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HEPARIN AND HEPARAN SULFATE INHIBIT EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION AND MYOCARDIAL CELL HYPERTROPHY INDUCED BY ENDOTHELIN-1

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KEY WORDS

\textit{c-fos; c-fos} SRE; extracellular signal-regulated kinase; Elk-1; Endothelin-1; myocardial cell hypertrophy

ABSTRACT

Heparan sulfate (HS) is one of the components of extracellular matrix and a potent antigrowth factor in various cells. Heparin has a similar structure to HS and is demonstrated to inhibit myocardial cell hypertrophy. We examined the intracellular signal mechanisms linking to the inhibitory effects of heparin and HS on endothelin-1(ET-1)-induced hypertrophy in cultured rat neonatal myocardial cells (MCs). Heparin inhibited ET-1-induced \textit{c-fos} mRNA expression. Heparin and HS inhibited ET-1-induced activation of \textit{c-fos} promoter/enhancer in MCs. Although heparin and HS inhibited ET-1-induced activation of the wild-type \textit{c-fos} serum response element (SRE), the activation of a mutated \textit{c-fos} SRE that contains an intact binding site for the serum response factor (SRF) but lacks the ternary complex factor (TCF) binding site, was not inhibited. In addition, heparin and HS inhibited the activation of TPA response element.
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(TRE). However, heparin did not inhibit the activation of cyclic AMP response element (CRE). Furthermore, heparin and HS inhibited ET-1-induced activation of extracellular signal-regulated kinase (ERK) and phosphorylation of Elk-1, which is one of the TCFs. These results indicate that heparin and HS inhibited ET-1-induced ERK activation, resulting in suppression of Elk-1 phosphorylation, and lead to inhibition of c-fos gene expression through SRF-independent manner. Moreover, heparin and HS inhibited ET-1-induced $[^{3}H]$Ieucine incorporation. These results suggest that heparin and HS inhibit ET-1 induced myocardial cell hypertrophy through the inhibition of gene expression and protein synthesis.

INTRODUCTION

In clinical studies, cardiac hypertrophy is an adaptational state before cardiac failure and an independent risk factor of cardiac morbidity and mortality. Therefore, it is important to inhibit the progression of myocardial cell hypertrophy. Myocardial cell hypertrophy has been shown to be caused by several stimuli, such as mechanical stress, growth factors linking to G-protein coupled receptor such as endothelin-1 (ET-1) and angiotensin II (ang II), and cytokines such as cardiotrophin-1 and leukemia inhibitory factor (LIF). However, little is studied about the endogenous factors that negatively regulate the progression of myocardial cell hypertrophy. Heparin and heparan sulfate (HS), which are the major components of extracellular matrix, inhibit growth proliferation and migration in several cell types. Heparin and HS are potent inhibitors of angiotensin II-induced myocardial cell hypertrophy and endogenous heparin-like substances negatively regulate myocardial cell hypertrophy. However, the mechanisms of HS- and heparin-mediated inhibitory effects on myocardial cell hypertrophy, especially the intracellular signal mechanisms, are not well understood.

The c-fos gene, an immediate-early gene, is transcriptionally activated rapidly and transiently within minutes of growth stimulation in a variety of cell types. In myocardial cells, c-fos gene is rapidly expressed upon hypertrophic stimuli such as hemodynamic forces and growth factors linking to G-protein coupled receptor. c-fos is implicated in transcriptional activation of various genes, which are induced in myocardial hypertrophy. Among G-protein coupled receptors, ET-1 is a potent stimulator for myocardial cell hypertrophy. Plasma ET-1 levels are elevated in heart failure and ET-1 production is increased in the failing heart. Long-term treatment with an endothelin-A (ET-A) receptor antagonist inhibits cardiac hypertrophy and improves the survival of rats with chronic heart failure.
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In the present study we aimed to explore the intracellular signal mechanisms linking to the inhibitory effects of heparin and HS on ET-1-induced myocardial hypertrophy. Our results demonstrate for the first time that heparin or HS inhibitor ET-1-induced activations of c-fos SRE and TRE but not CRE. Particularly in the c-fos SRE, TCF binding is inhibited by heparin and HS through inhibiting of at least at the level of ERK activation and subsequent suppression of Elk-1 phosphorylation.

