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PKC-MEK-MAPK-Dependent Signal Transduction Pathway Mediates the Stimulation of Lysyl Oxidase Expression by Serum and PDGF in Rat Aortic Smooth Muscle Cells

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Abstract Lysyl oxidase (LO) plays a critical role in the stabilization and insolubilization of fibrous structural proteins of the extracellular matrix and has been implicated in the suppression of Ras-induced tumorigenesis. Several prior reports demonstrate that the expression of this catalyst is strongly influenced by a variety of effectors of cell function and is responsive to the growth state of fibrogenic cells. Using specific inhibitors of components of signal transduction pathways, the present study reveals that a PKC-MEK-MAPK-dependent pathway is critical to the enhanced expression of the LO gene in response to variations in the levels of the serum component of the growth medium and in response to platelet-derived growth factor (PDGF). PDGF is shown to be the major component of fetal bovine serum, which stimulates the activity of a LO promoter construct. J. Cell. Biochem. 85: 775–784, 2002. © 2002 Wiley-Liss, Inc.

Key words: lysyl oxidase; smooth muscle cells; signal transduction; extracellular matrix

Lysyl oxidase (LO) (EC 1.4.3.13) is an enzyme that plays a key role in the formation of connective tissue matrices by catalyzing the post-translational oxidative deamination of peptidyl lysine residues within its elastin and collagen substrates. The peptidyl α-aminoadipic-δ-semialdehyde residues generated in this fashion undergo spontaneous condensation with nearby aldehydes and/or epsilon amino groups to form covalent crosslinks, which convert soluble forms of collagen and elastin to insoluble, fibrous networks [Kagan and Trackman, 1991; Smith-Mungo and Kagan, 1998]. Thus LO, which is expressed and secreted by fibrogenic cells, including vascular smooth muscle cells (VSMC) and fibroblasts, is essential to the development and repair of the cardiovascular, respiratory, skeletal, and other systems of the body. Changes in the levels of expression of LO are associated with a variety of fibrotic and genetic diseases. For example, levels of LO activity were significantly increased within aortic lesions in an animal model of atherosclerosis over those in healthy aortic tissue [Kagan et al., 1981].

Other investigations have revealed that LO may have additional and quite varied effects in a variety of cell types. It has been demonstrated that LO acts as a potent chemotactic agent for peripheral blood mononuclear cells [Lazarus et al., 1995], as well as for VSMC [Li et al., 2000]. This process requires enzyme activity, and can be attributed to the production of hydrogen peroxide stoichiometrically accompanying the enzymatic oxidation of lysine residues [Li et al., 2000]. Of further interest, LO appears to act as a repressor of the oncogenic activity of certain Ras species [Kenyon et al., 1991], while LO expression is downregulated in various malignantly-transformed human cells and is one of the primary genes induced in the reversion of these cells back to the nontransformed phenotype [Kuivaniemi et al., 1986; Hamalainen et al., 1995; Contente et al., 1999]. The expres-

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sion of LO in Ras-transformed 3T3 fibroblasts correlates with the decondensed state of chromatin and with the nontumorigenic states of the cells [Mello et al., 1995; Contente et al., 1999].

The existence of additional substrates and functions of LO are made more plausible by observations that the enzyme, normally secreted from fibrogenic cells, is also localized intracellularly, and specifically, within nuclei [Di Donato et al., 1997; Li et al., 1997]. Experiments with recombinant LO have furthered our understanding of possible intracellular roles of LO. Injection of recombinant LO into *Xenopus laevis* oocytes antagonized maturation of the oocytes induced by p21-Ha-Ras or by progesterone, thus implicating an inhibitory role for LO at a specific step of a Ras-induced process [Di Donato et al., 1997]. It has also been reported that intracellular LO activates transcription of collagen type III by enhancement of the binding of Ku antigen to an element within the collagen promoter. Ku antigen participates in the control of DNA repair processes, immunoglobulin recombination, and suppression of transformation [Giampuzzi et al., 2000]. Thus, the modulation of its activity by LO enhances the potential roles of LO in the modification of cell phenotypes.

