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Upregulation of Lysyl Oxidase in Vascular Smooth Muscle Cells by cAMP: Role for Adenosine Receptor Activation

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Abstract Lysyl oxidase (LO) is a key participant in the accumulation of insoluble fibers of elastin and collagen by virtue of its role in the initiation of the covalent cross-linkages between and within individual molecules comprising these fibers. In view of the essential role played by LO in the accumulation of the fibrotic components of occlusive arterial lesions in atherosclerosis, identification of the signaling molecules which can affect the expression of the LO gene in vascular smooth muscle is of considerable interest. In the present report, we describe evidence for the role of the second messenger, cAMP, in the modulation of the levels of LO in vascular smooth muscle cells. Elevated intracellular cAMP induces the transcription of the LO gene, as revealed by Northern blot analysis and nuclear run on assays. Transient transfection experiments performed with the wild-type LO promoter and with this promoter mutated at a consensus CREB site, located within the region −100 to −93 base pairs relative to the start of transcription, indicate that cAMP-induced transcriptional activation is partially due to the presence of this CREB site within the promoter. Activation of stimulatory adenosine receptors in vascular smooth muscle cells with 5′-N-ethylcarboxamido adenosine (NECA) increases cAMP, LO mRNA, and enzyme activity. These findings point to the importance of cAMP signaling, potentially initiated by a variety of physiological agents, in the upregulation of LO expression in vascular smooth muscle cells. J. Cell. Biochem. 75:177–185, 1999.

Key words: lysyl oxidase; cAMP; vascular smooth muscle cells

The vascular smooth muscle cell (VSMC) is actively involved in the development of neointimal lesions in atherosclerosis. These cells normally reside within the interlamellar regions of the vascular media, where they exhibit the contractile phenotype, modulating fluctuations in blood pressure. There is evidence that these cells migrate to the subendothelial intimal space in response to atherogenic stimuli, whereupon they adopt the synthetic phenotype, synthesizing, secreting, and depositing abundant quantities of the fibrous, extracellular matrix proteins, elastin and collagen. The continuing accretion of matrix, as well as of cellular and lipid deposits, in these lesions can ultimately lead to the occlusion of the affected arterial lumen with resulting infarct of the downstream tissue [Ross, 1993]

Lysyl oxidase (LO) (E.C. 1.4.3.13) is a key participant in the accumulation of insoluble fibers of elastin and collagen by virtue of its role in the initiation of the covalent cross-linkages between and within individual molecules comprising these fibers. This enzyme oxidizes specific lysine residues within these matrix macromolecules to peptidyl-α-aminoadipic-δ-semialdehyde. Subsequent spontaneous condensations of the aldehyde residues produce the cross-linkages that account for the insolubility of these fibrous proteins [Smith-Mungo and Kagan, 1998]. The expression of the LO gene is regulated at transcriptional and/or post-transcriptional levels by a number of agents or conditions, or both, relevant to the normal and/or diseased arterial wall. Among these, enzyme expression is significantly elevated by TGF-β1 [Gacheru et al., 1997; Roy et al., 1996], interleukin-1β (IL-1β) [Gacheru et al., 1997], PDGF [Green et al., 1995], hypoxia [Brody et

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al., 1979], and nutritional [Gacheru et al., 1997] and mechanical [Ando et al., 1996] stress, while prostaglandin E_2 (PGE_2)has been shown to block the up-regulation by TGF-β1 [Roy et al., 1996]. Cloning and sequence analysis of the human, rat, and mouse LO promoters reveal a significant degree of homology within the three species and a number of conserved elements representing putative binding sites for various transcription factors, including Sp1, Ap2, TFII/R, and the glucocorticoid receptor [Smith-Mungo and Kagan, 1998]. A functional consensus sequence for the binding of interferon regulatory factors (IRF-1 and IRF-2) was demonstrated in the mouse LO promoter [Tan et al., 1996]. Moreover, an additional element in the mouse promoter displays a significant degree of homology to a conserved sequence in the α1 (I) procollagen promoter although specific nuclear binding factors have not yet been identified [Jourdan-Le Saux et al., 1997].

