Increased leukotriene A_4 hydrolase expression in the heart of angiotensin II-induced hypertensive rat

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Abstract Leukotriene A_4 (LTA_4) hydrolase is essential for the conversion of LTA_4 to LTB_4, an inflammatory lipid mediator. We investigated whether LTA_4 hydrolase was regulated in the heart by angiotensin II (ang II) infusion. Continuous ang II infusion via an osmotic minipump for up to 7 days upregulated mRNA and protein levels of LTA_4 hydrolase (~3.5-fold of control) in the heart in a pressor-dependent manner. Immunohistochemistry demonstrated intense LTA_4 hydrolase staining in the myofibroblast as well as migrated monocytes/macrophages. These data suggest that the cardiac LTA_4 hydrolase-LTB_4 system plays a positive role in the promotion of cardiac inflammation in hypertension.

Key words: Hypertension; Angiotensin II; Leukotriene; Inflammation

1. Introduction

Leukotrienes (LTs) are biologically active lipids that are synthesized and released from leukocytes and non-leukocytic tissue. The pathway of LT synthesis is initialized by the release of arachidonic acid from membrane phospholipids by the action of phospholipase A_2 (PLA_2). Liberated arachidonic acid is then converted to LTA_4 through 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid by the enzyme 5-lipoxygenase (5LO) [1] with 5LO activating protein. LTA_4 is further converted to LTB_4 by the action of LTA_4 hydrolase or is conjugated to form LTC_4 [2]. Although 5LO is thought to be exclusively present in leukocytes, exogenously produced LTA_4 can be metabolized to LTB_4 by the action of LTA_4 hydrolase [3,4], in non-leukocytes via transcellular LTA_4 metabolism. Interestingly, exposure to substrate inactivates LTA_4 hydrolase as a consequence of the covalent binding of LTA_4 to the active site [5], implicating that LTA_4-LTB_4 conversion is a self-limiting reaction unless LTA_4 hydrolase is synthesized de novo.

LTs are implicated in inflammatory and immediate hypersensitive responses. Of these, LTB_4 has the strongest chemotactic and chemoattracting activities on leukocytes and has been postulated to play an important role in a variety of pathological conditions including rheumatoid arthritis, psoriasis and inflammatory bowel disease [6]. We have previously demonstrated that LTA_4 hydrolase is ubiquitously expressed in many tissues including heart, aorta, lung, intestinal tracts, liver [7] and kidney [8], as well as leukocytes. The wide distribution of LTA_4 hydrolase suggests a positive role of these non-leukocytic tissues in the generation of LTB_4 for the recruitment of leukocytes into the inflammatory lesion.

It has been shown that angiotensin II (ang II) administration induces a cardiac inflammatory response [9]. We hypothesized that the expression of LTA_4 hydrolase may be upregulated in the heart of rats receiving ang II. In this study, we demonstrate that LTA_4 hydrolase expression is upregulated in the heart of ang II-induced hypertensive rats and that myofibroblasts as well as migrated leukocytes in the inflammatory lesions show intense expression of this molecule.

2. Materials and methods

2.1. Animal models

To produce a rat hypertension model, an osmotic minipump (Alzet model 2001) (Alza) was subcutaneously implanted into Sprague-Dawley rats (Nippon Bio-Supply Center) as described previously [10]. Val^5-ang II (Sigma) was infused at a rate of 0.7 mg/kg/day for up to 7 days unless otherwise described. Systolic blood pressure and heart rate were measured in conscious rats by tail-cuff plethysmography (UR-5000) (Ueda Seisakusyo). In some experiments, the selective AT_1 receptor antagonist TA-606 (10 mg/kg/day) (a kind gift from Discovery Research Laboratory, Tanabe Seiyaku) or the non-specific vasodilator hydralazine (15 mg/kg/day) (Sigma) was given in the drinking water, beginning 2 days before pump implantation and during ang II infusion.

2.2. RNA isolation and Northern blot analysis

Total RNA was isolated from the homogenized heart by the acid guanidinium thiocyanate-phenol chloroform method using Isogen (WAKO). Human LTA_4 hydrolase cDNA was labelled with [α-^{32}P]-dCTP (DuPont NEN) using commercial kits (Nippon Gene). Hybridization was performed as described previously [10]. Hybridized bands were visualized and quantified using a bio-imaging analyzer (BAS 2000, Fuji Photo Film) and the band density was normalized to the intensity of ethidium bromide-stained 28S and 18S ribosomal RNA.

