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Inhibition of gene expression of heparin-binding epidermal growth factor-like growth factor by extracellular superoxide dismutase in rat aortic smooth muscle cells

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Abstract

Both extracellular superoxide dismsutase (EC-SOD) and heparin binding EGF like growth factor (HB-EGF) are produced in smooth muscle cells of the arterial wall, and are thought to play pathological roles in atherosclerosis with heparin binding characteristics. EC-SOD treatment clearly reduced the H_2O_2 induced expression of HB-EGF in rat aortic smooth muscle cells (RASMC). EC-SOD also inhibited the induction of HB-EGF by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in RASMC by 60%. Both H_2O_2 and TPA increased intracellular ROS levels, and EC-SOD inhibited ROS generation only for the case of H_2O_2 but not TPA. Treatment of the cells with heparin alone decreased HB-EGF expression by 20%, whereas EC-SOD alone and a co-incubation with EC-SOD and heparin suppressed the induction by 60 and 70%, respectively. These results suggest that EC-SOD is related to the EGF signaling in two ways, competition for HSPG with HB-EGF and as an ROS scavenger.

Keywords: *EC-SOD, Atherosclerosis, HB-EGF, EGFR signaling, ROS*

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in vessels may influence interactions among smooth muscle cells (SMC) and the vascular endothelium through direct or indirect pathways and are involved in the pathogenesis of atherosclerosis formation [1,2]. Extracellular superoxide dismutase (EC-SOD; SOD3) is a mammalian isoenzyme of SOD [3,4]. The primary structure of EC-SOD indicates that the enzyme consists of an N-terminal hydrophobic signal peptide for secretion, a CuZn-SOD-like domain in the middle portion, and a heparin-binding domain in the C-terminal portion, as well as one potential N-linked glycosylation site in the middle [4,5]. The heparin-binding domain, consisting of six sequential basic amino acids, provides the basis for its affinity for heparin analogues on the cell surface, a characteristic property of EC-SOD [4–6]. The affinity facilitates the binding of EC-SOD to the external surfaces of many cells and tissues. Therefore, in spite of the secretory nature of the enzyme, a major part of EC-SOD is typically located in the in extracellular matrix of tissues [7]. The identity of binding partner of EC-SOD in tissue is not known but heparan sulfate proteoglycans (HSPG) [6] and/or collagen [8] are potential candidates *in vivo* and *in vitro*.

The level of EC-SOD is greater than or equal to the level of other SOD isoenzymes in arterial walls [9,10], however, the content of EC-SOD is somewhat less than the other two SOD isoenzymes, cytosolic

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CuZn-SOD and mitochondrial Mn-SOD in most tissues [11,12], and immunohistochemical observations using a specific antibody have revealed that EC-SOD is localized on the vascular smooth muscle layer [4,7,10]. These observations suggested that the functional role of EC-SOD is as a modulator of nitric oxide (NO) to prevent its inactivation by ambient superoxide. While several lines of evidence suggest the existence of a relationship between vascular EC-SOD activity and atherosclerotic diseases [13–16], however, the molecular mechanism responsible for the protection of the vascular wall by EC-SOD is still unknown. In this context, the interaction between EC-SOD and mitogenic growth factor signaling in sooth muscle cells should be noted.

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a potent mitogen and chemotactic factor for SMC [17,18]. HB-EGF, a member of the EGF family, is synthesized in the form of a membrane-anchored precursor in several cell types including vascular SMC that can be processed to release the soluble mature form [19]. Mature HB-EGF induces the autophosphorylation of the EGF receptor [17] and switches on the subsequent downstream cascade. In plaque formation in atherosclerosis, a proliferative change in SMC in the arterial intima occurs, and significant amounts of HB-EGF are produced in SMC and macrophages of atherosclerotic plaques [20], suggesting that HB-EGF plays an important role in atherogenesis. Moreover, the expression of HB-EGF is induced by various stimuli including angiotensin, shear stress, hydrogen peroxide, serum, phorbol ester and reactive dicarbonyl metabolites [18,21–24].

The focus of this study was on the inhibitory effect of EC-SOD on the induction of HB-EGF gene expression in smooth muscle cells. This is the first report to demonstrate the involvement of EC-SOD in the gene expression and cell signaling of growth factors and may be of pathological significance in understanding the protective effect of EC-SOD against occurrence of atherosclerosis.