In view of the essential role played by LO in the accumulation of the fibrotic components of occlusive arterial lesions in atherosclerosis, knowledge of the signaling molecules that can affect the expression of the LO gene is of considerable relevance to the understanding of this process. In the present report, we have assessed the role of the second messenger, cyclic adenosine monophosphate (cAMP), in determining the levels of LO in VSMC. This is of particular interest since a variety of biological factors are known to modulate cAMP levels, including specific ligand binding to adrenergic receptors or adenosine receptors that modulate adenylyl cyclase activity [Belardinelli et al., 1989].

MATERIALS AND METHODS

Adult Rat Aorta Smooth Muscle Cell Cultures

VSMC were explanted from isolated medial layers of aortas of adult (6 months) Sprague-Dawley rats as described previously [McMahon et al., 1985]. Briefly, bacterial collagenase (10 mg; Sigma type I) and porcine pancreatic elastase (2.5 mg; Sigma type III) in 20 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) containing 3.7 g/L^{-1} sodium bicarbonate, penicillin (100 U/ml^{-1}) and streptomycin (100 $\mu g/ml^{-1}$) was added to minced medial layers of 4–6 aortas. Incubation was continued for 30–45 min at 37°C with stirring ; the resulting cell suspension was centrifuged at 400*g* for 5 min. The cell pellet was washed twice with DMEM supplemented with 20% fetal bovine serum (FBS) and then resuspended in 2–4 ml of fresh medium. The cells were then subcultivated into first passage after dispersion by incubation with 0.05% trypsin-0.02% EDTA (Gibco-BRL, Gaithersburg, MD) for 5 min at 37°C. The cells were subsequently seeded at 1.2×10^6 cells/100-mm dish and maintained with 10 ml of DMEM containing 10% FBS. The cultures were routinely monitored for smooth muscle cell morphology by phase contrast microscopy. Cell numbers were determined by counting with the aid of a hemocytometer.

Lysyl Oxidase Activity Assay

Cultures of VSMC were assayed for LO activity against a recombinant human tropoelastin substrate prepared and biosynthetically labeled with [4,5-^{3}H]-L-lysine (NEN, Boston, MA) as described [Bedell-Hogan et al., 1993]. LO enzyme activity was detected only at 10–16 days of incubation of the cells in culture. At shorter incubation times, when matrix has not been yet accumulated, the assay does not appear to be sufficiently sensitive to detect enzyme activity. Cells were cultured in DMEM supplemented with 10% FBS for 8 days or 14 days, then treated for 48 h with 10 μM forskolin or 10 μM NECA, respectively, before the determination of LO activity. In preparation for assays of enzyme activity, the medium was decanted from cell cultures, the cell layer rinsed in 16 mM potassium phosphate, 150 mM NaCl, pH 7.8, and then extracted in 4 M urea, 16 mM potassium phosphate, pH 7.8, with a Dounce homogenizer. Aliquots of the extracts (300 μl) or conditioned medium (600 μl) were incubated in triplicate for 2 h at 37°C against 125,000 cpm of the tritium-labeled tropoelastin substrate in assays brought to a final volume of 800 μl with 100 mM sodium borate and 150 mM NaCl, pH 8.0. Tritiated water released during the incubation was isolated by distillation in vacuo and quantified by liquid scintillation spectroscopy. All activities are presented as β-aminopropionitrile-inhibitable tritium release, using this irreversible inhibitor of LO to ensure specificity of the measured enzyme activities [Tang et al., 1983]. Enzyme activities were expressed per mg protein, determined by the BioRad assay [Zhao et al., 1997b].

DNA Synthesis

[^{3}H]Thymidine incorporation was used as a measure of DNA synthesis using a modification

of the technique described [Zhao et al., 1997a]. The VSMC were distributed into 96 well plates (2×10^4 cells/cm^2), cultured for 2 days and then subcultured into the various conditions defined in 200 µl of fresh medium containing 20 µCi ml^{-1} [H]thymidine (1 µCi/well). The cultures were then incubated for 4 h, washed with Puck's saline, the cell layers were treated with 0.05% trypsin in 0.02 % EDTA, and the cells were then hypotonically lysed with distilled water. DNA was collected directly onto Mash II glass fiber filters (grade 934AH, Bioproducts, Walkersville, MA) using a Mash II harvester (Bioproducts) and tritium incorporation was determined by liquid scintillation spectrometry. Cell numbers were determined as previously reported [Zhao et al., 1997b].

