Heparan sulfate proteoglycan is essential to thrombin-induced calcium transients and nitric oxide production in aortic endothelial cells

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Summary
Thrombin induces Ca\(^{2+}\) transients and subsequent nitric oxide (NO) production in vascular endothelial cells. Thrombin cleaves protease-activated receptors, resulting in activation of intracellular signals, but it is not clarified how the extracellular thrombin stays around the cells to exert its enzyme activities. This study aimed to investigate the possible involvement of heparin sulfate proteoglycan (HSPG) in the effects of thrombin on vascular endothelium. Heparinase III completely removed the polysaccharide chain of HSPG in bovine aortic endothelial cells (BAECs). Thrombin induced Ca\(^{2+}\) transients in control BAECs, but not in heparinase III-treated BAECs. In contrast, ATP-induced Ca\(^{2+}\) transients both in control and heparinase III-treated BAECs. Thrombin that was pre-incubated with heparin also failed to induce Ca\(^{2+}\) transients in BAECs. Furthermore, thrombin-induced NO production, as assessed with DAF-2 fluorescence, was suppressed in heparinase III-treated BAECs and by the pre-incubation of thrombin with heparin. ATP-induced NO production was, however, not affected in heparinase III-treated BAECs. These results indicate that it is essential for thrombin to bind to the polysaccharide chain of HSPG for inducing Ca\(^{2+}\) transients and NO production in BAECs.

Keywords
Calcium, endothelial cells, heparan sulfate proteoglycan, nitric oxide, thrombin

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Introduction
Thrombin plays a significant role both in vascular physiology and pathophysiology. In intact endothelial cells thrombin increases the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) that leads to the production of nitric oxide (NO) (1). In the injured vessels thrombin plays more pathological roles such as the proliferation of smooth muscle cells (2). Thrombin shows these effects via protease-activated receptor (PAR) families, especially PAR-1 subtype in vascular endothelial cells (3). Thrombin is an endolytic serine protease that cleaves PAR receptors and activates intracellular signals, but it is not clarified how the extracellular thrombin stays around the cells to exert its enzyme activities.

Heparan sulfate proteoglycan (HSPG) is an ubiquitously expressed extracellular matrix that is composed of core proteins and polysaccharide side chain, and plays a significant role in the binding of basic growth factor (4) and mechanosensation (5) in endothelium. The polysaccharide chain of HSPG contains heparin regions in its structure (6), so ideally every substance that has a heparin-binding site would be able to bind to HSPG. For instance, heparin-binding site of antithrombin III was shown to bind to neutrophil HSPG, and the interaction between these molecules was suggested to be involved in preventing neutrophil activation (7). Since thrombin has a heparin-binding exosite (8), it would therefore be possible that thrombin binds to abundantly expressed HSPG, and the interaction of these molecules may play a role in thrombin-induced responses.

This study aimed to investigate the possible role of polysaccharide chain of HSPG in the thrombin-induced responses in BAECs. We used two methods for tackling this issue, i.e. enzymatic degradation of polysaccharide chain of HSPG with heparinase III, and masking of the heparin-binding exosite of thrombin by pre-incubation with heparin. Obtained results have clarified for the first time that the binding of thrombin to HSPG plays an essential role in thrombin-induced Ca\(^{2+}\) transients and NO production in BAECs.