Materials and Methods

Cell culture

Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aorta of a Wistar rat (body weight ~200 g) as described previously [25] and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin sulfate (100 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured to about 80% confluence and further incubated with fresh medium containing the above reagents. Throughout these experiments, the cells were used within passages 6–10.

Materials

12-*O*-tetradecanoylphorbol-13-acetate (TPA) and hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical Industry (Osaka, Japan). Heparin sodium salt was purchased from Nacalai Tesq (Kyoto, Japan). Other chemicals were of the highest grade commercially available.

Recombinant mouse EC-SOD

Recombinant mouse EC-SOD was prepared from the conditioned medium of a stably EC-SOD expressing cell line CHO-EK [26]. Typically, after reaching confulency, CHO-EK cells were culutured in 2% FCS supplemented DMEM/F-12 medium for an additional 2–3 days. The conditioned medium obtained was added to a Heparin-Cellulofine column (Chisso corp.) equilibrated with 20 mM Tris–HCl, pH 7.4. After a thorough washing with the equilibration buffer, the bound EC-SOD was eluted by a linear-gradient of NaCl from 0 to 1 M. The active fraction was concentrated on a Centriplus apparatus (Millipore) and then fractionated through Superose6 column (GE Healthcare Bio-Sciences Corp). The concentration of the recombinant protein was determined by BCA assay using bovine serum albumin (BSA) as a standard.

Cell Treatment

RASMC were cultured in 6 cm diameter dishes in DMEM supplemented with 10% FCS and antibiotics. Just prior to use, the cells were washed with serum-free DMEM twice then cultured with serum free DMEM containing the indicated concentration of recombinant EC-SOD for the indicated times. After the culture, cells were washed with PBS twice and then treated with the stimulant in serum-free DMEM for 1 h.

Expression analysis

Cellular RNA was extracted using TriZol Reagent (Invitrogen). A measure of 4 μg for HB-EGF or 1 μg for GAPDH of the total RNA were fractionated through a formaldehyde degenerating 1.2% agarose gel. After blotting onto a HybondN + (GE Healthcare Bio-Sciences Corp), hybridization was carried out using DIG-labeled rat HB-EGF cRNA probe in an EasyHyb solution (Roche) at 68°C overnight. A GAPDH DIG-cRNA probe was used to control hybridization. The hybridized DIG probe was visualized using a chemiluminescence detection kit (Roche) with CDP-star as an alkaline phosphatase substrate and exposed onto BioMax films (Kodak) for several minutes. Densitometorical analyses were performed using the ImageJ software

(http://rsb.info.nih.gov/ij/index.html) and expressed as a percentage of the control value.

Western blotting

After incubating with various concentrations of recombinant EC-SOD, RASMC cultured in 10 cm diameter dishes were washed with ice-cold PBS three times, and harvested with a rubber policeman and the cytosolic fraction was then extracted using a Cell lytic NuCLEAR Extraction kit (Sigma). One-tenth of the cytosolic fraction of RASMC from the 10 cm dish culture was used in a 12.5% SDS-PAGE run. After electroblotting onto an Immobilon-P membrane (Millipore), the membrane was incubated with anti-mouse EC-SOD antibody [27] or anti-beta-tubulin antibody (Sigma). The signals were visualized using HRP-conjugated secondary antibody and ECL-plus (GE Healthcare Bio-Sciences Corp) and exposed to BioMax films.

Immunocytochemical detection

RASMC, after culturing on a circular cover glass in a 12 well dish, were analyzed by an immunocytochemical method as follows: the cultures were fixed with 3.7% formaldehyde/PBS (pH 7.3) for 10 min at room temperature, permeabialized with 0.1% Triton X-100/PBS for 10 min, and then blocked with 2% (v/v) FCS/PBS. For indirect immunofluorescence analysis, the cells were incubated with the anti-mouse EC-SOD IgG in 2% FCS/PBS for 1 h at RT. Immunoreactivity was visualized using an FITC-conjugated anti-rabbit IgG IgG (DAKO). Three washes with PBS were carried out between each step.

Analysis of intracellular peroxide

To assess the levels of intracellular peroxides, flow cytometric analyses were carried out using an oxidation sensitive fluorescent probe, DCFH-DA (Molecular Probe) as described previously [28]. After the treatment, RASMC were incubated in 10-cm diameter dishes with DCFH-DA (5 μM at final concentration) for 20 min. The cells were washed twice with ice-cold PBS then harvested with trypsin/EDTA followed by two additional washings with PBS. Fluorescence was measured using FACScan (Becton Dickinson).