RNA Isolation and Hybridization

Total RNA was obtained from cultured VSMC by guanidinium isothiocynate extraction [Chomczynski and Sacchi, 1987]. The RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose filters, and then immobilized on the filters by ultraviolet (UV) irradiation. The filters were hybridized in Quikhyb buffer (Stratagene, La Jolla, CA) according to the manufacturer's protocol with a randomly primed, [^{32}P]-labeled LO rat cDNA probe corresponding to the 1.6-kb fragment released from plasmid pBSCOD with *Sal*I restriction nuclease, as described [Trackman et al., 1992]. The hybridized filters were washed twice in 0.325 M NaCl, 0.03 M sodium citrate, 0.1% sodium dodecyl sulfate (SDS), pH 7.0, buffer at room temperature, and then twice with 32.5 mM NaCl, 3.6 mM sodium citrate, 0.1% SDS, pH 7.0, at 50°C. The filters were exposed to Kodak XAR film at −80°C for 24 h. To correct for possible differences in loading of RNA samples to the gels, the filters were hybridized with a probe for 18 s mRNA, a transcript not expected to change significantly under the conditions of these experiments [Tso et al., 1985]. Films were developed and the image density of the bands was quantified using a Molecular Dynamics Image Quant gel scanner.

Nuclear Run-On Analysis

After incubation for 24 h in DMEM supplemented with 10% serum in the presence or absence of forskolin (2 µM), rat VSMC were lysed and nuclei isolated as described [Greenberg and Ziff, 1984]. Nuclei were suspended and incubated at 30°C for 20 min in reaction buffer (200-µl final volume) containing 10 mM Tris-HCl, pH 8.0, 5 mM magnesium chloride, 300 mM potassium chloride, 0.5 mM each of CTP, ATP, and GTP, and 150 µCi of [^{32}P] UTP (Dupont, NEN). Transcripts were prepared from the reaction mixtures, using the TriZol reagent according to the manufacturer's suggestion (Gibco-BRL). The RNA pellet was washed with cold 70% ethanol, resuspended in reaction buffer, and RNA re-precipitated by adding ammonium acetate to 2.5 M and 70% ethanol. The radioactive RNA pellets were isolated by centrifugation, washed with 70% ethanol, and finally resuspended at 6.5×10^6 cpm ml^{-1} in 10 ml of Quickhyb hybridization buffer (Stratagene, La Jolla, CA). Denatured cDNA probes (10 µg total) previously slot-blotted onto nitrocellulose filters were hybridized with equal quantities of radioactivity of the [^{32}P]-labeled RNA products at 50°C for 3 days. The filters were washed as described for Northern blot preparation and exposed to Kodak XAR film at −80°C. The collagen I type I cDNA and Ubiquitin cDNA were a gift of Dr. Gail Sonenshein (Boston University School of Medicine, Boston, MA).

Lysyl Oxidase Promoter Constructs

A human genomic lung fibroblast library (WI38 cell line-lambda Fix library; Stratagene, La Jolla, CA) was screened with an α-^{32}P-labeled random primed cDNA probe derived from the 5′-*Pst* fragment of human LO cDNA. One clone containing an 8-kb insert was digested with *Xho*I/*Sac*I to yield two fragments of 5450 and 2500 base pairs (bp), which were subcloned into the luciferase reporter vector PGL2-basic (Promega, Madison, WI). Sequencing showed that the 5450-bp clone contained 245 bp of the 5′-UTR of human LO, as well as 5205 bp 5′ of the putative start of transcription, as described previously [Hamalainen et al., 1991]. Neonatal rat VSMC transiently transfected with this PGL2-LO promoter construct exhibited significant luciferase activity as compared with cells transfected only with the PGL2-basic vector or the PGL2 vector containing the 2500-bp fragment [Gacheru et al., 1997]. The −639, −520, and −308 deletion constructs of the LO promoter that were created using convenient restriction sites also exhibited significant luciferase activity [Gacheru et al., 1997]. In the

current study, the plasmid carrying the mutation in the CREB site located within −100 to −93 bp upstream to the transcriptional start (from TTACGTGA to TTAATACC) was created by the hook-up polymerase chain reaction (PCR) [Ravid et al., 1991], as follows: a primer hybridizing approximately 100 bp upstream and another hybridizing 100 bp downstream to the CREB site were each used along with primers bearing the mutation in CREB. A second round of PCR with only the distal 5′ and 3′ primers yielded a mutated fragment. This was subcloned into the −520 construct by restriction digest replacement. The accuracy of the resulting mutant construct was confirmed by DNA sequencing. After incubation under the specified conditions, the cells were extracted and supernatants assayed for luciferase activity according to the commercially supplied protocol (Promega, Madison, WI). Luciferase activity was normalized for transfection efficiency, determined by assays for β-galactosidase activity deriving from a co-transfected β-galactosidase expression vector.

