, and RNase protection assay was performed using an RPAII kit (Ambion) [25]. Briefly, total RNA (20 μg) was hybridized with 32P-labeled RNA probes overnight at 42 °C in the 80% formamide hybridization buffer, followed by digestion with RNase A and RNase T1 at 37 °C for 30 min. After the RNases were inactivated, the protected fragments were separated in 6% polyacrylamide–urea denaturing gels by electrophoresis. Relative signal intensities were determined using the Fujix BAS 2000 a bio-imaging analyzer. Protection of rat EL transcripts resulted in a labeled fragment of 184 nucleotides, and protection of cyclophilin transcripts resulted in a labeled fragment of 103 nucleotides.

Fig. 1. Primary sequence of rat EL. The deduced amino acid sequence of rat EL (rEL) is aligned and compared with murine EL (mEL) and human EL (hEL). Identical amino acids are boxed. The GXSXG lipase motif is indicated by a dashed line. The signal peptide cleavage site of EL is indicated by a vertical arrow. The conserved cysteines are indicated by dots, and the catalytic triad is indicated by asterisks. Heparin binding clusters are indicated by dashed boxes. The hydrophobic sequences are delineated by solid bars. The lid region is indicated by a horizontal bar.
The relative signal intensity of EL mRNA expression was standardized with that of cyclophilin mRNA.

2.6. Statistical analyses

Data were expressed as mean ± S.E.M. Statistical analyses were performed by unpaired Student’s t-test or one-way analysis of variance followed by the Bonferroni’s test for multiple comparisons. A P value of less than 0.05 was considered significant.

3. Results

3.1. Characterization of rat EL

We have isolated a cDNA encoding the rat homologue of EL by RT-PCR. Two independent clones were obtained from the rat kidney and cultured RASMC, and the full-length sequences were determined by dideoxy sequencing. The sequences between clones were completely identical and this sequence was identified as the rat homologue of EL cDNA (GenBank, accession number AY916123). The sequence analysis indicated that the open reading frame of the rat EL sequence was 1479 nucleotides, and encoded a highly conserved 493-amino acid protein, including the initial ATG (Fig. 1). The primary amino acid sequence of rat EL had 91.9% identity to murine EL and 80.9% identity to human EL. A hydrophobicity analysis of the predicted amino acid sequence revealed a hydrophobic leader peptide with putative signal peptide cleavage site located 18 amino acids downstream of the translation initiation site, as predicted by the rules of von Heijne [26]. The mature rat EL protein thus consists of 475 amino acids, which is 8 amino acids shorter than murine and human EL. Alignment with mouse and human EL amino acid sequences revealed the characteristic ‘GXSSXG’ lipase motif (169–172), conservation of the catalytic residues serine (Ser-171), aspartate (Asp-195) and histidine (His-276), as well as 10 cysteine residues, which are implicated in the disulfide bonds necessary for enzymatic activity of the other lipases. Similarly, two hydrophobic amino acid regions (163–172 and 274–283) adjacent to the catalytic serine and histidine, which are thought to represent lipid-binding regions, were also conserved. Rat EL has a lid, which serves to confer substrate specificity, consisting of 19 amino acids, three residues shorter and less similar than those in LPL and HL. Because the rat EL lid has a high sequence homology with murine and human EL, rat EL might hydrolyze the same substrate(s) as human and mouse EL. In contrast, the EL lid region has minimal sequence homology with other lipases, which is consistent with the EL substrate differing from the other lipases. In addition, the EL sequence contains conserved positively charged clusters involved in heparin binding sites. Finally, five potential N-linked glycosylation sites (two of which are conserved and the others are unique to EL) are predicted by the presence of the universal acceptor sequence. These glycosylation sites might modulate the heparin binding properties of EL.