2.3. Protein purification and Western blot analysis

Protein was isolated by homogenizing samples in the lysis buffer (50 mmol/l HEPES, 5 mmol/l ethylenediaminetetraacetic acid (EDTA) and 50 mmol/l NaCl; pH 7.5) containing protease inhibitors (10 μg/ml aprotinin, 1 mmol/l PMSF and 10 μg/ml leupeptin). Blots were incubated with affinity-purified anti-human LTA_4 hydrolase antibody at a concentration of 2 μg/ml and subsequently incubated with horseradish-conjugated secondary antibody (Jackson ImmunoResearch) at a 1/2000 dilution. The ECL Western blotting system (Amersham Life

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Sciences) was used for detection. Bands were visualized and quantified using a lumino-analyzer (LAS-1000, Fuji Photo Film).

2.4. Immunohistochemistry

Immunohistochemistry was performed as described previously [11]. Briefly, deparaffinized sections were preincubated with 10% horse serum. Sections were then incubated with antibodies against macrophage/monocyte (ED1; Chemicon International, Temecula, CA, USA), human α-smooth muscle actin (α-SM actin, Sigma) and human LTA_4 hydrolase at 1/200, 1/1000 and 1/75 dilutions, respectively, at 37°C for 1 h. Next, the slides were washed and incubated with biotinylated secondary antibodies. After treating the slides with Elite ABC kit (Vector Laboratories), antigens were visualized with the 3,3-diaminobenzidine tetrahydrochloride (DAKO) system. Counterstaining was performed with methyl green (DAKO).

2.5. LTA_4 hydrolase assay

LTA_4 hydrolase activity was measured as described previously with minor modifications [8]. Frozen samples were homogenized with three volumes of phosphate-buffered saline containing 10 mmol/l EDTA and homogenized using a glass grinder. Then, 40 μl of $100\,000\times g$ supernatant was mixed with 10 μl of 0.1 mol/l Tris-HCl buffer (pH 7.6). After pre-warming at 37°C, 1 μg of LTA_4 in ethanol was added to the samples and reaction was stopped after 1 min. After centrifugation at $10\,000\times g$, the samples were injected onto high pressure liquid chromatography. The conditions were as follows: column, TSK-ODS 80TM, 0.46×15 cm; column temperature, 35°C; UV monitor and 270 nm. Prostaglandin B_2 (PGB_2) and LTB_4 were eluted at approximately 7 and 11 min, respectively. The LTA_4 hydrolase activity was calculated from the peak ratio of LTB_4/PGB_2. In a preliminary study, we have confirmed that LTB_4 formation was dependent on the amount of microsomal fraction of the heart samples in this assay.

2.6. Statistical analysis

Data were expressed as mean ± S.E.M. ANOVA followed by a multiple comparison test for comparisons on initial data before expression as a percentage of the control. A value of $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Effect of ang II infusion on hemodynamics and LTA_4 hydrolase expression

Continuous infusion of ang II for 5 and 7 days significantly increased the systolic blood pressure and heart rate (Fig. 1A). Northern blot analysis showed that LTA_4 hydrolase mRNA was significantly upregulated in the heart of hypertensive rats receiving ang II (Fig. 1B,C). The detected mRNA size showed good agreement with that described in the previous report [12]. LTA_4 hydrolase mRNA expression in the heart of normotensive rats had relatively wide variation. This may be due to the minor contamination of the blood cells in the heart samples irrespective of the extensive wash-out of the blood. In any case, upregulation of LTA_4 hydrolase mRNA expression after ang II administration was consistently observed throughout the study. Subsequently, we carried out Western blot analysis using affinity-purified anti-human LTA_4 hydrolase antibody [13]. As depicted in the previous paper [8,13], an immunoreactive band of approximately 70 kDa protein, presumably LTA_4 hydrolase protein [12], was detected (Fig. 2A, left panel). Though another band with the approximate size of 80 kDa was also detected, the identity of this molecule was unknown. To test the possibility that these two bands originated from different cell populations in the heart, protein samples of cultured cells were also tested. Bands of the same size were also detected in the cultured vascular smooth muscle cells (VSMC) and cardiomyocytes of the primary culture (CMC) (Fig. 2A, right panel), indicating that both