cAMP Measurement

Rat VSMC were cultured as described above in the absence or presence of 1 μM forskolin (Sigma, St. Louis, MO), or 10 μM 5′-N-ethylcarboxamido adenosine (Sigma), or 50 μM N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (Dib-cAMP; Sigma) for 24 h. Intracellular cAMP concentration was then determined, using a radioimmunoassay (RIA) kit (DuPont), as described previously [Zhao et al., 1997b].

RESULTS

Effects of Elevation of Intracellular cAMP on Smooth Muscle Cell Proliferation

Adult VSMC were incubated under conditions known to modulate intracellular cAMP levels. Cells were cultured in the presence of forskolin, which directly activates adenylyl cyclase, or in the presence of the cell-permeable cAMP analogue, N^6,2′-O-Dibutyryladenosine 3′:5′-cyclic monophosphate (Dib-cAMP), and DNA synthesis and growth rates were determined. As shown in Figure 1A, forskolin increased intracellular levels of cAMP and forskolin and Dib-cAMP each decreased the growth rate by about 45% (Fig. 1B). Corresponding analyses of [^{3}H]thymidine uptake showed that closely similar degrees of change occurred in DNA synthesis in response to these effectors (Fig. 1C).

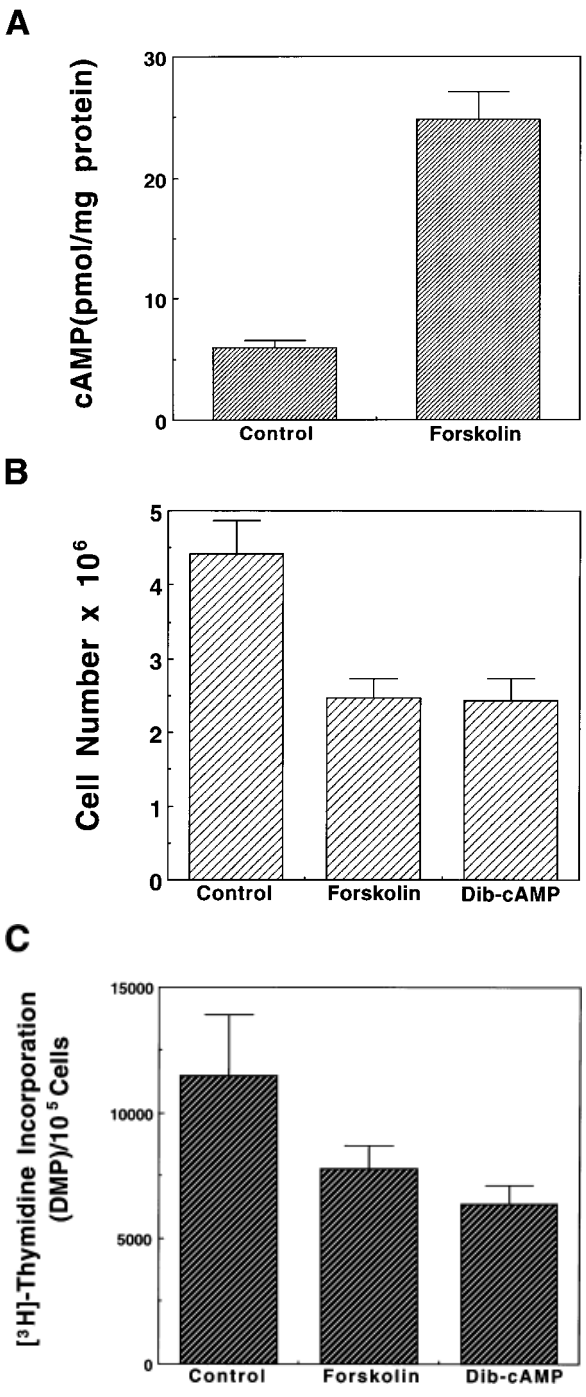

Fig. 1. cAMP levels and DNA synthesis is vascular smooth muscle cells. Rat vascular smooth muscle cells were cultured in the presence of 1 μM forskolin, or 50 μM N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (Dib-cAMP) for 24 h before cAMP determination **(A)**, cell number determination **(B)**, and measurement of DNA synthesis during a pulse of 3 h with [^{3}H] thymidine **(C)**, as described under Materials and Methods. The data shown represent averages of three experiments with standard deviations indicated by vertical bars.

Response of Steady-State mRNA and cAMP Levels of VSMC to Effectors