3.2. Expression of EL in rat tissues

To evaluate EL expression in rat tissues, we performed Northern blotting using total RNA from several rat tissues. In rats, EL transcripts were detected at high levels in gut, lung, kidney, and spleen, and at low levels in skeletal muscle, thoracic aorta, and liver (Fig. 2A). EL mRNA was barely detectable in the brain and heart. Therefore, polyA+ RNAs from rat tissues were used for Northern blotting and EL mRNA was then detected at low levels in the heart and brain (Fig. 2B). Overall, this expression pattern seems similar to that identified for human EL [13]. In the rat, however, EL mRNA expression in the lung and gut was expressed at higher levels compared with that in human as reported previously.

3.3. Angiotensin II and PMA increased rat EL expression in cultured RASMC

To test whether EL expression is regulated in vascular smooth muscle cells, we examined the effects of Ang II and PMA on EL mRNA expression. Cultured RASMCs were stimulated with Ang II (100 nM) or PMA (100 nM) for 4 to 24 h, and rat EL expression was evaluated by Northern blotting with a rat EL-specific probe. Rat EL mRNA expression was detected at low levels in quiescent RASMCs, and the expression was markedly upregulated.
by Ang II and PMA in a time-dependent manner (Fig. 3). Maximum expression was achieved 24 h after Ang II and PMA treatment with 2.9- or 3.3-fold increases, respectively. These results indicated that EL expression is regulated in cultured vascular smooth muscle cells by Ang II, which might promote the process of vascular remodeling in hypertension.

3.4. EL expression was upregulated in rat models of hypertension

To clarify whether EL expression is regulated in vivo, two rat models of hypertension were used to evaluate EL expression in this study. The SHR-SP had a higher systolic blood pressure than the control WKY rats (240.4 ± 9.1 vs. 125.4 ± 2.8 mm Hg, *p* < 0.001; Table 1). In Ang II-treated rats, systolic blood pressure was elevated to 168.9 ± 12.4 mm Hg, compared to 118.3 ± 2.1 mm Hg in control rats (*p* < 0.001; Table 2). Plasma total cholesterol levels were significantly lower in SHR-SP compared to WKY control rats (84.4 ± 1.6 vs. 101.8 ± 4.5 mg/dl, *p* < 0.01; Table 1).

![Fig. 3. EL mRNA is upregulated by Ang II and PMA in vascular smooth muscle cells.](image-url)

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=5)</th>
<th>SHR-SP (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>125.4±2.8</td>
<td>230.4±9.1***</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>101.8±4.5</td>
<td>84.4±1.6*</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>80.6±4.2</td>
<td>79.6±5.9</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>65.4±2.6</td>
<td>52.0±2.6*</td>
</tr>
</tbody>
</table>

WKY: Wistar–Kyoto rat; SHR-SP: stroke-prone spontaneously hypertensive rat.

*p* < 0.01 vs. WKY rats.

**p** < 0.001 vs. WKY rats.

![Fig. 4. EL expression in the rat models of hypertension.](image-url)
Plasma HDL cholesterol levels were also reduced in SHR-SP compared to WKY control rats (52.0±2.6 vs. 65.4±2.6 mg/dl, p<0.01; Table 1). In contrast, plasma triglyceride levels were not different in either SHR-SP or WKY control rats (Table 1). There were no significant differences in the lipid profile such as total cholesterol, triglyceride, and HDL cholesterol between Ang II- and vehicle-treated groups. The tissues were excised from these animals and EL expression in each tissue was determined by RNase protection assays. Because EL mRNA was expressed at low levels in the aorta and heart, an RNase protection assay was used to compare the expression level of EL hypertensive rats and normotensive controls. EL mRNA levels were upregulated in tissues that are exposed to high blood pressure in SHR-SP and Ang II-stimulated rats (Fig. 4). In the aorta, the expression was increased by 2.1- and 2.3-fold in SHR-SP and Ang II-infused hypertensive rats, respectively, compared to corresponding controls. Similarly, EL mRNA expression was upregulated by approximately 2-fold in the heart from SHR-SP and Ang II-treated rats. EL expression was increased 1.9-fold in the lung from Ang II-rats. A modest increase in the EL level was observed in the lung from SHR-SP, although the difference was not statistically significant. The expression levels, however, were not significantly increased in the kidney (Fig. 4) and liver (data not shown). The results indicate that EL expression is altered in tissues from hypertensive rats.