MATERIALS AND METHODS

Materials

Sprague-Dawley rats were purchased from Japan SLC Co. Ltd. The standard culture medium was Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12, 1:1 [vol/vol], GIBCO BRL). For luciferase assay, a luciferase assay kit (TOYO Ink MFG. Co. Ltd.) was used. Other materials and chemicals were obtained from commercial sources.

Plasmids

The c-fos promoter/enhancer linked to the luciferase gene (c-fos luciferase), SRE CAT (chloramphenicol acetyltransferase), CRE CAT, TRE CAT, pTKCAT (thymidine kinase CAT) and RSV Luciferase (Rous sarcoma virus promoter linked to luciferase reporter gene) expression plasmids were generous gifts from Dr. K. Kaibuchi (Nagoya University, Japan). pTKCAT or RSV Luciferase was used as an internal control to standardize the transfection efficiency. 2x SRE CAT, 2x SRE.L CAT, and glutathion S-transferase (GST) Elk-1 (307-428) expression plasmids were generous gifts from Dr. Richard Treisman (Imperial Cancer Research Fund, UK). 2x SRE CAT contains two copies of the wild-type c-fos SRE. 2x SRE.L CAT contains two copies of the mutated c-fos SRE that has the intact binding site for SRF but lacks the TCF binding site.

Cell culture

Single-cell cultures were prepared from neonatal rat hearts. The culture medium was DMEM/F-12 supplemented with 5% calf serum and penicillin-streptomycin (0.02 unit/ml and 0.02 µg/ml, respectively). In 24 hours after seeding, the culture medium was changed to DMEM/F-12 supplemented with 0.1% bovine serum albumin (BSA) and ITS (10 µg/ml insulin, 10 µg/ml transferrin, and 10 ng/ml selenious acid) as described previously.
Amino Acid Incorporation

The relative amount of protein synthesis was determined by assessing the incorporation of the radioactivity into a trichloroacetic acid (TCA)-insoluble fraction as described previously. After 48 hours in the serum depleted medium, MCs were stimulated with ET-1 (100 nM) in the presence or absence of heparin (10 µg/ml) and HS (10 µg/ml) for 24 hours. 0.5 μCi/ml [3H] leucine was added 2 hours before harvesting. MCs were quickly rinsed twice with ice-cold PBS and incubated for 30 minutes on ice with 5% TCA. After being washed twice with ice-cold 5% TCA, MCs were solublized in 0.1 N NaOH. Total TCA-insoluble radioactivity of each well was determined by liquid scintillation counting.

Northern blot analysis

After 48 hours in the serum depleted medium, MCs were stimulated with ET-1 (100 nM) with or without heparin (10 µg/ml) and HS (10 µg/ml) for 24 hours. Total RNA (15 µg) was subject to this analysis as described previously.

Transfection, Luciferase assay and CAT assay

The medium was changed 24 hours after seeding the cells to DMEM-12 supplemented with 0.1% BSA, ITS and 30 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)(HEPES)(pH 7.5). MCs in duplicate dishes were transfected with reporter plasmids by using the modified calcium phosphate precipitation method as described previously. The DNA/CaP04 precipitates in each dish (5.0 ml) contained the following: 5.0 µg of c-fos luciferase, SRE CAT, CRE CAT, TRE CAT, 2x SRE CAT, or 2x SRE.L CAT reporter plasmids; 4.0 µg of pTKCAT or 0.5 µg of RSV Luciferase as an internal control for variations in the transfection efficiency, and variable amounts of pcDSRα expression plasmid to adjust total DNA to 13 µg. Precipitates were removed 2 hours after, and then the cells were maintained in DMEM/F-12 supplemented with 0.1% BSA and ITS for 48 hours. For the last 10 hours of the incubation period, MCs were pretreated (30 minutes) with heparin or HS, and then stimulated with various agents, ET-1 (100nM), forskolin (10nM) and Ro 20-1724 (10nM) for 9 hours. After the stimulation, MCs were harvested by scraping. Luciferase and CAT assay were done as described previously. c-fos luciferase expression in MCs treated with each agent was divided by CAT expression in MCs co-transfected with pTKCAT in the same experiment. Similarly, SRE, CRE, TRE, 2 x SRE or 2x SRE.L CAT expression in MCs treated with each agent was divided by luciferase expression in MCs co-transfected with RSV Luciferase in the same experiment. Luciferase or CAT experiments are expressed relative to the basal condition without stimulation.
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(control) value (1.0). Under these conditions, both assays were within the linear range. There was no difference in CAT expression derived from pTKCAT between control and various agent-treated MCs. Similarly, there was no difference in RSV Luciferase expression between control and various agent treated MCs.