Since our previous studies indicated that the levels of LO mRNA are inversely related to cell proliferation [Gacheru et al., 1997], VSMC were incubated in the presence or absence of forskolin or Dib-cAMP and total mRNA was isolated from the cells and analyzed by Northern blotting. As shown (Fig. 2A), both forskolin and Dib-cAMP increased the steady-state levels of LO mRNA relative to the untreated control. Notably, the increases in the 4.8-kb and 5.6-kb LO mRNAs appeared to parallel the increases seen in cAMP in response to these two effectors. 18S Ribosomal mRNA was probed in the experiments of Figure 2A as a control transcript. Comparison of the densities of LO and 18 S RNA bands indicates that the observed effects of these agents on LO mRNA levels are not due to artifacts of sample loading. These results indicate that the effect of forskolin on LO mRNA reflects elevation in intracellular cAMP, and are supported by the observation that Dib-cAMP was able to mimic the forskolin effect.

Transcription Assays

The effect of the presence of forskolin in VSMC cultures on the rate of transcription of the LO gene relative to that of control cells was assessed by performing nuclear run-on analyses. As shown (Fig. 2B), exposure to forskolin strongly stimulates the transcription of LO. The empty vector (pUC19) control is appropriately negative, while the expression of ubiquitin gene, not expected to be changed under these conditions, was minimal at basal levels and not altered significantly by forskolin. Thus, these results further indicate that the stimulation of LO expression in response to cAMP levels involves control at the level of gene transcription. Interestingly, the rate of transcription of an additional matrix protein, collagen αI type I, was also increased in forskolin-treated cells. In the current study, however, we elected to focus on the mechanism of transcriptional upregulation of LO gene by cAMP. Examination of the sequence of the 5′-flanking region of the LO gene reveals the presence of a putative CREB element within the region −100 to −93 relative to the start of transcription. We note with interest that the increase in rate of transcription (approximately 8-fold) is greater than