4. Discussion

In the present study, we cloned the rat homologue of EL cDNA from cultured aortic smooth muscle cells and kidney of SD rats, and investigated EL mRNA expression levels in two rat models of hypertension. The deduced amino acid sequence of rat EL encoded all of the expected lipase motifs, including the catalytic triad of serine, aspartate, and histidine, as well as heparin and lipid binding domains. The lid region of rat EL, which contributes to substrate specificity, had high sequence homology with those of mouse and human EL, suggesting the same substrate specificity [13]. In most tissues, the EL expression pattern in rats was similar to that in human. EL mRNA levels in the lung and gut, however, were high in rat. The functional roles of EL in these organs are not fully understood. Further studies are required to address this question.

EL is expressed by endothelial cells, smooth muscle cells, and macrophages in the arterial vessel wall [27]. Cell culture experiments indicate that EL expression is increased by biophysical forces such as shear stress and cyclic stretch, and inflammatory cytokines in endothelial cells [18,19]. In the present study, rat EL expression was also upregulated by Ang II and PMA in vascular smooth muscle cells. Ang II is a potent stimulator of proliferation and migration of vascular smooth muscle cells. PKC, which is activated by Ang II, is a common and important intracellular signal mediator that transduces a variety of extracellular stimuli to cellular responses.

EL expression was increased in tissues from rat models of hypertension. SHR-SP is a rat model of hypertension, and WKY is accepted as its normotensive control [22,28,29]. In SHR-SP, EL expression was upregulated in the heart and aorta where the tissues were exposed to elevated blood pressure. Treatment of mice with Ang II resulted in elevated blood pressure and increased EL expression levels in the lung, heart, and aorta. There are two plausible mechanisms for the EL upregulation; the direct action of Ang II and mechanical forces to the vessel wall. Previous data demonstrated that mechanical stimuli, such as shear stress and cyclic stretch, upregulated EL mRNA in cultured endothelial cells [18]. In the lung, however, EL expression was increased in AngII-treated rats, but not in SHR-SP. This result seems reasonable because the lung is not the organ that is exposed to systemic blood pressure. In AngII-treated rats, therefore, we hypothesize that Ang II directly increases EL mRNA expression in the lung, which is consistent with the in vitro experiments. In both rat models of hypertension, EL mRNA was not upregulated in the kidney, although the kidney is a target organ in hypertension. The reason for this result remains unclear. One possibility is that EL is expressed not only by the vessel wall, but also by the renal tubule, where the cells are not exposed to blood pressure (Azumi et al., unpublished data). Further studies are required to address this question.

The functional significance of upregulated EL in hypertension remains unclear. Considering that there is a correlation between functional EL expression levels in mice [14,16], EL upregulation could affect lipoprotein metabolism in the hypertensive rats. In this study, plasma total and HDL cholesterol levels were lower in SHR-SP than in WKY, consistent with previous studies [30,31]. The increased EL expression might account for part of the reduced HDL cholesterol in SHR-SP. Lipoprotein metabolism, however, was not affected by Ang II-infusion. It is possible that the dose and/or duration of Ang II infusion in this study were not sufficient to alter the lipoprotein metabolism. Recently, we demonstrated that EL was upregulated by inflammatory stimuli including LPS treatment in mice, and can act as a bridging molecule between endothelial cells and monocytes/macrophages [21]. Given the potential role of EL in the uptake of lipoproteins [32,33], it can be hypothesized that an upregulation of its expression might have direct proatherogenic effects in animal models with hypertension.

Acknowledgements

This work was supported in part by the Grants in Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports, Science and Technology of Japan, the Grant for 21st Century COE Program from Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Grant-in-Aid for JSPS Fellows.
Japan, the Suzuken Memorial Foundation, the Takeda Medical Research Foundation, the Mitsubishi Pharma Research Foundation, the Ono Medical Research Foundation, and Japan Research Promotion Society for Cardiovascular Diseases.

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