Am J Physiol Heart Circ Physiol
281: H1621–H1629, 2001.

Vasoactive factors and growth factors alter vascular smooth muscle cell EC-SOD expression

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Received 5 November 1999; accepted in final form 24 May 2001

Strålin, Pontus, and Stefan L. Marklund. Vasoactive factors and growth factors alter vascular smooth muscle cell EC-SOD expression. *Am J Physiol Heart Circ Physiol* 281: H1621–H1629, 2001.—Oxygen free radicals have been suggested to play important roles in atherogenesis and other pathological processes in the blood vessel wall. The vascular wall contains large amounts of extracellular superoxide dismutase (EC-SOD), which is produced and secreted to the extracellular space by smooth muscle cells. In this study, we investigated the influence of factors regulating tension and proliferation of vascular smooth muscle cells and of some interstitial matrix components on EC-SOD expression. The expression and secretion of EC-SOD were upregulated by histamine, vasopressin, oxytocin, endothelin-1, angiotensin II, serotonin, heparin, and heparan sulfate and were downregulated by platelet-derived growth factors-AA and -BB, acidic and basic fibroblast growth factors, and epidermal growth factor. The responses were slow and developed over several days. The findings suggest that various physiological and pathological conditions might markedly influence EC-SOD expression, significantly altering the susceptibility of the vascular wall to effects of the superoxide radical.

oxygen radicals; atherosclerosis; vascular smooth muscle cells; glycosaminoglycans; heparin; extracellular superoxide dismutase

THERE IS EVIDENCE for increased levels of superoxide radical (O_2^-) in the extracellular space of the blood vessel wall in a number of pathological processes, including atherosclerosis, diabetes, and hypertension (9, 27, 39). Superoxide is secreted from activated endothelial cells (29), activated smooth muscle cells (SMCs) (8), and activated macrophages (3). It is also produced by xanthine oxidase bound to the extracellular matrix and cell walls (38). The increased levels of superoxide radicals may contribute to low-density lipoprotein oxidation (10) and to other degenerative oxidative processes in the vessel wall. O_2^- reacts momentarily with nitric oxide, forming toxic peroxynitrite (17) and causing nitric oxide dysfunction. The superoxide radical may thus play a significant proatherogenic role in the vessel wall. The human arterial wall contains very large amounts of the secreted superoxide dismutase (SOD) isoenzyme extracellular SOD (EC-SOD) (24, 30, 34), whereas the contents of cytosolic Cu- and Zn-containing SOD (Cu/Zn-SOD) and mitochondrial matrix Mn-containing SOD (Mn-SOD) are low compared with other tissues. EC-SOD was found to be evenly distributed in the wall, including large amounts in the intima (34). The major source of EC-SOD in healthy vessels are SMCs (34), whereas in atherosclerotic vessels the enzyme is produced by both SMCs and macrophages (6, 23). The strategic extracellular location and high levels suggest that EC-SOD exerts an important protective role against the superoxide radical in the vascular wall.

In the present study, we examined the effects of growth factors, vasoactive factors, and glycosaminoglycans on EC-SOD expression by human arterial SMCs. We found large effects, suggesting that these factors may significantly alter the secretion of EC-SOD in vivo and thus the response of the vascular wall to superoxide radicals.

MATERIALS AND METHODS

Cell culture and regulation experiments. Arterial SMC lines were initiated from pieces of human uterine artery as described (34). Cell lines from five different people were established (Au-1 to Au-5) and were used between the fifth and eighth passages. Uterine arteries were found to contain ~33 μg/g wet wt of EC-SOD, a value similar to those previously found in the human coronary artery and aorta (34). The cells were maintained in Waymouth MB 752/1 medium containing 15% fetal calf serum (FCS), 10^5 U/l bensylpenicillin, 100 mg/l streptomycin, 2 mmol/l glutamine, and 1 mmol/l sodium pyruvate.

For synthesis regulation experiments, SMC lines were cultured for 4 days with media containing active substances that were exchanged and collected each day. Because vascular SMC lines are heterogeneous and show heterogeneous responses (5), the effects of the growth and vasoactive factors were mostly tested on four different cell lines. Before the experiments, cells were seeded into 12-well culture plates (bottom area 3.80 cm^2) and grown to near confluence. During the experiments, culture media were supplemented with either 15% FCS or 1% BSA as indicated. When supplemented with 1% BSA, the medium was exchanged twice to medium with 1% BSA ~20 h before the start of the experiments. The experiments were started by exchange to 0.5 ml of medium

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with either 1% BSA or 15% FCS containing the indicated concentrations of factors or medium with only 1% BSA or 15% FCS (controls). Every 24 h, media were collected and replaced with fresh media containing active factors. At the end of the experiments, after 4 days, media were collected and the wells were washed three times with 0.15 mol/l NaCl. To collect and homogenize the cells, 0.5 ml of ice-cold 50 mmol/l sodium phosphate (pH 7.4) containing 0.3 mol/l KBr, 10 mmol/l diethylene triamine pentaacetic acid, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 10^7 IU/l aprotinin (the latter three additions to inhibit proteases) was added to the wells. After sonication in the wells, with the plate bathing in ice water, the homogenates were centrifuged (20,000 *g* for 10 min), and the supernatants were collected for analysis. All samples were kept at −80°C until assay. A heparin wash step was introduced in experiments related to heparin and other glycosaminoglycans. In these experiments, the SMCs were incubated for 10 min at 37°C with culture media containing 10^5 IU/l heparin followed by three washes with 0.15 mol/l NaCl and homogenization as described above.