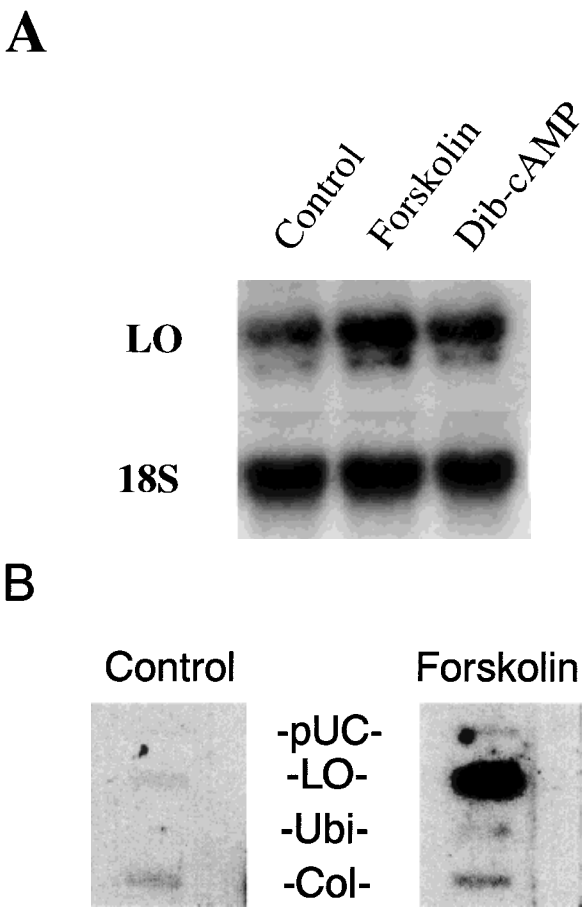

Fig. 2. Effects of elevated cAMP on lysyl oxidase (LO) gene expression. **A:** Rat vascular smooth muscle cells were cultured in the presence of 1 μM forskolin or 50 μM N^6,2′-O-Dibutyryladenosine 3′:5′-cyclic monophosphate (Dib-cAMP) for 24 h before RNA preparation and Northern blot analysis. The blots were probed with LO cDNA, yielding an expected doublet of mRNAs of approximately 4.8 kb and 5.6 kb, or with a probe recognizing the 18S ribosomal RNA, to confirm equal loading (15 μg RNA/lane). No other LO mRNA species were detected in the adult vascular smooth muscle cells. The data shown are representative of the results of three experiments. Similar data were obtained when cells were cultured with 2 μM forskolin for 48 h, indicating that the selected concentration and time of incubation yielded maximal changes in LO mRNA content (not shown). **B:** Rat vascular smooth muscle cells, cultured in the presence of 2 μM forskolin for 24 h, were also subjected to nuclear run-on analysis, as described under Materials and Methods. Rates of transcription of LO, ubiquitin (Ubi), collagen αI type I (Col) or, as a control, pUC19 (pUC) were followed.

that estimated by Northern blotting (2.5-fold) (Fig. 2A). The steady-state LO mRNA level reflects degradation of LO mRNA, which has been shown to have a half-life of 3 h in proliferating VSMC [Gacheru et al., 1997]. RNA synthesis in the nuclear run-on assay is followed for only 20 min, as described under Materials and

Methods, permitting the detection of LO mRNA accumulated in response to stimuli.

Effects of Forskolin on LO Promoter Activity

A 5450-bp fragment and restriction fragments derived therefrom of the 5′-region of the human LO gene have been shown to exhibit promoter function in neonatal VSMC, which is modulated by cytokines and nutrient conditions known to alter the expression of LO [Gacheru et al., 1997]. Luciferase-coupled fusion constructs were generated from the −5205, −639, and −520 promoter fragments, with numbering relative to the start site of transcription [Hamalainen et al., 1991]. These promoter fragments and putative sites for transcription factors within them were described in detail elsewhere [Gacheru et al., 1997; Smith-Mungo and Kagan, 1998]. In the current study, a mutant of the −520-bp construct was generated in which the putative CREB site (TTACGTGA) at −100 to −93 bases from the start of transcription in the LO promoter was mutated (to TTAATACC) to assess for the participation of the wild-type sequence in cAMP-dependent effects. As shown in Figure 3, forskolin increased luciferase expression/activity in adult VSMC to similar, prominent degrees (approximately 2.5-fold) in the wild-type −639 and −520 constructs. This forskolin-induced increase in promoter activity, as reflected by assaying for steady-state levels of luciferase activity 2 days after the addition of the stimuli, is lower than the one observed in the nuclear run-on assay (Fig. 2). This is expected because in the latter assay the newly formed LO mRNA is followed over a period 20 min, during which time the degradation of this RNA is limited. The agonist-induced stimulation of activity of the −520 mutant construct is significantly decreased, as compared with wild-type −520 construct, but not eliminated. This indicates that sites, other than the consensus CREB element, may play a role in mediating cAMP effect on LO promoter activity. Mutation of the CREB site in the −520-bp construct did not result in reduction of basal activity, suggesting that the intracellular cAMP concentration in the control (non stimulated) cells is not sufficient for activation of the CREB site. Although some stimulation is seen in the −5205 construct in response to forskolin (Fig. 3), these effects are smaller in magnitude than those seen in the shorter, wild-type constructs, raising the possibility that suppressor elements or secondary structures, or both, may occur in the longer construct that attenuate the responsiveness of downstream elements. Nevertheless, our data point to the conclusion that the putative CREB element in the LO promoter is functional and responsive to cAMP-dependent effects in these VSMC.