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Figure 1: Effects of heparinase and heparin on thrombin-induced Ca$^{2+}$ transients in bovine aortic endothelial cells (BAECs). A) a: Immunofluorescence staining of the polysaccharide chain of HSPG in control BAECs. Cells were treated with anti-HSPG antibody for 15 min. Cell-bound anti-HSPG antibody was then visualized with FITC-labeled secondary antibody. Note the abundant amounts of polysaccharide chain of HSPG on the cell surface. Scale, 30 µm. b: Thrombin (3 U/ml) induced Ca$^{2+}$ transients in control BAECs. Data from 13 cells in a coverslip are shown. Data are representative of nine repeated experiments. B) a: Cells were treated with 30 mU/ml heparinase III for 2 h, and anti-HSPG antibody was applied to visualize the polysaccharide chain of HSPG. Note that traces of HSPG are stained as dots. Scale, 30 µm. b: Thrombin (3 U/ml) did not induce Ca$^{2+}$ transients in heparinase III-treated BAECs. Cells were treated for 2 h with 30 mU/ml heparinase III. Data from 12 cells in a coverslip are shown. Data are representative of seven repeated experiments. C) Effects of pre-incubation with heparin on thrombin-induced Ca$^{2+}$ transients. Thrombin (30 U/ml) was pre-incubated with 3 U/ml (a) or 30 U/ml (b) of heparin for 1 h at room temperature, and perfused to untreated BAECs after one-tenth dilution (final concentrations; thrombin 3 U/ml, heparin 0.3 U/ml in a and 3 U/ml in b). Data from 25 (a) and 24 (b) cells in a coverslip are shown. Data are representative of seven (a) and six (b) repeated experiments.
Materials and methods

Cell culture
BAECs were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum as previously described (9). The present study was performed with BAECs from five aortae.

Immunofluorescent staining of HSPG
Polysaccharide chain of HSPG was stained immunologically with a monoclonal anti-heparan sulfate antibody (clone HepSS-1, 4 μg/ml, Seikagaku Corp., Tokyo, Japan) as described previously (5).

Measurement of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was measured from non-confluent BAECs with fura-2 by using a fluorescence microscopy system (Aquacosmos; Hamamatsu Photonics, Hamamatsu, Japan). Cells cultured on a coverslip were loaded with the acetoxyethyl-ester form of fura-2 (fura-2 AM, 1 M, Molecular Probes, Eugene, OR, USA) for 20 minutes (min) at 37°C. The coverslip with fura-2-loaded cells was placed on a chamber of 0.5 ml volume and mounted on an inverted-microscope (IX70, Olympus, Tokyo, Japan). The cells were excited with two alternative wavelengths, 340 and 380 nm, and the emitted fluorescent intensities at 505 nm (F\(_{340}\) and F\(_{380}\), respectively) were recorded with CCD camera (C6790, Hamamatsu Photonics) and calculated into the fluorescence ratio (R), F\(_{340}/F_{380}\).

Measurement of intracellular NO production
To determine the intracellular NO production in BAECs, a NO-sensitive fluorescent dye DAF-2 (10) was used. Non-confluent cells grown on coverslip were incubated with a diacytlated form of DAF-2 (10 μM, Daiichi Pure Chemicals, Co. Ltd., Tokyo, Japan) for 20 min at 37°C. DAF-2 fluorescence was measured by using Aquacosmos fluorescence system as above but with a different filter set (excitation at 490 nm and emission at 515 nm). DAF-2 fluorescence increased almost linearly with the NO concentration (10). Therefore, we expressed the intracellular NO production as the DAF-2 fluorescence relative to its initial value. All solutions used for NO measurement contained 3 mM L-arginine.

Treatment of BAECs with heparinase III
Polysaccharide chain of HSPG was digested by incubating BAECs with 30 μU/ml heparinase III (Sigma, St. Louis, MO, USA) for 2 hours (h) at 37°C in culture medium. Cells were washed twice with fresh culture medium and immediately used for each assay.

Pre-incubation of thrombin with heparin
Thrombin (30 U/ml) dissolved in Krebs solution was pre-incubated with 3 or 30 U/ml heparin for 1 h at room temperature. If the pre-incubation mixture was not used immediately, it was kept on ice until just prior to the experiment. The solution was then perfused to the cells after being diluted to 3 U/ml of thrombin and 0.3 or 3 U/ml of heparin with Krebs solution.