Results

EC-SOD attenuated HB-EGF expression induced by ROS in RASMC

It is well known that various agents and growth factors, including HB-EGF itself, induce HB-EGF-mRNA expression in vascular cells. To test the effect of EC-SOD to the cell response, we investigated the induction of HB-EGF mRNA in RASMC by exposure to H_2O_2. As shown in Figure 1A, exposure to H_2O_2 for 2 h induced HB-EGF mRNA in RASMC in a dose dependent manner, whereas 500 μM of H_2O_2 caused obvious damage to the cell. The time course for the induction is shown in Figure 1B. The induction was occurred within 30 min and maximum expression was observed at 1 h. Longer exposure times led to a decrease in induction. Preincubation of the cells with EC-SOD at 20 μg/ml for 4 h (Figure 1C, lanes 5–10) clearly reduced the induction of HB-EGF by 100 μM H_2O_2 (lanes 9, 10) compared to the mock incubation (lanes 3, 4), while EC-SOD itself had no effect on HB-EGF gene transcription levels (lanes 5, 6). To confirm the interaction between EC-SOD and RASMC, an immunocytochemical study, and a Western blot analysis were carried out (Figure 2). Substantial levels of granular deposits could be seen on RASMC (Figure 2A) and EC-SOD was detected in RASMC cytosolic fractions in a dose-dependent manner (Figure 2B).

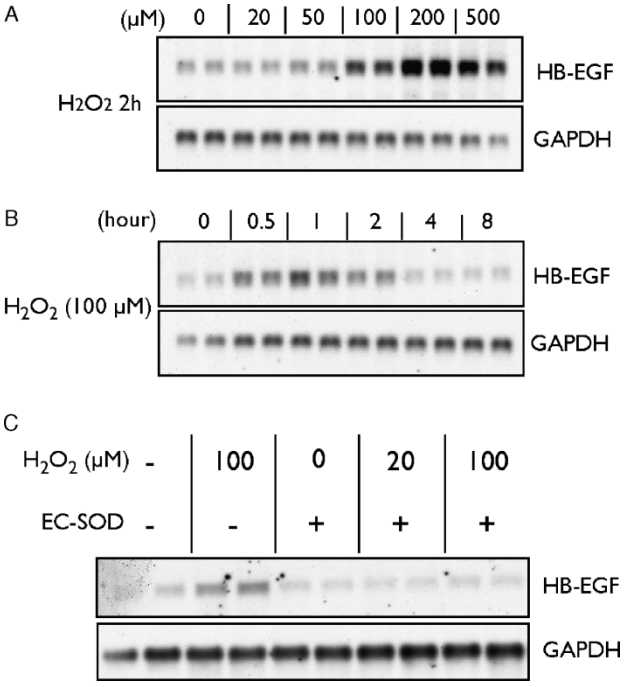

Figure 1. EC-SOD attenuated HB-EGF expression induced by ROS in RASMC through the interaction with cells. RASMC was treated with the indicated concentrations of H_2O_2 for 2 h (A) or at 100 μM for the indicated periods (B). Preincubation of RASMC with EC-SOD (20 μg/ml, 2 h) spoiled HB-EGF induction by H_2O_2 treatment. Lanes 1 and 2: control without H_2O_2 and EC-SOD; lanes 3 and 4: positive control with H_2O_2 without EC-SOD; lanes 5 and 6: negative control without H_2O_2 with EC-SOD; lanes 7 and 8: stimulated with H_2O_2 (20 μM) following the preincubation with EC-SOD (20 μg/ml for 4 h); lanes 9 and 10: stimulated with H_2O_2 (100 μM) following to the preincubation. (C) A measure of 4 μg (for HB-EGF) or 1 μg (for GAPDH) of total RNA were submitted to Northern blot analysis using rat HB-EGF or human GAPDH cRNA probes for the hybridization.