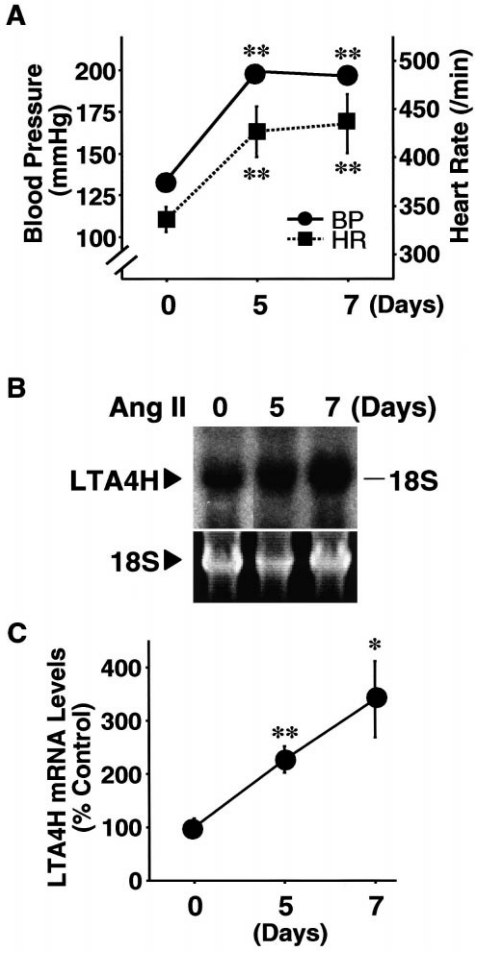

Fig. 1. Effect of infusion of ang II on the hemodynamics and expression of LTA_4 hydrolase (LTA_4H) mRNA. A: Time course of blood pressure and heart rate of ang II-infused rats ($n=9$). B,C: Effect of continuous ang II infusion on LTA_4 hydrolase mRNA levels in the heart. B: Representative Northern blot analysis. C: Data from 4–6 animals are summarized in a line graph. $^*P<0.05$ and $^{**}P<0.01$ vs. sham-operated control.

VSMC and CMC have ∼70 and ∼80 kDa immunoreactive proteins. Ang II infusion for 7 days resulted in an approximately 3.5-fold increase in LTA_4 hydrolase protein over the control. An immunoreactive band at ∼80 kDa was also increased by ang II infusion, though less markedly. As expected, LTA_4 hydrolase activity was significantly increased in the heart of ang II-infused rats (Fig. 3). Compared to the increase of LTA_4 hydrolase protein, increase of its activity was relatively small. This may be partially due to the substrate-dependent irreversible enzyme inactivation of LTA_4 hydrolase [5].

3.2. Effects of anti-hypertensive drugs on ang II-induced LTA_4 hydrolase upregulation

To investigate the mechanism of ang II-induced LTA_4 hydrolase upregulation, some rats were given TA-606, a specific AT_1 receptor inhibitor [14], and hydralazine, a non-specific vasodilator. Both TA-606 and hydralazine effectively normalized the ang II-induced increase in blood pressure. TA-606 did but hydralazine did not normalize the ang II-induced increase in heart rate (Fig. 4A). Northern blot analysis showed that both drugs, when administered to rats not receiving ang II,