Analysis of EC-SOD. EC-SOD protein in culture media and cell homogenates was determined with an ELISA as previously described (15).

Turnover of EC-SOD in cell cultures. To assess the rate of uptake of EC-SOD by cultured cells, conditioned media from SMCs containing EC-SOD were incubated with human cell lines not producing EC-SOD. Thus Waymouth medium supplemented with 1% BSA was incubated with confluent SMCs for 4 days, after which it was diluted with unconditioned medium to ~3 μg/l EC-SOD, a typical level in the SMC experiments (c.f. Fig. 1). Diluted medium (0.5 ml) was then added to triplicate wells with different human cell lines in 12-well culture plates. The cell lines were MG-251, a glioma cell line; Hep-G2, a hepatoma cell line; and PL-3 and DU-145, prostate cancer cell lines. The concentrations of EC-SOD in the media were determined at intervals over 24 h.

Protein and DNA analysis. For protein analysis, Coomassie brilliant blue G-250 was employed (1), standardized with human serum albumin. The DNA concentration was determined with fluorimetry as a complex with bisbenzimidazol (Hoechst 33258) (20) using calf thymus DNA as a standard.

Incorporation of [^{35}S]methionine into protein. SMCs were cultured in 12-well culture plates (3.8 cm^2) for 4 days as described above. At the end of the experiment, the cells were incubated with [^{35}S]methionine for 1 h followed by homogenization, protein precipitation with 10% trichloroacetic acid,