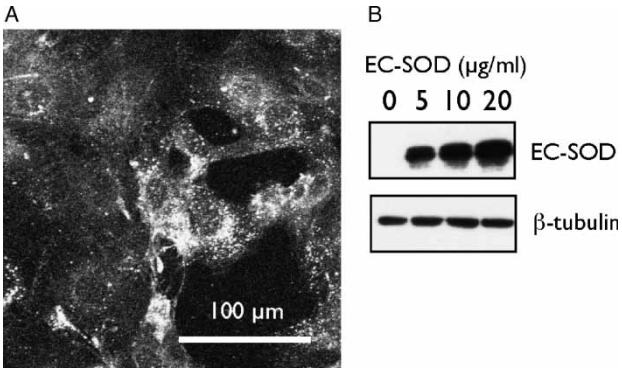

Figure 2. Binding of recombinant EC-SOD to RASMC. (A) Immunocytochemical examination of RASMC using an anti-mouse EC-SOD antibody [26] and an FITC-conjugated secondary antibody. Bar indicates 100 μm. (B) After 4 h incubation with indicated concentrations of EC-SOD, RASMC was harvested and cytosolic fraction was submitted to SDS-PAGE. The Western blot analysis was performed using anti-mouse EC-SOD antibody. (Upper panel) and anti-beta-tublin antibody (lower panel)

Partial inhibition of TPA induced HB-EGF expression by EC-SOD

Next we observed the effect of EC-SOD on HB-EGF expression induced by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a well-known inducer of HB-EGF expression in RASMC. Cells were preincubated with EC-SOD for 2 h followed by exposure to 200 nM TPA for 1 h and HB-EGF mRNA levels were then assayed. As shown in Figure 3, the stimulants induced HB-EGF expression (lanes 1 and 2) and the pretreatment with EC-SOD reduced the induction (lanes 3–6). The pretreatment of RASMC with EC-SOD (20 μg/ml, 2 h) had essentially no effect on HB-EGF expression (Figure 3A; control). A densitometric analysis showed an approximately 60% reduction in HB-EGF expression as the result of the EC-SOD treatment (Figure 3B).

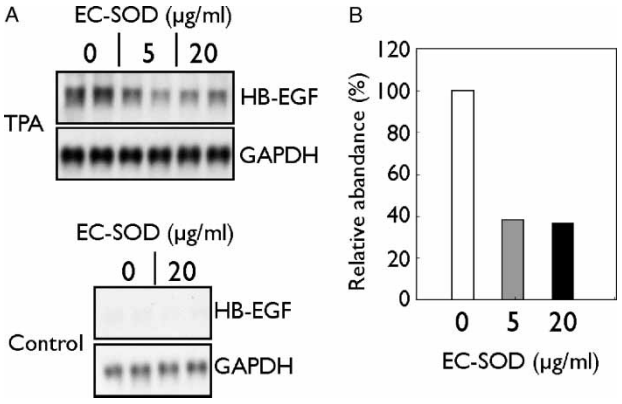

Figure 3. Effect of EC-SOD on HB-EGF mRNA expression induced by various stimuli and involvement of ROS. The effect of EC-SOD incubation on H_2O_2 (200 μM), the TPA (200 nM) and serum (5% v/v) induced induction of HB-EGF mRNA. (A) RASMC were incubated with the indicated concentrations of EC-SOD for 2 h followed by the treatment with H_2O_2 (200 μM). TPA (200 nM) or serum (5% v/v) for 1 h, and then harvested. A measure of 4 μg (for HB-EGF) or 1 μg (for GAPDH) of total RNA were analyzed by Northern blotting and hybridized with rat HB-EGF or human GAPDH cRNA probes. In the control experiment, the stimulant was omitted. (B) Densitometoric analysis of HB-EGF expression from panel A. Values are expressed as the relative abundance of the control value.

Intracellular ROS level

Intracellular peroxide formation in RASMC was evaluated by a flowcytometoric analysis using DCFH-DA as an oxidation sensitive fluorescent probe (Figure 4). Exposure to both H_2O_2 and TPA increased the intracellular peroxide levels (solid lines) and a pretreatment with EC-SOD inhibited the generation of the fluorescence only against H_2O_2, but not against TPA (dotted lines) compared to the control (gray areas).