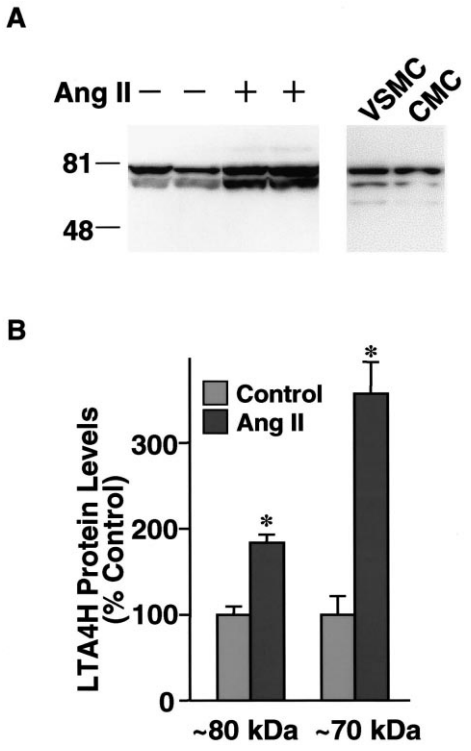

Fig. 2. Effect of infusion of ang II on expression of LTA_4 hydrolase (LTA_4H) protein. Ang II was infused continuously at a rate of 0.7 mg/kg/day for 7 days and the hearts were harvested. Immunoblot was performed using affinity-purified anti-LTA_4 hydrolase antibody. A: Left panel, representative immunoblot; right panel, immunoblot analysis of LTA_4 hydrolase using cultured rat VSMC and CMC. In each lane, two major bands were detected at the sizes of ~70 and ~80 kDa. B: Data from 5–6 animals are summarized in the bar graph. $*P < 0.01$ vs. sham-operated control.

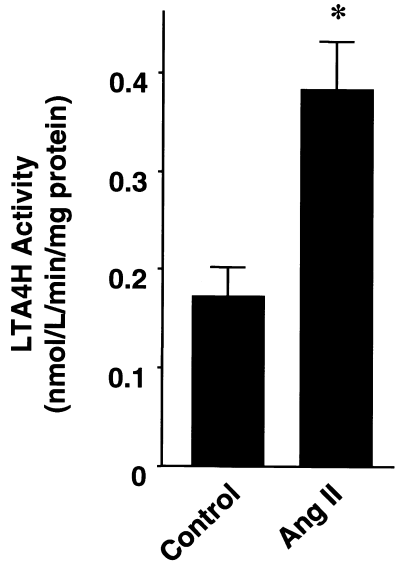

Fig. 3. LTA_4 hydrolase (LTA_4H) activity in the heart. After discarding the membrane and microsomal fractions, cytosolic fraction of the heart from control and ang II-infused rats was prepared and LTA_4 hydrolase activity was determined using high pressure liquid chromatography. The amount of formed LTB_4 was calculated from the peak ratio of LTB_4/PGB_2 (see Section 2). Data from 4–5 animals are summarized in the bar graph. $*P < 0.05$ vs. sham-operated control.