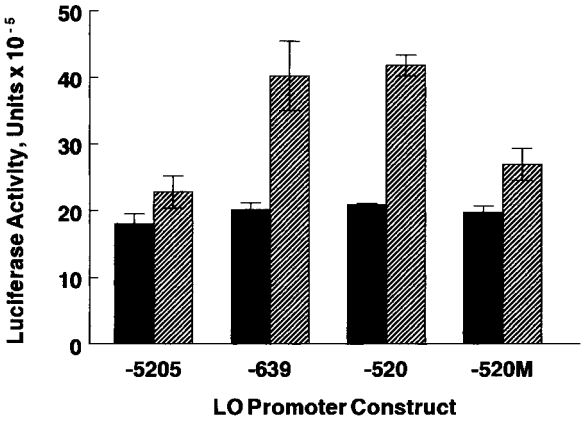

Fig. 3. Analysis of lysyl oxidase (LO) promoter activity in transfected adult rat vascular smooth muscle cells. Three different promoter constructs containing various LO promoter lengths (5205, 639, and 520 bases upstream to the transcriptional start) fused to a luciferase reporter gene were constructed. In addition, a putative CREB site within the −520-bp region of the LO promoter was mutated. After transfection into adult rat vascular smooth muscle cells, and a subsequent 48 h incubation with 1 M forskolin, cell extracts were prepared and assayed for luciferase reporter gene activity. The results were normalized against β-galactosidase activity from a co-transfected CMV-β-galactosidase vector. The data are presented as averages of three determinations with standard deviations indicated. Solid bars represent control cells treated with growth media (Dulbecco's modied Eagle's medium [DMEM] + 10% fetal bovine serum [FBS]); hatched bars represent forskolin-treated cells in identical media.

Effects of Forskolin on LO Enzymatic Activity

LO activity in VSMC was assayed against a [^{3}H]lysine-labeled recombinant human tropoelastin substrate, as described previously [Kagan et al., 1981]. As shown in Figure 4, forskolin significantly increased the level of LO activity primarily in cell extracts, with significant but lesser increases seen in the enzyme remaining soluble in the media.

Effects of 5′-N-ethylcarboxamido Adenosine on LO

It has been reported previously that rat VSMC possess A2-type adenosine receptors which activate adenylyl cyclase [Jonzon et al., 1985; Zhao et al., 1997b]. Interestingly, NECA,

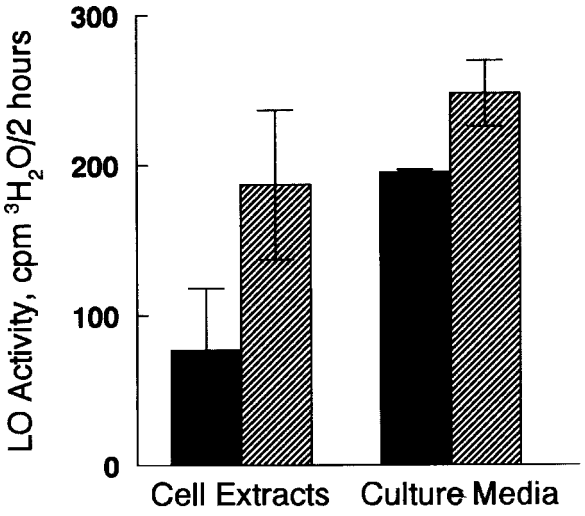

Fig. 4. Analysis of lysyl oxidase (LO) activity in cell extracts and medium from adult rat vascular smooth muscle cells. LO activity was determined in cell extracts or media, as described under Materials and Methods. Activities were normalized against the concentration of total protein in the cell extracts. Solid bars represent controls; hatched bars, cells treated with 1 μM forskolin.