Effect of heparin on HB-EGF induction

As reported previously, heparin competitively interferes with the interaction of EC-SOD [4] and HB-EGF [29] to HSPG on the cell surface. To better

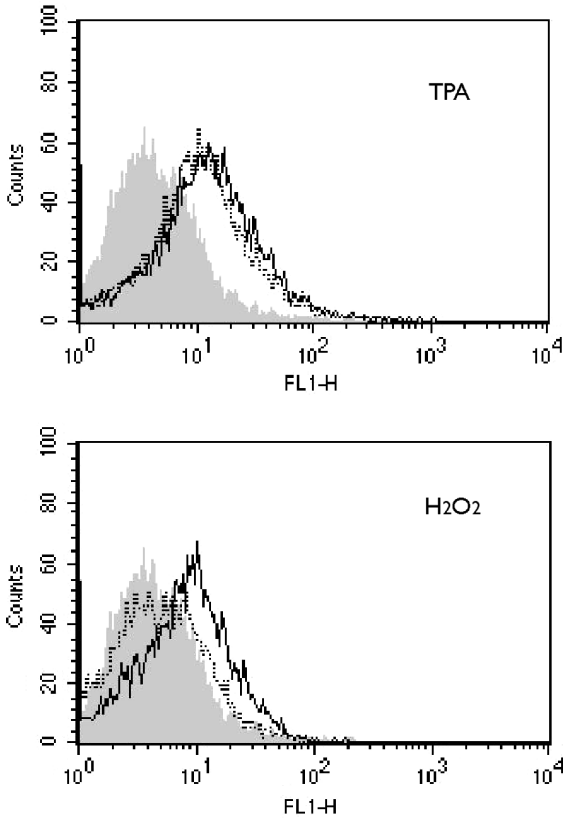

Figure 4. Effect of EC-SOD on intracellular ROS levels. Intracellular ROS levels of RASMC by treatment of H_2O_2 (200 μM) or TPA (200 nM) for 1 h are depicted in solid lines. Effect of preincubation with EC-SOD (20 μg/ml for 4 h) was shown by dotted lines. Control experiment was performed without EC-SOD and stimulant (gray area).

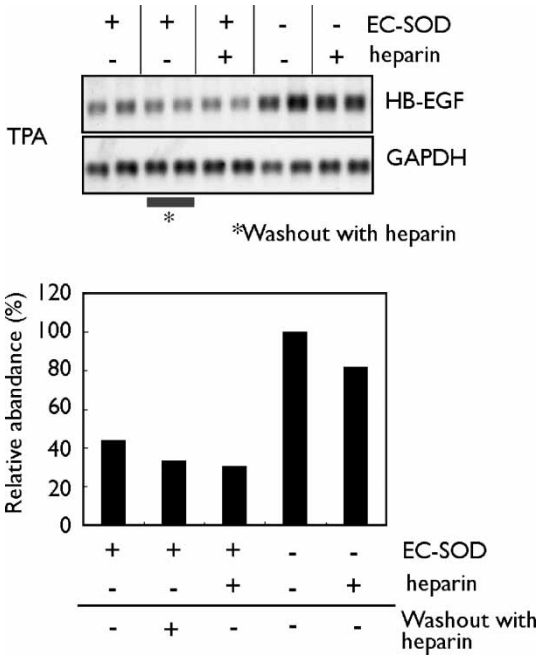

Figure 5. Effect of heparin on the HB-EGF expression. RASMC were preincubated with EC-SOD for 2 h with or without heparin (50 μg/ml). After washing three times with PBS, cells were treated with TPA (100 nM) for 1 h. A measure of 4 μg (for HB-EGF) or 1 μg (for GAPDH) of total RNA were analyzed by Northern blotting using rat HB-EGF or human GAPDH cRNA probes (upper panel). Densitometoric analyses of HB-EGF expression is depicted in panel B. Values are expressed as the relative abundance of the control value (lower panel).:

understand the significance of these interactions, the effect of heparin on TPA stimulation was investigated. TPA treatment induced HB-EGF within 1 h (Figure 5, lanes 7, 8), and preincubation with heparin alone decreased HB-EGF expression by 20% (lanes 9, 10). Preincubation with EC-SOD alone (10 μg/ml, 4 h) suppressed the induction by 60% (lanes 1, 2), whereas washing out the cell surface bound EC-SOD with heparin (lanes 3, 4) and the simultaneous preincubation with EC-SOD and heparin (lanes 5, 6) decreased the expression by 70%.