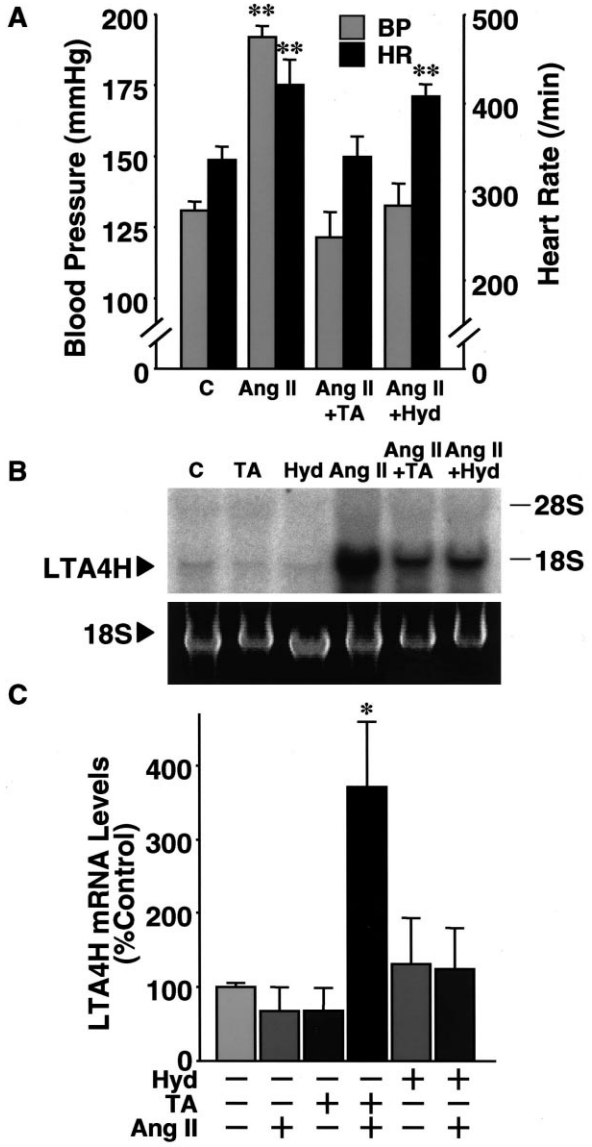

Fig. 4. Effects of AT_1 receptor inhibitor, TA-606 (TA), and non-specific vasodilator, hydralazine (Hyd), on hemodynamics and LTA_4 hydrolase (LTA_4H) expression in ang II-induced hypertensive rats. A: Both TA-606 and hydralazine normalized the ang II-induced increase in blood pressure. However, hydralazine only partially normalized the ang II-induced increase in heart rate. B,C: Both TA-606 and hydralazine suppressed ang II-induced upregulation of LTA_4 hydrolase mRNA. Though either TA-606 or hydralazine slightly decreased LTA_4 hydrolase mRNA when administered to normotensive rats, these effects were not statistically significant. B: Representative Northern blot. C,B: Data from 4–6 animals are summarized in the bar graph. $*P < 0.05$ and $**P < 0.01$ vs. sham-operated control rats, respectively.

slightly decreased the baseline expression of LTA_4 hydrolase mRNA, though this difference was not statistically significant. Both drugs suppressed ang II-induced LTA_4 hydrolase mRNA upregulation, suggesting that ang II increased LTA_4 hydrolase expression in a pressor-dependent manner.

3.3. Immunohistochemical analysis of LTA_4 hydrolase

To examine the localization of LTA_4 hydrolase in the heart, immunohistochemistry was performed. Ang II infusion resulted in the development of an inflammatory lesion, charac-