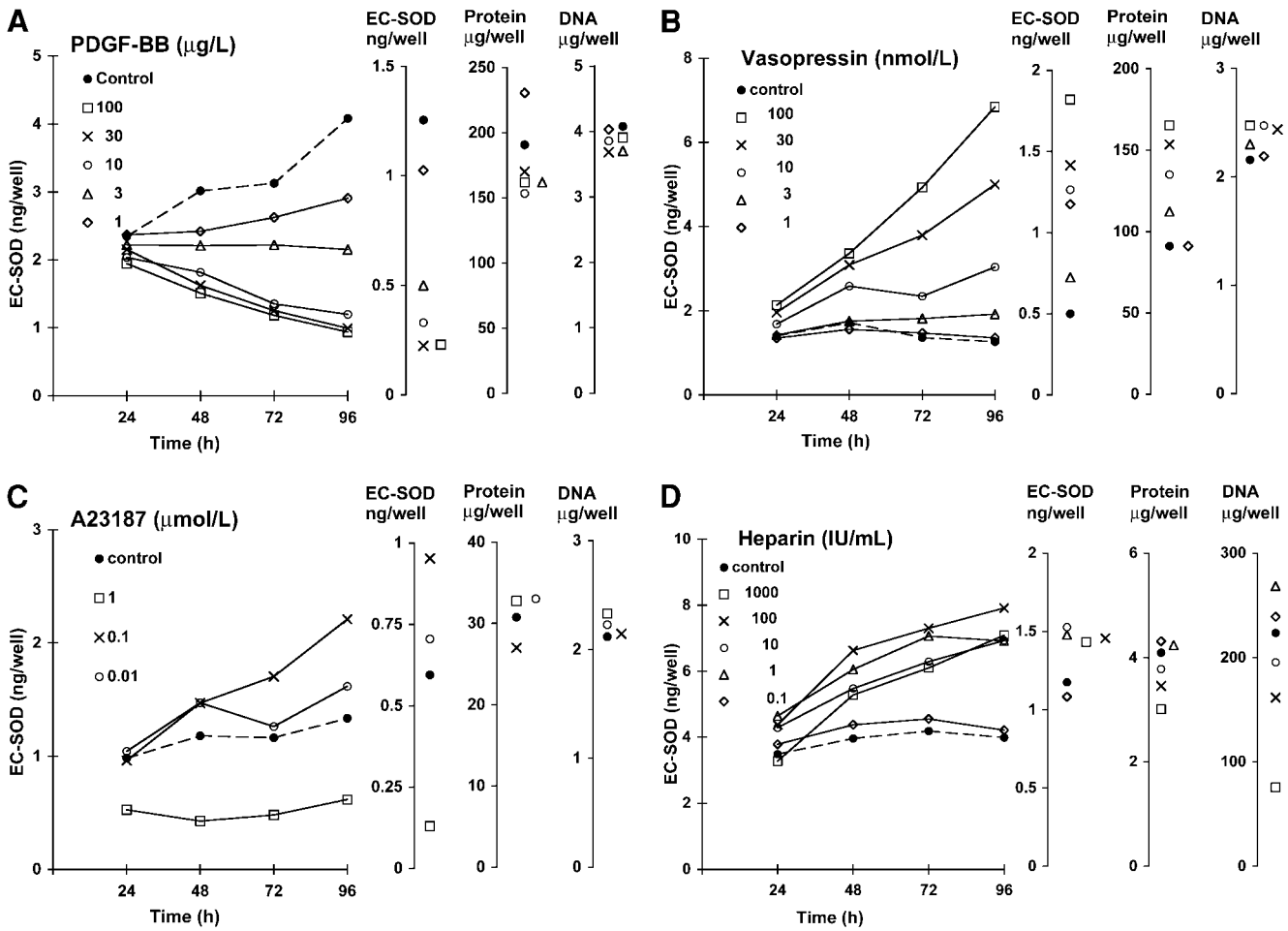

Fig. 1. Effects of some factors on extracellular superoxide dismutase (EC-SOD) expression in the Au-1 smooth muscle cell line. The cells were cultured in 3.8-cm^2 wells, and culture media (0.5 ml) containing factors at indicated concentrations were exchanged daily for 4 days. At the end of the experiment, the cells were homogenized, and analyses were made on culture media (*left*) and cell homogenates (*right*). EC-SOD content was determined by ELISA as described in MATERIALS AND METHODS. The data presented are the means of results from 2 wells. *A* and *D*: effects of platelet-derived growth factor (PDGF)-BB (*A*) and heparin (*D*) on cells with media supplemented with 15% fetal calf serum. *B* and *C*: effects of vasopressin (*B*) and A-23187 (*C*) on cells with media supplemented with 1% BSA.

and analysis of [^{35}S]methionine incorporation into protein as previously described (25). Aliquots of the cell homogenates were also analyzed for EC-SOD, protein, and DNA as described above.

RNA extraction and Northern blot analysis. Total cellular RNA was isolated from SMCs using the TRIzol reagent (GIBCO-BRL Life Sciences; Gaithersburg, MD). RNA (10 μg/lane) was electrophoresed in formaldehyde-containing 1.2% agarose gels, transferred to nylon filters (Hybond N, Amersham), and immobilized by ultraviolet linkage. The filters were then prehybridized for 15 min and hybridized for 1 h with Quickhyb solution (Stratagene; La Jolla, CA) at 65°C in a hybridization oven. For EC-SOD detection, a DNA probe was used that corresponded to nucleotides 1,018–1,211 in the cDNA sequence (11). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection, a 1,100-bp cDNA sequence (Clontech; Palo Alto, CA) was used. ^{32}P-labeling was achieved by random priming (Megaprime DNA-labeling systems, Amersham). To allow rehybridization, boiling 0.1% SDS was poured twice onto the filters. Radiolabeled filters were exposed to imaging screens for 3–4 days and analyzed in a Molecular Imager (Bio-Rad Life Technologies; Hercules, CA).

Statistics. Experiments were carried out on two to six wells for each data point. All major findings were replicated at least once on at least two cell lines. For analysis of significance levels, the individual results from each well were normalized to the mean of the controls of that experiment. Student's *t*-test was then applied to the set of relativized values from all the experiments on that data point (Table 1).

Materials. Heparin was obtained from Lövens Läkemedel (Malmö, Sweden). Fragmin was purchased from Pharmacia Upjohn (London, UK). Chondroitin sulfate A, isolated from bovine nasal septa (containing 1.0 sulfate residues/disaccharide unit), was kindly donated by Dr. Å. Wasteson (Linköping, Sweden). Heparan sulfate, derived from pig intestinal mucosa, was kindly supplied by Dr. U. Lindahl (Uppsala, Sweden). All other factors were obtained from Sigma (St. Louis, MO). Waymouth cell culture medium and FCS were purchased from Flow (Irvine, UK) and GIBCO-BRL Life Technologies.

RESULTS

General observations. The effects of the analyzed factors on EC-SOD expression and total cell protein were found to naturally group into three patterns of responses (Table 1). The first group, hereafter called the "growth factor" group, included acidic (aFGF) and basic fibroblast growth factors (bFGF), platelet-derived growth factors (PDGF)-AA and -BB, and epidermal growth factor (EGF). The second group, hereafter called the "vasoactive factor" group, included histamine, vasopressin, oxytocin, angiotensin II (ANG II), endothelin-1 (ET-1), and serotonin. The third group, hereafter called the "heparan sulfate" group, included heparin and some other glycosaminoglycans. Characteristic for all factors was a slow development of effects on EC-SOD in culture media over the 4-day experiments and a correspondence between results for EC-SOD in media and in cell homogenates. The four cell lines tested generally responded in similar ways to factors tested. All active responses found were reproduced several times with two of the SMC lines, mostly Au-1 and Au-2. Additional confirmatory two-well dose-response experiments (c.f. Fig. 1) were carried out with two additional cell lines.

Responses to growth factors. All factors in this group decreased the levels of EC-SOD secreted to the medium and in the cell homogenates in cultures supplemented with 15% FCS. The cell protein and DNA content in the cultures were in some cases to a moderate extent influenced. The levels of EC-SOD protein in the 4-h day media corrected for cell protein and relative to controls were between 0.3 and 0.8 in cell cultures exposed to the factors (Fig. 1*A* and Table 1).

In the absence of serum, the responses varied. PDGF-AA and -BB tended to stimulate overall protein synthesis and downregulate EC-SOD, whereas aFGF, bFGF, and EGF influenced the cells less. The cell response was strongest to PDGF-BB, with approximately a doubling in the protein level after 4 days of exposure concomitant with a downregulation of EC-SOD (Table 1). The increases in DNA content of the cultures were overall less pronounced.