Solutions and drugs
The standard extracellular solution was a modified Krebs solution containing (in mM): 132 NaCl, 5.9 KCl, 1.2 MgCl\(_2\), 1.5 CaCl\(_2\), 11.5 glucose, 11.5 Hepes; pH adjusted to 7.3 with NaOH. All other drugs were purchased from Sigma.

Data analysis
Data are given as mean ± standard error of the mean, and statistical significance was assessed with Student’s unpaired t-test. Probabilities less than 5% (p<0.05) were regarded as significant.

Results
Heparinase and heparin inhibit thrombin-induced Ca\(^{2+}\) transients in bovine aortic endothelial cells (BAECs)
Firstly we confirmed that heparinase III properly degraded the polysaccharide chain of HSPG in BAECs. The cell surface of control BAECs is covered with a dense expression of HSPG (Fig. 1Aa), which was mostly digested by a 2 h-treatment with 30 μU/ml heparinase III (Fig. 1Ba). Thrombin (3 U/ml) induced Ca\(^{2+}\) transients in control cells (Fig.1Ab), but not in heparinase III-treated BAECs (Fig. 1Bb).

When thrombin (30 U/ml) was pre-incubated with 3 U/ml heparin for 1 h at room temperature, diluted thrombin/heparin mixture (final concentrations; thrombin 3 U/ml, heparin 0.3 U/ml) induced smaller degree of Ca\(^{2+}\) transients in control BAECs (Fig. 1Ca). Furthermore, when the concentrated thrombin/heparin mixture was prepared with a higher concentration of heparin (30 U/ml), its dilution (final concentrations; thrombin 3 U/ml, heparin 3 U/ml) did not induce Ca\(^{2+}\) transients (Fig. 1Cb).

These results suggest that the binding of thrombin to the heparin region of HSPG plays a critical role in thrombin-induced Ca\(^{2+}\) transients.

Effects of heparinase III on ATP-induced Ca\(^{2+}\) transients in BAECs
As we previously reported (11), low concentration of ATP (0.3 μM) induced Ca\(^{2+}\) oscillation in control BAECs (Fig. 2Aa), and a higher concentration of ATP (10 μM) induced phasic increase in [Ca\(^{2+}\)], (Fig. 2Ab). Heparinase III-treated BAECs also showed Ca\(^{2+}\) oscillation (Fig. 2Ba) and phasic increase in [Ca\(^{2+}\)], (Fig. 2Bb) in response to 0.3 and 10 μM ATP, respectively. This indicates that HSPG is not required for the ATP-induced Ca\(^{2+}\) transients in BAECs.

Effects of heparinase III on thrombin- and ATP-induced NO production in BAECs
Thrombin (1, 12) and ATP (13) induce NO production in endothelium in a Ca\(^{2+}\)-dependent manner. We measured NO production with an NO-sensitive fluorescent dye DAF-2 (10).

Thrombin (3 U/ml) induced gradual increase in DAF-2 fluorescence in control BAECs (Fig. 3Aa). In contrast, BAECs that were treated with 30 μU/ml heparinase III for 2 h did not show the thrombin-induced increase in DAF-2 fluorescence (Fig. 3Aa). Furthermore, thrombin/heparin mixture, which was prepared by incubating 30 U/ml thrombin with 30 U/ml heparin for 1 h at room temperature and diluted into one-tenth concentration (final concentrations; thrombin 3 U/ml, heparin 3 U/ml), failed.

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to induce the increase in DAF-2 fluorescence in untreated cells (Fig. 3Aa). Since the binding of DAF-2 with NO is irreversible (10), net increment of DAF-2 fluorescence corresponds to the total amount of NO production. As shown in Figure 3Ab, the amount of thrombin-induced NO production was significantly suppressed in heparinase III-treated cells and heparin-treated thrombin.

ATP-induced NO production, however, was not affected significantly by the treatment with heparinase III in BAECs (Fig. 3B).