**Kinase Assay**

The medium was changed 24 hours after seeding the cells to DMEM/F12 supplemented with 0.1% BSA, ITS, and then the cells were maintained in the same medium for 48 hours. MCs were pretreated with heparin (10 µg/ml) or HS (10 µg/ml) for 30 minutes, and then stimulated with ET-1 (100 nM) for various periods. After the stimulation, MCs were washed twice in ice-cold phosphate-buffered saline and scraped in 200 µl of DMEM/F12 containing 0.1% BSA, ITS, and then incubated in the same medium for 48 hours. MCs were pretreated with heparin (10 µg/ml) or HS (10 µg/ml) for 30 minutes, and then stimulated with ET-1 (100 nM) for various periods. After the stimulation, MCs were washed twice in ice-cold phosphate-buffered saline and scraped in 200 µl of 20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA (pH 8.0), 20 mM β-glycerophosphate, 0.05% Triton X-100, 0.5 mM diethylthreitol (DTT), 0.1 mM sodium orthovanadate, 2 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). In-gel kinase assay for ERK activation and solid-phase kinase assay for Elk-1 phosphorylation were done as described previously.

Statistical procedures

All values were expressed as means ±S.E.M. Experimental results were compared by one-way ANOVA and Bonferroni test for samples. Values were considered to statistically at p<0.05.

**RESULTS**

**Heparin and HS inhibit ET-1-induced amino acid incorporation in MCs.**

Heparin and HS are shown to inhibit amino acid incorporation in MCs associated with an increase in their cell surface area stimulated by angiotensin II. Therefore we examined the effect of heparin and HS on the amino acid incorporation. As shown in Fig.1 (a), treatment with ET-1 for 24 hours increased protein synthesis as evaluated by [3H] leucine incorporation into MCs. Heparin and HS inhibited ET-1 induced increase in the amino acid incorporation.

**Heparin inhibits c-fos m-RNA stimulated by ET-1 in MCs.**

Busch et al reported that heparin selectively inhibits AP-1-mediated gene expression in
FIG. 1. (a) Effect of HS on ET-1-induced increase in [3H] leucine incorporation in MCs. Cultured MCs were preincubated with or without 10µg/ml HS for 30 min. Cells were subsequently treated with vehicle or 100nM ET-1 for 24 hours. 0.5µCi/ml [3H] leucine was added 2 hours before harvesting. Total radioactivity of incorporated [3H] leucine was determined by liquid scintillation counting. The bar graphs shows liquid scintillation counting analysis of [3H] leucine incorporation as a relative percentage of control unstimulated cells. The values are means ± S.E.M. of five independent experiments. (b) Northern blot showing the inhibition of ET-1-induced c-fos mRNA expression by heparin. Cultured neonatal MCs were pretreated with 10µg/ml heparin for 30 minutes and subsequently stimulated with 100nM ET-1. After the stimulation, the cells were lysed. And total RNA (15µg) was hybridized with the c-fos DNA probe as described in Materials and Methods. (c) Bar graph showing the effect of heparin and HS on ET-1-induced c-fos luciferase expression. c-fos luciferase expression vector was transfected to cultured MCs. MCs were pretreated with heparin (10µg/ml) or HS (10µg/ml) for 1 hour and subsequently stimulated with ET-1(100nM) for 9 hours. Luciferase expression was assayed as described in Materials and Methods. The values are means ± S.E.M. of three independent experiments.

vascular smooth muscle cells. 24) c-fos gene expression is important for myocardial cell hypertrophy and AP-1 is a complex of Fos and Jun. Then, we examined the effect of heparin and HS on c-fos gene expression by Northern blot analysis. Heparin suppressed the expression of c-fos mRNA by approximately 40% (Fig. 1(b)).