The responses of EC-SOD mRNA levels were measured by Northern analysis for bFGF, PDGF-BB, and EGF. The responses on EC-SOD mRNA levels relative to GAPDH mRNA were similar to the responses seen in EC-SOD protein expression (Fig. 2*B*)

Responses to vasoactive factors. Responses to this group were discrete in cell cultures supplemented with 15% FCS (Table 1). In cell cultures supplemented with 1% BSA, increases in the amount of EC-SOD protein in both culture media and cell homogenates were observed (Fig. 1*B* and Table 1). The factors often also caused an increase in general protein synthesis as measured by the Bradford technique and by [^{35}S]methionine incorporation (Table 1; data on [^{35}S]methionine incorporation not shown), but the increases in EC-SOD protein relative to total protein were most often significant. There was also often a minor proliferative effect, as indicated by increases in the DNA content of the cultures.

For ANG II, the response was found to differ between the Au-1 and Au-2 cell lines. In repeated experiments on the Au-1 cell line, there was no increase in the amount of EC-SOD in the medium and in cell homogenates relative to protein level, whereas repeated experiments displayed increases in the Au-2 cell line (Table 1).

Northern blot experiments showed increases in the levels of EC-SOD mRNA relative to GAPDH mRNA for all factors tested in this group (Fig. 2*B*).

For histamine and serotonin, experiments were performed to determine which receptor subtypes mediated the responses. The addition of 10 μmol/l diphenhydramin, a selective H1 receptor antagonist, markedly diminished the responses to histamine in the Au-2 cell line, whereas addition of 10 μmol/l cimetidin, a selective H2 receptor antagonist, did not affect the histamine response, indicating a H1 receptor-mediated histamine response. Also, the cells did not respond to 10 μmol/l dimaprit, a selective H2 receptor agonist (Table 2).

Table 1. *Collection of data for factors influencing EC-SOD expression*

Cell Line	No. of Experiments	No. of Wells	EC-SOD		DNA	Protein
			In media	In cells		
Media supplemented with 1% BSAL						
ANG II (10 μmol/l)						
Au-1	2	7	0.82 ± 0.16*	1.27 ± 0.48	1.17 ± 0.05‡	1.91 ± 0.27‡
Au-2	2	8	2.00 ± 0.13†	2.56 ± 0.43‡	1.15 ± 0.06‡	1.31 ± 0.07‡
Au-4	1	2	2.1; 2.2	2.0; 2.0	1.2; 1.2	1.4; 1.9
Au-5	1	2	1.5; 1.8	1.4; 1.6	1.4; 1.5	1.2; 1.6
Serotonin (10 μmol/l)						
Au-1	5	16	1.66 ± 0.70†	1.42 ± 0.30‡	1.08 ± 0.08†	1.32 ± 0.17‡
Au-2	4	13	1.40 ± 0.27‡	1.63 ± 0.42‡	1.06 ± 0.09*	1.13 ± 0.16†
Au-4	1	2	1.0; 1.1	0.93; 1.2	1.1; 1.1	1.3; 1.4
Au-5	1	2	1.3; 1.4	1.0; 1.0	1.2; 1.4	1.3; 1.5
Histamine (10 μmol/l)						
Au-1	6	17	2.26 ± 1.62†	1.92 ± 0.52‡	1.17 ± 0.09‡	1.55 ± 0.29‡
Au-2	4	13	1.52 ± 0.32‡	2.33 ± 1.30†	1.06 ± 0.08*	1.22 ± 0.16‡
Au-4	1	2	1.4; 2.2	1.5; 2.5	1.2; 1.2	1.2; 2.0
Au-5	1	2	2.0; 2.3	1.4; 1.5	1.4; 1.4	1.5; 1.7
Vasopressin (100 nmol/l)						
Au-1	3	6	2.75 ± 1.32†	2.43 ± 0.64‡	1.14 ± 0.05‡	1.61 ± 0.30†
Au-2	3	6	2.02 ± 0.10‡	2.31 ± 0.62‡	1.13 ± 0.12*	1.57 ± 0.27‡
Au-4	1	2	3.9; 4.8	2.3; 2.8	1.1; 1.2	1.1; 1.4
Au-5	1	2	1.7; 1.9	1.4; 1.7	1.0; 1.1	1.0; 1.2
Oxytocin (1 nmol/l)						
Au-1	3	7	1.40 ± 0.40*	1.56 ± 0.26‡	1.12 ± 0.03‡	1.72 ± 0.34‡
Au-2	3	7	1.52 ± 0.78	1.74 ± 1.13	1.02 ± 0.04	1.25 ± 0.17†
Au-4	1	2	2.3; 2.3	1.7; 1.7	1.1; 1.2	1.1; 1.2
Au-5	1	2	1.5; 1.8	1.3; 1.4	0.93; 1.0	1.0; 1.1
Endothelin-1 (1 μmol/l)						
Au-1	4	13	1.71 ± 0.70†	1.51 ± 0.56†	1.13 ± 0.10‡	1.67 ± 0.76†
Au-2	3	11	1.13 ± 0.26	2.30 ± 1.28†	1.06 ± 0.07*	1.22 ± 0.28*
Au-4	1	2	2.2; 2.5	2.5; 2.6	1.2; 1.2	1.2; 1.6
Au-5	1	2	1.6; 1.7	1.6; 1.6	1.3; 1.3	1.4; 1.4
PDGF-AA (50 μg/l)						
Au-1	1	2	0.57; 0.88	0.81; 1.1	1.1; 1.1	1.4; 1.6
Au-2	1	2	0.68; 0.83	1.0; 1.3	1.2; 1.2	1.4; 1.8
PDGF-BB (50 μg/l)						
Au-1	2	5	0.66 ± 0.38	0.78 ± 0.44	1.39 ± 0.15‡	2.02 ± 0.27‡
Au-2	2	5	0.44 ± 0.05‡	0.52 ± 0.15‡	1.36 ± 0.08‡	2.25 ± 0.24‡
aFGF (50 μg/l)						
Au-1	1	2	0.43; 0.45	0.63; 0.69	1.1; 1.2	1.5; 1.7
Au-2	1	2	0.75; 0.81	1.0; 1.2	1.0; 1.1	1.1; 1.1
bFGF (100 μg/l)						
Au-1	1	3	1.36 ± 0.11†	1.28 ± 0.21	0.99 ± 0.12	0.95 ± 0.13
Au-2	1	2	0.84; 0.95	0.95; 1.0	1.0; 1.1	1.1; 1.2
EGF (10 μg/l)						
Au-1	2	5	1.09 ± 0.22	1.11 ± 0.25	1.07 ± 0.05	1.13 ± 0.15
Au-2	2	5	0.90 ± 0.22	0.91 ± 0.15	1.13 ± 0.03‡	1.11 ± 0.19
Heparin (10^5 IU/l)						
Au-1	3	7	1.12 ± 0.26	0.56 ± 0.05‡	1.03 ± 0.05	1.05 ± 0.16
Au-2	3	7	1.40 ± 0.27†	1.15 ± 0.11†	0.95 ± 0.06	0.94 ± 0.10
Media supplemented with 15% FCS						
ANG II (500 nmol/l)						
Au-1	1	2	0.92; 1.0	0.90; 1.0	1.0; 1.0	1.1; 1.1
Au-2	1	2	1.0; 1.1	1.2; 1.3	0.76; 0.79	0.78; 0.83
Serotonin (10 μmol/l)						
Au-1	1	2	0.72; 0.94	0.81; 1.0	1.0; 1.0	1.1; 1.2
Au-2	1	2	0.94; 1.0	0.93; 1.1	1.1; 1.1	1.0; 1.0
Histamine (10 μmol/l)						
Au-1	1	2	1.0; 1.0	1.1; 1.2	0.92; 0.95	0.93; 1.0
Au-2	1	2	0.83; 1.0	1.1; 1.3	1.0; 1.1	1.1; 1.2
Vasopressin (100 nmol/l)						
Au-1	1	2	1.0; 1.0	1.0; 1.0	1.0; 1.0	1.0; 1.1
Au-2	1	2	1.2; 1.4	1.1; 1.3	0.95; 1.0	0.87; 0.95
Oxytocin (1 nmol/l)						
Au-1	1	2	0.82; 1.0	1.0; 1.1	0.93; 1.0	1.0; 1.1
Au-2	1	2	1.2; 1.4	1.4; 1.8	0.93; 1.0	0.92; 0.93