These results indicate that the polysaccharide chain of HSPG plays an essential role in thrombin-induced, but not in ATP-induced, NO production in BAECs.

**Discussion**

The present study showed for the first time that HSPG plays a significant role in the regulation of thrombin-induced responses in endothelial cells. We have observed that the thrombin-induced Ca$^{2+}$ responses and subsequent NO production were inhibited in heparinase III-treated BAECs (Figs. 1Bb and 3A). We confirmed that heparinase III completely degrades the polysaccharide chain of HSPG (Fig. 1Ba). Thus we suppose that the effects of heparinase III can be attributed to the removal of polysaccharide chain of HSPG. Thrombin-induced Ca$^{2+}$ responses are initiated by the production of IP$_3$ (14), and ATP-induced Ca$^{2+}$ transients mediated by P$_2Y$ receptors are also obtained via IP$_3$ production (15). Since ATP-induced Ca$^{2+}$ transients were not affected in heparinase III-treated BAECs (Fig. 3B), we have concluded that removal of polysaccharide chain of HSPG does not affect the cellular Ca$^{2+}$ responsiveness to IP$_3$ in BAECs. Furthermore, intracellular NO productivity remains intact after the treatment with heparinase III, since ATP-induced NO production was also not different between control and heparinase III-treated BAECs (Fig. 3B). Therefore, these results indicate that the polysaccharide chain of HSPG plays a role in the binding of thrombin to PAR receptors but not in the intracellular signaling pathways from IP$_3$ to NO production.
In the experiments using a thrombin/heparin mixture, heparin was pre-incubated with thrombin for 1 h at room temperature, so it can be postulated that the heparin-binding exocite of thrombin was blocked by heparin during the incubation period. In these assays, heparin inhibited thrombin-induced $\text{Ca}^{2+}$ transients in a concentration-dependent manner in BAECs (Fig. 1C). Furthermore, as in case of heparinase III-treated BAECs, heparin-pretreated thrombin failed to induce NO production in untreated cells (Fig. 3A). Therefore, this strongly indicates that the binding between polysaccharide chain of HSPG and heparin-binding exocite of thrombin plays a critical role in the actions of thrombin on endothelial cells.

It is well established that HSPG is a proangiogenic extracellular molecule that promotes the activity of growth factor (4). Furthermore, the removal of HSPG with heparinase blocked mechanosensitive NO production in the endothelium (5). Involvements of HSPG in the pathogenesis of cystic fibrosis (16) and leukocyte-endothelium interaction (17) were also suggested. This study revealed for the first time that HSPG may play an another important role in endothelial pathophysiology, i.e. the regulation of thrombin-induced responses. Since thrombin plays an important role in vascular pathophysiology such as the control of vascular tones and proliferation (3, 12, 18), the present study may also suggest HSPG as a potential target of cardiovascular disorders (19).

Figure 3: Intracellular NO production induced by thrombin and ATP in BAECs. A) Thrombin (3 U/ml) was applied to control (open circles) and heparinase-treated (closed circles) BAECs. Thrombin/heparin mixture, which was prepared by incubating 30 U/ml thrombin with 30 U/ml heparin for 1 h at room temperature, was also applied to untreated BAECs after one-tenth dilution (open triangles; final concentrations, thrombin 3 U/ml and heparin 3 U/ml). DAF-2 fluorescence was measured every 30 seconds. Relative DAF-2 fluorescence obtained from 20–30 cells in a coverslip were averaged for each data point, and the mean ± SEM. values from six repeated measurements are shown (a). Statistical analysis of maximal elevation of DAF-2 fluorescence is shown in b (n=6). * p<0.05 vs. control. B) ATP (10 $\mu$M)-induced increase in DAF-2 fluorescence was measured in control (open circles) and heparinase III-treated (closed circles) BAECs. Data from six repeated experiments were averaged (a). Statistical analysis of maximal elevation of DAF-2 fluorescence is shown in b (n=6). n.s., p>0.05.
References