**Heparin and HS inhibit activation of c-fos promoter/enhancer stimulated by ET-1 in MCs.**

Next, to examine whether the inhibitory effect of heparin and HS on the c-fos mRNA depends on the transcription, we performed the transfection assay. As shown in Fig.1 (c), heparin and HS inhibited the activation of c-fos promoter/enhancer by ET-1.

**Heparin and HS inhibit activation of c-fos SRE stimulated by ET-1 in MCs.**

It is known that c-fos promoter region contains major inducible cis-acting elements, such as SRE and CRE. We examined the effects of heparin and HS on SRE activation in MCs. As shown in Fig. 2(a), ET-1 activated SRE CAT, and heparin and HS inhibited its activation. SRE consists of Ets motif and serum response factor (SRF) binding site. Ets motif is known to bind
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**FIG. 2.** Effects of heparin or HS on 2xSRE and 2xSRE.L CAT expression. 2xSRE or 2xSRE.L CAT was transfected to cultured neonatal MCs. MCs were pretreated with heparin (10µg/ml) or HS (10µg/ml) for 1 hour and subsequently stimulated with ET-1 (100nM) for 9 hours. CAT expression was assayed as described in Materials and Methods. (a) Bar graphs showing the effect of heparin or HS on 2xSRE CAT expression. The values are means ±S.E.M. of four independent experiments. (b) Bar graphs showing the effect of heparin or HS on 2xSRE.L CAT expression. The values are means ±S.E.M. of four independent experiments.

Heparin and HS inhibit activation of TRE stimulated by ET-1, but not CRE in MCs.

Since Fos cooperates with Jun to stimulate the TRE, we examined the effect of heparin and HS on TRE activation stimulated by ET-1. Heparin and HS inhibited ET-1-induced TRE activation. However, heparin had no inhibitory effect on the activation of CRE by Forskolin, adenylate cyclase activator or Ro 20-1724, phosphodiesterase inhibitor (data not shown). These results indicated that heparin inhibit the activation of TRE, but not CRE.

**Heparin and HS inhibit activation of ERK and phosphorylation of Elk-1 stimulated by ET-1 in MCs.**

Since ERK plays a critical role on c-fos SRE activation, we examined the effect of heparin and HS on ERK activation. As shown in Fig. 3, ET-1 induced activation of ERK as assessed by in gel kinase assay, and heparin and HS inhibited the activation. Then we examined the effect of heparin and HS on the phosphorylation of Elk-1, one of the ternary complex factors belonging to Ets domain proteins. ERK activation induced Elk-1 phosphorylation, which results in
FIG. 3. Effects of heparin or HS on ERK activation stimulated by ET-1. Cultured neonatal MCs were pretreated with heparin (10µg/ml) or HS (10µg/ml) for 30 minutes and subsequently stimulated with ET-1 (100nM) for various periods. ERK activation was assayed as described in Materials and Methods. (a) An autoradiogram of in-gel ERK assay showing the effect of heparin or HS on ERK activation. (b) Radioactivities of phosphorylated MBP at the positions of ERK-2 were quantitated and plotted as a percentage of control. The values are means ± S.E.M. of three independent experiments.

activation of c-fos SRE. As shown in Fig. 4, ET-1 stimulation resulted in Elk-1 phosphorylation, which was inhibited by heparin and HS.

DISCUSSION

In the present study, we showed that heparin and HS decrease the amino acid incorporation in MCs stimulated by ET-1. Then we demonstrated that heparin and HS inhibit ET-1 induced c-fos gene expression. Using c-fos promotor/enhancer-linked luciferase and CAT assay, we revealed that heparin and HS transcriptionally inhibit c-fos gene expression through SRE and TRE.