Continued

Table 1.—*Continued*

Cell Line	No. of Experiments	No. of Wells	EC-SOD		DNA	Protein
			In media	In cells		
			Media supplemented with 15% FCS—Continued			
Endothelin-1 (1 μmol/l)						
Au-1	1	2	0.88; 1.1	1.0; 1.3	0.93; 0.93	1.1; 1.1
Au-2	1	2	1.1; 1.3	1.2; 1.4	1.0; 1.1	1.0; 1.2
PDGF-AA (10 μg/l)						
Au-1	2	4	0.60 ± 0.14†	0.54 ± 0.09‡	0.99 ± 0.06	1.07 ± 0.15
Au-2	2	4	0.67 ± 0.15*	0.80 ± 0.14*	1.39 ± 0.24*	1.31 ± 0.19*
PDGF-AA (50 μg/l)						
Au-4	1	2	0.48; 0.54	0.52; 0.60	1.1; 1.2	1.2; 1.2
PDGF-BB (100 μg/l)						
Au-1	2	4	0.30 ± 0.06‡	0.25 ± 0.05‡	1.00 ± 0.06	0.88 ± 0.06
Au-2	2	4	0.31 ± 0.11‡	0.38 ± 0.12‡	1.52 ± 0.07‡	1.73 ± 0.73
PDGF-BB (50 μg/l)						
Au-4	1	2	0.31; 0.36	0.43; 0.61	1.0; 1.1	0.83; 0.85
Au-5	1	2	0.28; 0.29	0.42; 0.42	1.2; 1.3	1.2; 1.3
aFGF (50 μg/l)						
Au-1	2	4	0.30 ± 0.04‡	0.33 ± 0.03‡	1.15 ± 0.15	1.18 ± 0.11*
Au-2	2	4	0.38 ± 0.09†	0.44 ± 0.10‡	1.31 ± 0.07‡	1.32 ± 0.13*
Au-4	1	2	0.47; 0.49	0.47; 0.54	1.2; 1.2	1.0; 1.1
bFGF (100 μg/l)						
Au-1	3	6	0.48 ± 0.10‡	0.41 ± 0.11‡	1.08 ± 0.05	1.06 ± 0.28
Au-2	3	8	0.88 ± 0.17	0.94 ± 0.28	1.10 ± 0.25	0.98 ± 0.15
Au-4	1	2	0.70; 0.71	0.75; 0.90	1.1; 1.1	0.88; 0.92
Au-5	1	2	0.64; 0.71	0.75; 0.77	1.0; 1.1	1.0; 1.1
EGF (10 μg/l)						
Au-1	4	10	0.54 ± 0.33‡	0.50 ± 0.35‡	1.11 ± 0.11*	0.96 ± 0.10
Au-2	5	11	0.61 ± 0.16‡	0.59 ± 0.14‡	1.10 ± 0.08†	1.09 ± 0.13
Au-4	1	2	0.43; 0.47	0.59; 0.65	0.90; 0.93	0.76; 0.76
Au-5	1	2	0.36; 0.37	0.45; 0.50	1.1; 1.1	1.0; 1.1
Heparin (10^5 IU/l)						
Au-1	3	7	2.73 ± 0.35‡	1.27 ± 0.34	0.83 ± 0.07‡	0.75 ± 0.10‡
Au-2	4	9	2.08 ± 0.46‡	1.36 ± 0.78	0.79 ± 0.07‡	0.75 ± 0.10‡
Au-4	1	2	2.3; 2.6	1.5; 1.7	0.60; 0.61	0.65; 0.67
Au-5	1	2	2.1; 2.2	1.4; 1.4	0.78; 0.81	0.70; 0.79