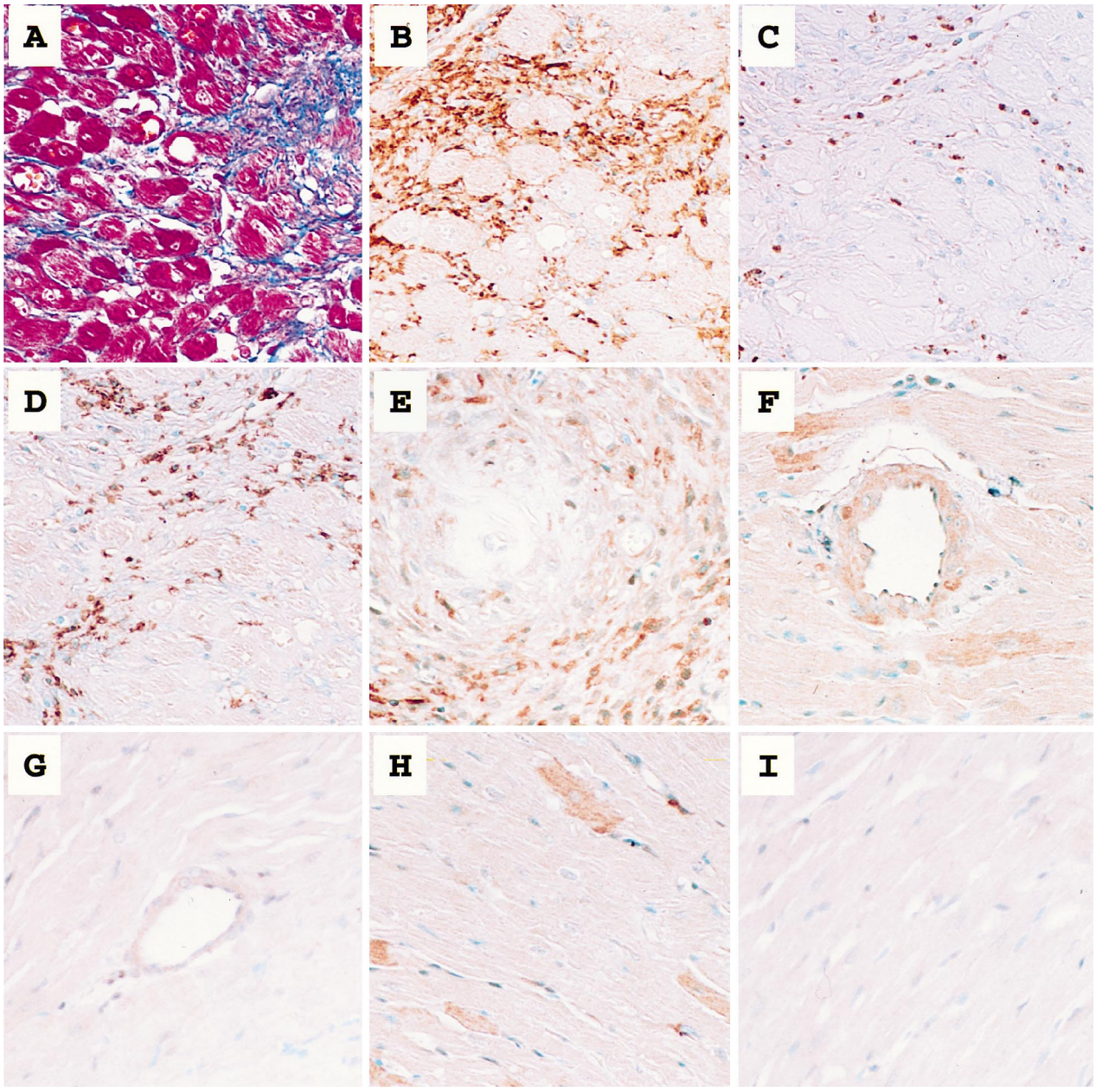

Fig. 5. Immunohistochemical analysis of the heart of normotensive and ang II-induced hypertensive rats. Ang II was continuously infused for 7 days. A: Masson's trichrome staining. Inflammatory changes (granulation, fibrosis) are seen. B: α-SM actin staining. Spindle-shaped non-cardiomyocytes were strongly α-SM actin positive and, thus, considered to be myofibroblasts. C: ED-1 staining depicting infiltration of monocytes/macrophages in the granulation tissue. D: LTA_4 hydrolase staining. Infiltrated monocytes/macrophages, as well as some myofibroblasts, were strongly positive for LTA_4 hydrolase. A–D are from the same region of the one specimen and both A-B and C-D are serial sections, respectively. E: LTA_4 hydrolase staining of myofibroblasts. Some of the spindle-shaped, and thus not leukocytic cells, myofibroblasts were expressing high levels of LTA_4 hydrolase. F–I: Staining of LTA_4 hydrolase in the heart from normotensive rats (G,I) and hypertensive rats (F,H). Increased expression of LTA_4 hydrolase was seen in the coronary arteries and cardiomyocytes after ang II infusion.

terized by granulation tissue formation (increased fibrosis (Fig. 5A) and proliferation of α-SM actin positive fibroblasts (myofibroblasts) (Fig. 5B)) and inflammatory leukocytic infiltration (Fig. 5C). LTA_4 hydrolase was highly expressed in these inflammatory lesions. Intense staining of LTA_4 hydrolase was seen in spindle-shaped myofibroblasts as well as migrated monocytes/macrophages (Fig. 5D). Intense expression of LTA_4 hydrolase in myofibroblasts was more obvious in Fig. 5E. Faint staining of LTA_4 hydrolase was seen in the coronary arteries (Fig. 5G) and cardiomyocytes (Fig. 5I) in the heart of normotensive rat. LTA_4 hydrolase staining in these regions was more intensely demonstrated in the heart of hypertensive rats in the same regions (Fig. 5F,H).

In previous experiments, we found that granulation tissue was formed in the heart of the ang II-induced hypertensive rat model 7 days after ang II infusion (Ishizaka, N., unpublished data). In those studies, ang II-induced granulation formation was inhibited by AT_1 receptor blocker (100%) and hydralazine ($>82\%$). Therefore, both TA-606 and hydralazine may have suppressed ang II-induced LTA_4 hydrolase upregulation,

at least partially, through inhibiting granulation tissue formation and inflammatory cell migration.

Intense expression of LTA_4 hydrolase in the myofibroblast in the granulation tissue where monocytes/macrophages exist suggests that the myofibroblast plays a positive role in recruiting leukocytes and that once leukocytes are recruited, LTB_4 will be further generated by intercellular LTA_4 transfer from leukocytes to myofibroblasts. It was previously suggested that LTB_4 may be an important proinflammatory mediator of leukocyte invasion into the heart in the setting of myocardial infarction [15]. Our data also suggested the possible role of LTA_4-LTB_4 axis in the pathophysiology of hypertensive heart disease. Whether or not the LTA_4 generating system (by the successive actions of PLA_2 and 5LO) and the LTB_4 effector system are present in non-leukocytic cells with high LTA_4 hydrolase expression in the heart should be elucidated in future studies.

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