Activation of c-fos promoter/enhancer is known to be regulated by c-fos SRE. c-fos SRE is activated by the phosphorylation of TCF by ERK and also by SRF binding. Although heparin and HS inhibited ET-1-induced activation of the wild-type c-fos SRE, activation of the mutated c-fos SRE, which contains an intact binding site for SRF but lacks TCF binding site, was not inhibited. These results indicate that heparin and HS do not inhibit the binding of SRF to SRF binding site, and that the inhibitory effect of heparin and HS on c-fos gene, expression depends on the ERK-TCF pathway. This also reveals that the inhibitory effects of heparin and HS on signaling molecules are not non-specific. Heparin and HS inhibited ERK dependent Elk-1 phosphorylation. Thus, heparin and HS inhibited ET-1 induced ERK activation, which
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FIG. 4. Effect of heparin or HS on phosphorylation of Elk-1 induced by ET-1. Cultured neonatal MCs were pretreated with heparin (10µg/ml) or HS (10µg/ml) for 30 minutes and subsequently stimulated with ET-1 (100nM) for various periods. (a) An autoradiogram of solid-phase kinase assay using GST Elk-1 at the substrate showing the effect of heparin or HS on phosphorylation of Elk-1. (b) Radioactivities of phosphorylated Elk-1 were quantitated and plotted as a percentage of control. The values are means ± S.E.M. of three independent experiments.

resulted in suppression of Elk-1 phosphorylation and lead to inhibition of c-fos gene expression.

It is demonstrated in a previous report that heparin does not inhibit the binding of ang to angiotensin-receptor. We did not examine the binding of ET-1 to endothelin-receptor. However, heparin and HS did not inhibit ET-1-induced activation of c-fos SRE mutant. This result indicated that heparin and HS do not inhibit the binding of ET-1 to endothelin-receptor.

Heparin and HS inhibited ET-1-induced amino acid incorporation. The mechanisms of myocardial cell hypertrophy still remain not fully clarified. It has been reported that activations of small GTP-binding protein such as Ras, Rho, and Rac, protein kinase C (PKC), STAT, phosphatidylinositol 3-kinase (PI 3-kinase), ERK, and calcineurin are involved in the mechanisms. We previously reported that ERK activation is necessary for ET-1-induced myocardial cell hypertrophy. We also demonstrated that complete inhibition of ERK activation decreases about 50% of amino acid incorporation by ET-1 stimulation. Collectively, the present results suggest that the inhibitory effect of heparin and HS on myocardial hypertrophy is, at least partly, occurred through the inhibition of ERK and the subsequent attenuation of amino acid incorporation. However our data not exclude the possibility that these reagents exert the anti hypertrophic effect through a mechanism other than the ERK-mediated
pathway. Further studies are required to clarify the inhibitory mechanisms of heparin and HS on myocardial cell hypertrophy.

We also showed that heparin and HS inhibit ET-1-induced activation of TRE. These results indicated the possibility that heparin and HS inhibit the gene expression which has TRE in the promoter. The finding that heparin inhibits TRE activation is in accordance with previous reports demonstrating that heparin suppress AP-1-mediated gene expression in some cell types. Fos and Jun form a dimeric complex (AP-1) binding to TRE and activate TRE. Thus inhibition of c-fos expression would further suppress gene expression regulated by TRE. Since TRE-mediated transcriptional regulation is suggested to play a role in the mechanisms of myocardial hypertrophy, heparin and HS may inhibit hypertrophy also through the inhibition of TRE activation.

In conclusion, Heparin and HS inhibit ET-1-induced ERK activation and c-fos gene expression in MCs. The inhibition of ERK and the subsequent c-fos gene expression partly account for the inhibitory action of heparin and HS. HS may act as a negative regulator of myocardial cell hypertrophy.

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HEPARIN INHIBITS ENDOTHELIN-INDUCED MYOCARDIAL CELL HYPERTROPHY