Values are means ± SD, and *t*-test significance levels were calculated on the relative values and are presented when the number of wells is ≥3. If the number of wells = 2, the 2 relative values are presented. The extracellular superoxide dismutase (EC-SOD) contents of culture media and cell homogenates were determined by ELISA as detailed in MATERIALS AND METHODS (see also Fig. 1). The levels of EC-SOD content are shown from 4-day culture media and from cell homogenates relative to levels in control wells and corrected for cell protein. The protein and DNA in the cell homogenates relative to levels in control wells are also presented. The concentrations of the active factors presented are those that yielded close to maximal effects on EC-SOD synthesis. ANG II, angiotensin II; PDGF-AA and -BB, platelet-derived growth factors-AA and -BB, respectively; a- and bFGF, acidic and basic fibroblast growth factors, respectively; EGF, epidermal growth factor. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

Addition of 1 μmol/l ritanserin, a selective 5-HT-2 receptor antagonist, completely abolished the response to 10 μmol/l serotonin in the Au-2 cell line, indicating a 5-HT-2 receptor-mediated serotonin response. Cells also responded to 10 μmol/l *dl*-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride, a selective 5-HT-2 receptor agonist, similarly to serotonin (Table 2).

To evaluate the possible involvement of Ca^{2+} in EC-SOD regulation, the response to the Ca^{2+} ionophor A-23187 was studied on the Au-1 and Au-2 cell lines. The cells responded to 0.1 μmol/l A-23187 with an increase in the EC-SOD levels to ~1.7 compared with controls, whereas the general protein synthesis was not affected. At the concentration of 1 μmol/l, the EC-SOD levels were more than halved, whereas the protein levels remained unchanged (Fig. 1*C*).

Responses to glycosaminoglycans. In cell cultures supplemented with 15% FCS, the level of EC-SOD in culture media relative to controls was approximately doubled when cells were exposed to 10^5 IU/l heparin for 4 days (Table 1 and Fig. 1*D*). Cell protein levels and DNA levels were slightly decreased at 10^5 IU/l heparin and higher concentrations tested (Table 1 and Fig. 1*D*). In cell cultures supplemented with 1% BSA, responses were less pronounced on culture media levels of EC-SOD. Because heparin and heparan sulfate may cause release of EC-SOD from glycosaminoglycans on the cell surfaces and matrix of SMC cultures, a heparin wash step was introduced in the preparation of the cell homogenates to distinguish between EC-SOD bound to the glycosaminoglycans on the cell surfaces and intracellular EC-SOD (see MATERIALS AND METHODS). As expected, EC-SOD levels in the heparin wash solutions were reduced in cultures exposed to heparin for 4 days compared with controls in both cultures supplemented with 15% FCS and 1% BSA (data not shown).

Northern blot analysis of cells exposed to heparin for 4 days in the presence of 15% FCS showed an increase in EC-SOD mRNA relative to GAPDH mRNA (Fig. 2*A*).