Discussion

Several investigators have commented on EC-SOD with regard to its protective effect against atherosclerosis because it has been reported to be a major antioxidant enzyme in arterial walls [9,10] and because it modulates the lifetime of endothelial-derived nitric oxide by eliminating superoxide radicals [4]. In fact, Wang et al. [15] reported that lower plasma EC-SOD levels was associated with a history of myocardial infarction (MI) and Landmesser et al. [16] reported that vascular EC-SOD levels were reduced in the patients with coronary artery disease. Fukai et al. [30] also reported that lipid-laden macrophages express EC-SOD in atherosclerotic lesions. These observations constitute proofs of a relationship between vascular diseases and a pathological role of EC-SOD, however the molecular mechanism involved in vascular wall protection by EC-SOD has remained unclear. In the present study, we demonstrate a novel role of EC-SOD in the signaling pathway of growth factors to reduce the proliferation of smooth muscle cells.

Kayanoki et al. [23] reported that increased intracellular ROS levels stimulated HB-EGF expression induced by HB-EGF itself or thrombin in both RASMC and HUVEC. However, a direct effect of H_2O_2 on HB-EGF induction was found only in HUVEC. As shown in Figure 1, H_2O_2 exposure increased HB-EGF expression in RASMC in a dose-dependent manner and EC-SOD suppressed the induction. A substantial production of HB-EGF was detected in SMC and macrophages of atherosclerotic plaques along with EGF receptors on SMC [21] and several stimuli including oxidative stress, TPA and HB-EGF itself were found to induce HB-EGF expression in SMC [18,23]. HB-EGF is initially synthesized in the form of a membrane-anchored precursor (proHB-EGF), and conversion by the ADAM-mediated ectodomain shedding process gives the mature soluble form, which has potent chemoattractic and mitogenic activities to vascular SMC [19,21,31]. Certain stimuli have also been demonstrated to lead to the conversion of proHB-EGF to the soluble form, with the simultaneous activation of ADAM proteases [31]. Therefore, careful consideration should be focused on whether the induction is dependent on the direct effect of H_2O_2 because the oxidative stress also induces the activation of ADAM proteases, leading to the ectodomain shedding of proHB-EGF in human tumor cells [32]. In our results, EC-SOD obviously bound to and was incorporated into the cell (Figure 2), and normalized the intracellular peroxide level evoked by the H_2O_2 exposure (Figure 4), suggesting that EC-SOD may carried out its role in cell protection as the result of the exogenous given oxidative stress.

As shown in Figure 4, a TPA stimulus led to the generation of intracellular ROS and EC-SOD treatment had no effect on ROS levels, whereas EC-SOD reduced HB-EGF mRNA expression by 60% (Figure 3). ROS accumulation is dependent on PKC-β activation by TPA and drives the MEK kinase-1/SAPK pathway [33], which binds to the AP-1 site in the HB-EGF promoter with Ets-2 simultaneously activated by the Raf-MEK-MAPK pathway, to induce HB-EGF gene expression [34]. On the other hand, not only TPA but also ROS activates EGF receptor signaling via two distinct pathways, the PKC-δ dependent activation of non-receptor tyrosine kinases such as JAK2 and PYK2, and the metalloprotease dependent shedding of proHB-EGF [35], resulting in the marked induction of HB-EGF gene

expression. EC-SOD may participate in these signaling pathways in two possible steps. One possible step is an interaction between HB-EGF and HSPG molecules on the cell surface because the interaction of HB-EGF, via the heparin-binding domain, with cell surface HSPG is essential for its optimal binding to the EGF receptor [29] and EC-SOD may compete for HSPG with HB-EGF. The other possible step is activation of the signaling pathway by ROS. Oxidative stress was reported to activate metalloprotease activity [31,35] and PKC-δ [35,36], although the involvement of superoxide radicals in these activations remains to be elucidated. As shown in Figure 5, the inhibition of TPA induction by heparin alone was increased up to 20%, besides inhibiting EC-SOD by 60%. Moreover, washing out of the extracellular bound EC-SOD failed to reduce the inhibition, suggesting that the contribution by the intracellular ROS scavenging effect of EC-SOD was more than equal to the extracellular HSPG-dependent inhibition.

In conclusion, the findings herein show that EC-SOD participates in EGFR signaling by reducing the expression of HB-EGF in RASMC. This finding may provide a new insight into the physiological role of EC-SOD in controlling the generation of atherosclerosis by preventing of proliferative change in vascular smooth muscles, as well as the protection of extracellular matrices from the oxidative stress and the modulation of nitric oxide bioavailability.

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