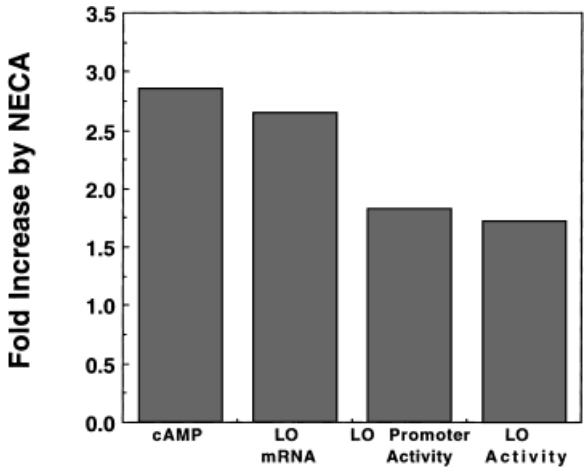

Fig. 5. Effects of 5′-N-ethylcarboxamido adenosine on LO expression in adult rat vascular smooth muscle cells. Adult rat vascular smooth muscle cells were cultured in the presence of 10 μM 5′-N-ethylcarboxamido adenosine (NECA), and intracellular cAMP concentrations were then determined, as well as Northern blot analysis of LO mRNA, LO promoter activity (luciferase activity driven by the −520-bp construct as in Fig. 3), and LO enzyme activity were determined and described in the legends to Figures 1 and 2 and under Materials and Methods. The intensity of the bands in the Northern blots was quantitated using a Molecular Dynamics Image Quant gel scanner. The data are averages of two experiments.

an adenosine analogue, which elevates cAMP levels in VSMC via binding to stimulatory adenosine receptors [Zhao et al., 1997b], also elevated LO mRNA, as well as LO promoter and enzyme activities (Fig. 5).

DISCUSSION

This article demonstrates that cAMP stimulates the expression of LO in adult VSMC. This effect is seen at the level of steady-state message, in the relative rates of transcription, in transfection assays of the response of the LO promoter, and at the level of enzyme activity.

The present evidence that the cAMP inhibits the proliferation of VSMC is consistent with findings of previous reports [Dubey et al., 1996]. It is of interest in this regard that the expression of LO is markedly elevated upon reduction of VSMC proliferation in culture either by withdrawal of serum or by the addition of TGF-β [Gacheru et al., 1997]. Whether overlapping or unique signal transduction pathways account for the apparently coordinated inverse relationship between LO gene expression and cell proliferation remains to be established. Clearly, however, the responses seen in the present study are accompanied (in the case of forskolin) and/or brought about (in the case of Dib-cAMP) by elevations in cAMP. In further support of the role of cAMP in LO regulation, evidence was also obtained for the presence of a functional CREB element in the LO promoter, as shown by the marked reduction of the forskolin-enhanced promoter activity by mutation of this element in the −520 LO promoter-luciferase reporter construct.

Adenosine and its analogues can inhibit PDGF-induced proliferation of vascular smooth muscle cells as well as collagen- and ADP-induced platelet aggregation [Ferrer et al., 1992; Haslem et al., 1987]. In view of these cardiovascular effects, apparently dependent on the activation of adenylyl cyclase, it has been proposed that adenosine and its receptors may participate in the attenuation of atherosclerosis [Ferrer et al., 1992]. In the cardiovascular system, aggregating platelets and endothelial cells can release adenine nucleotides, which can then be metabolized to adenosine in the extracellular space [Frick and Lowenstein, 1976; Itoh et al., 1986]. The actions of adenosine and its analogues are mediated through guanine-nucleotide (G) protein-coupled receptors subtypes of which, A1, A2a, A2b, and A3, have been identified. It is of particular relevance to the present report that both the A2b adenylyl cyclase stimulatory receptor [Dubey et al., 1996] and the A3

adenylyl cyclase-inhibitory adenosine receptor [Zhao et al., 1997b] have recently been identified in rat aorta smooth muscle cells. As seen in the current study, activation of the A2-type receptors by an adenosine analogue elevates cAMP and, in parallel, increases LO mRNA, LO promoter activity and enzyme activity.

Although the effects of cAMP on LO gene expression have not been previously recorded, cAMP-dependent regulation of extracellular matrix gene expression, including lamin A, B1, and B2 chains, as well as collagen types I, III, and IV, have been reported in Schwann cells and in glomerula mesanglial cells [Yamamoto et al., 1994; Ziyadeh et al., 1995]. Fibronectin gene expression in lung fibroblasts, NIH 3T3 cells, and other cell types has also been found to be upregulated by cAMP [Dean et al., 1989; Kreisberg and Kreisberg, 1995; Lee et al., 1997]. In the current study, we report the importance of cAMP-dependent signaling for activation of LO gene expression in vascular smooth muscle cells. In view of the essential role played by LO in the accumulation of the fibrotic components of occlusive arterial lesions in athersoclerosis, these results contribute to the more complete understanding of vascular mechanisms of regulation of this enzyme which might be exploited in efforts to modulate the fibrotic component of arterial disease.

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