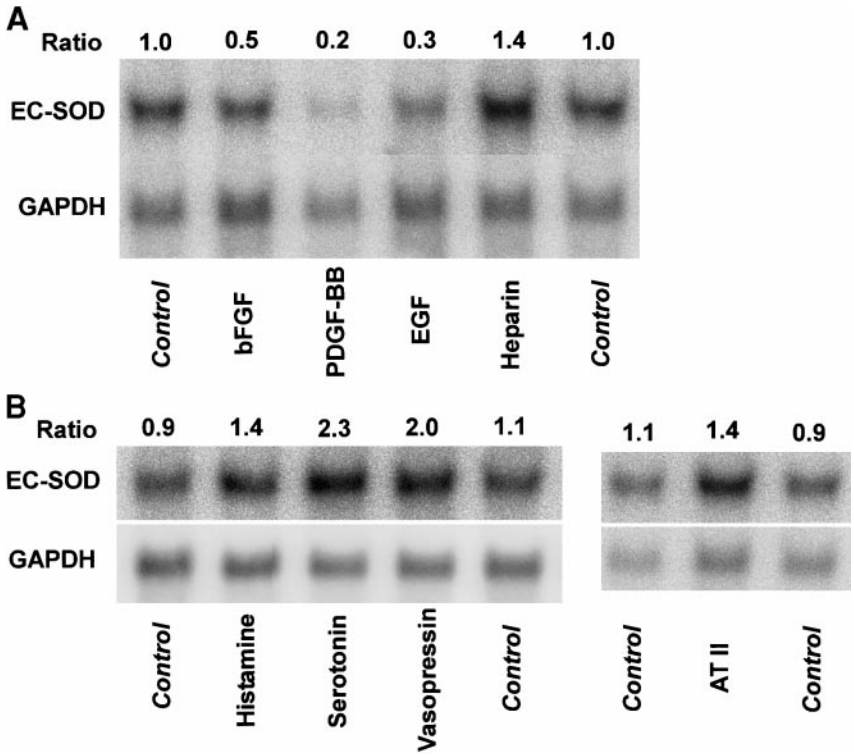

Fig. 2. Effects of growth and vasoactive factors on EC-SOD mRNA levels. Smooth muscle cells were cultured in media supplemented with 15% FCS (*A*) and 1% BSA (*B*) and were exposed to active factors for 4 days. The Au-1 cell line was used for all experiments, with the exception of the ANG II study, where the Au-2 cell line was employed. RNA was extracted, and mRNA levels were determined as described in MATERIALS AND METHODS. The following factors were used: basic fibroblast growth factor (bFGF; 30 μg/l), PDGF-BB (30 μg/l), epidermal growth factor (EGF; 30 μg/l), heparin (10^5 IU/l), histamine (10 μmol/l), serotonin (10 μmol/l), vasopressin (100 nmol/l), and ANG II (ATII; 10 μmol/l). The EC-SOD-to-glyceraldehype-3-phosphate dehydrogenase (GAPDH) ratios relative to the mean of the controls are indicated.

The response to low-molecular-weight heparin, Fragmin (10–10^6 IU/l), was very similar to that of heparin (data not shown). Another set of glycosaminoglycans, including heparan sulfate (0.01–1,000 mg/l), chondroitin sulfate A, B, and C (each 200 mg/l), and hyaluronic acid (200 mg/l), was also tested. Of these, only heparan sulfate showed an effect similar to that of heparin on EC-SOD synthesis (data not shown).

Turnover of EC-SOD in cell cultures. When medium conditioned to contain EC-SOD was added to cultured cells not expressing the enzyme, uptake of the enzyme was found. Thus, with cell densities expressed as protein per well, DU-145 (285 μg protein/well), HEP-G2 (140 μg protein/well), MG-251 (135 μg protein/well), and PL-3 (50 μg protein/well) internalized 33%, 24%, 21%, and 18%, respectively, of the EC-SOD in the medium over 24 h.

Table 2. *Histamine- and serotonin-related agonists and antagonists effects on the Au-2 cell line*

	EC-SOD			
	In media	In cells	DNA	Protein
Histamine	2.44	2.14	1.17	1.42
Diphenhydramin	1.03	1.02	1.04	1.04
Cimetidin	0.98	1.24	1.06	1.16
Histamine + diphenhydramin	1.34	1.22	1.14	1.13
Histamine + cimetidin	3.02	1.95	1.06	1.24
Dimaprit	0.81	0.97	1.11	1.23
Serotonin	2.43	2.53	1.06	1.16
Ritanserin	0.96	1.06	1.02	0.94
Serotonin + ritanserin	0.78	1.25	1.07	1.29
DOI	2.04	1.61	1.08	1.27

Data presented are the means of results from 2 wells. The EC-SOD contents of culture media and cell homogenates were determined by ELISA as detailed in MATERIALS AND METHODS. Culture media were supplemented with 1% BSA. For ritanserin, a concentration of 1 μmol/l was used; for all other drugs, a concentration of 10 μmol/l was used. Levels of EC-SOD content are shown from 4-day culture media and from cell homogenates relative to controls and corrected for protein. Protein and DNA levels from cell homogenates relative to controls are also shown.

DISCUSSION

It has been previously found that EC-SOD expression is regulated by cytokines such as interferon-γ, tumor necrosis factor-α, interleukin-4 and transforming growth factor-β in SMCs (35) and fibroblasts (25). In this study, we showed an extensive regulation of SMC EC-SOD synthesis by vascular growth factors, vasoactive factors, and heparin and other glycosaminoglycans.

The effects of the various factors on EC-SOD and general protein and DNA synthesis naturally patterned into three groups. The first group included classic growth factors: aFGF, bFGF, PDGF-AA, PDGF-BB, and EGF. The response to these factors was a decrease in EC-SOD levels relative to general cell protein levels. The effects were more pronounced if culture media were supplemented by 15% FCS. All these factors act via tyrosine kinase-coupled receptors (28). Specific receptors for PDGF, FGF, and EGF have been shown on vascular SMCs (28). PDGF-A and -B gene expression is increased in SMCs and macrophages of atherosclerotic lesions (28). Likewise aFGF and bFGF are expressed in the atherosclerotic setting (22). Even though there are no reports on EGF expression in atherosclerotic lesions, production of the related peptide heparin-binding EGF-like growth factor, which acts on the EGF receptor, has been found in

macrophages in atherosclerotic lesions (32). The growth factors have been considered proatherogenic mainly by way of inducing SMC chemotaxis and hyperplasia (28). PDGF-BB also induces superoxide production and secretion in aortic SMCs (26). The downregulation of EC-SOD synthesis by these factors may contribute to their proatherogenic character.

The second group contained a set of vasoactive factors: histamine, vasopressin, oxytocin, ANG II, ET-1, and serotonin. The response to this group was an increase in EC-SOD levels relative to total cell protein as well as an increase in total cell protein levels. Effects on DNA were smaller. This response was only seen when culture media were supplemented with 1% BSA but not with 15% FCS. For ANG II, a heterogeneity was seen on effects on different cell lines. The heterogeneity could have a genetic basis or represent different samples from generally heterogeneous SMC populations in the vessel wall (5). The factors of this group act on SMCs via various G protein-coupled receptors. The H1 receptor indicated in our study to mediate the EC-SOD response to histamine has been previously identified in large vessels and is known to mediate SMC contraction (7). The 5-HT-2 receptor indicated in our study to mediate the serotonin response has been previously identified in the human uterine artery (14). Signs of increased mast cell degranulation have been noted in fatty streaks (18), suggesting that histamine may occur in significant amounts in atherosclerotic lesions. Atherosclerotic vessels have been found to respond with augmented contraction to serotonin (36). Vasopressin receptors of the V1a type (2) and oxytocin receptors (40) have been found on human vascular SMCs. Vasopressin has been shown to stimulate rat vascular SMC hypertrophy (37). ANG II has recently been found to upregulate EC-SOD expression in the blood vessel wall in mice and in cultured human aortic SMCs (6). In the present study, we confirmed this finding in human uterine artery SMCs. ANG II also induces superoxide production from SMCs (8). This mechanism has been shown to contribute to the development of hypertension after ANG II infusion in rats (21). Both the expression of and effects of ET-1 in atherosclerotic plaque may be enhanced (19). The upregulation of EC-SOD may have an important function in counterbalancing an increased superoxide production stimulated by ET-1 (4) and ANG II.

A common theme of the signal transduction of the vasoactive factors is an intracellular increase in Ca^{2+}, resulting in a contractile response in SMCs (2, 12, 14, 40). The stimulating effect on EC-SOD synthesis of an intermediate concentration of A-23187 (Fig. 1*C*) suggests that the release of Ca^{2+} may be involved in mediating this effect of the factors.

The third group included heparin and the closely related proteoglycan heparan sulfate. There was no response to chondroitin sulfates or hyaluronic acid. The characteristic response was an increase in EC-SOD expression and a decrease in the amount of total protein in SMC cultures in media supplemented with 15% FCS. EC-SOD has a high affinity for heparin and heparan sulfate and binds in vivo to the latter compound, which exists in the interstitial matrix and on cell surfaces in the tissues (16). The addition of heparin or heparan sulfates to the media in our experiments led to release of EC-SOD bound to the cell layers because of competition for binding. The loss of EC-SOD from the cell surfaces is probably not involved in the mechanism of the observed heparin effects because this loss occurred in cultures supplemented with both 15% FCS or 1% BSA, whereas upregulation of EC-SOD was only seen in the presence of 15% FCS. Also, the addition of Cu/Zn-SOD (3–300 mg/l) to the cultures did not downregulate EC-SOD expression (data not shown). Heparin and heparan sulfates have been reported to exert antiproliferative effects on vascular SMCs (33). Specific heparin receptors, responding to heparin and heparan sulfates, have been characterized on SMCs, possibly coupled to activation of protein kinase C (31). It is interesting to note that the coronary artery concentration of heparan sulfate tends to decrease with age and particularly so in atherosclerotic lesions, whereas the content of chondroitin sulfate increases (41). These changes may tend to reduce EC-SOD synthesis and may also alter the distribution of EC-SOD in the arterial wall.

When medium conditioned to contain EC-SOD was added to cultured cell lines lacking EC-SOD expression, an uptake of ~25% of the enzyme over 24 h was found. We cannot formally exclude that some of the tested factors also influenced the EC-SOD turnover of the SMCs. However, the correspondence between the Northern blot analysis (Fig. 2) and the analysis of EC-SOD levels in the 4-day culture media (Table 1 and Fig. 1) suggest that the major part of the effects of the tested substances is caused by influences on the synthesis of the enzyme.

The responses of the SMC lines to the groups of factors differed markedly depending on the presence and absence of 15% FCS. From a study (13) of the expression of 8,600 genes in fibroblasts, it was concluded that serum induced a pattern expected in wound repair. It is possible that serum induces a more proliferative "response to injury" phenotype in SMCs, whereas in its absence the cells are modulated toward a more contractile phenotype. One might speculate that growth factors should be more efficient in reducing EC-SOD synthesis in intimal atherosclerotic lesions, as would the decreased amount of heparan sulfate. Conversely, vasoactive factors should be more efficient in inducing EC-SOD synthesis in normal SMCs in the media.

The present findings together with the previous demonstration of EC-SOD synthesis regulation by inflammatory cytokines (35) suggest that EC-SOD content and protection against superoxide radicals in the vascular wall may be markedly altered in various pathological situations. The regulation is complex, suggesting the possibility of different responses in EC-SOD synthesis and distribution in different stages of atherosclerotic lesions and other vascular diseases. Indeed, reduced levels of EC-SOD have been measured in

advanced human connective tissue-rich atherosclerotic lesions, whereas the levels were increased in early cell-rich lesions in the rabbit (23).

The skillful technical assistance of E. Bern, K. Hjertkvist, L. Nilsson, A. Öberg, and E. M. Öhman is gratefully acknowledged. We thank Peter Nilsson for valuable advice on the Northern blot experiments.

This study was supported by Swedish Medical Research Council Grant 12566.

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