LO expression is regulated in VSMC both transcriptionally and post-transcriptionally by specific cytokines, growth factors, and conditions that can affect the normal or diseased arterial wall. Transforming growth factor β-1 (TGF-β1) increases LO expression in rat VSMC primarily by stabilizing LO mRNA as this growth factor inhibits the proliferation of these cells [Gacheru et al., 1997]. The elevation of transcription of the LO gene by activation of adenosine receptors in VSMC is mediated by increased levels of endogenous cAMP levels in these cells [Ravid et al., 1999]. Elevation of cAMP has been related to the growth arrest and maintenance of the contractile phenotype of VSMC [Feng et al., 1996]. In contrast to the apparent correlation between the upregulation of LO expression by these effectors, which reduce VSMC proliferation, platelet-derived growth factor (PDGF), an activator of smooth muscle cell (SMC) proliferation and migration [Bornfeldt et al., 1994; Graf et al., 1997; Nelson et al., 1998; Pukac et al., 1998], induces LO mRNA in quiescent human adult VSMC as do angiotensin II and 10% serum [Green et al., 1995]. Although, these and other regulatory effects on LO expression have been documented, little is known about the intracellular signal transduction pathways mediating these effects. In the present study, we sought to identify specific intracellular signal transducing molecules that modulate LO expression in SMCs. We initially assessed for the participation of protein kinase C (PKC), as this is a key protein kinase known to be involved in mediating many cellular processes such as proliferation, differentiation, tumorigenesis, and apoptosis [Gomez et al., 1999; Black, 2000; Musashi et al., 2000]. The present study provides the first evidence of which we are aware that LO expression is regulated by a PKC-dependent-mitogen-activated protein kinase (MAPK) pathway in VSMC, a pathway, which may be activated by PDGF.

MATERIALS AND METHODS

Neonatal Rat Aortic SMC Cultures

Neonatal rat aorta SMCs (NRASMC) were explanted from isolated medial layers of aortas from neonatal (2–3-day-old) Sprague–Dawley rats as previously described [Gacheru et al., 1997]. Approximately 20 minced aortas were digested with bacterial collagenase and porcine pancreatic elastase for 30 min at 37°C with continual stirring. The resulting cell suspension was centrifuged at 400*g* for 5 min, and the pellet washed twice with Dulbecco's Minimal Essential Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). Following initial plating, the primary cultures were subcultivated into first passage following dispersion by incubation with 0.05% trypsin–0.02% EDTA for 4 min at 37°C. Confluent cultures in first passage were then seeded into tissue culture dishes/plates for various experimental procedures. Cells designated as second passage were seeded at 1.2×10^6 cells in 100-mm dishes for RNA isolation and Western blotting or at 1.2×10^5 in each well of six-well plates for transfection studies. Cell numbers were determined with the aid of a hemocytometer.

RNA Isolation and Hybridization

Adherent NRASMC cultures were washed twice in cold Puck's saline. Total RNA was obtained from cultures using the TriZol (GIBCO-BRL, Gaithersberg, MD) reagent according to the manufacturer's recommendation. Electrophoretic Northern analyses were

performed on 12 μg samples of RNA per lane of formaldehyde–agarose gels, the bands were transferred onto MagnaGraph nylon membranes (Osmonics, Inc., Minnetonka, MN), and then immobilized by UV crosslinking. Membranes were hybridized in Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's recommendation. A 1.6-kb rat LO cDNA fragment [Trackman et al., 1990, 1991] was labeled with ^{32}P by the random primer method and used as the hybridization probe to detect LO mRNA. Following detection of LO mRNA bands, the membranes were stripped and reprobed with a ^{32}P-labeled 1.5-kb EcoR1 fragment from plasmid HHCSA65 (ATCC), which hybridizes to 18S ribosomal RNA, using the densities of the 18S band to assess for any differences in loading of RNA samples to the gel. Membranes were autoradiographed at -80°C for various lengths of time and developed. The image densities of the developed films were determined by densitometry.

Transfection of LO Promoter Constructs

The preparation of a series of nested deletions of the LO promoter inserted upstream of the luciferase reporter gene within the pGL2-basic vector has been described previously [Gacheru et al., 1997]. SmaI/XbaI restriction digestion of the full-length promoter construct was used to isolate an additional construct containing 1,485 bases of LO promoter 5′ of the transcription start site. These constructs were transfected into NRASMC either by the calcium phosphate method or by using the LipofectaminePLUS reagent (GIBCO-BRL) under the conditions specified herein. Following experimental treatment, the cells were lysed with reporter lysis buffer and luciferase activity determined using commercially available substrate according to the supplier's protocol (Promega, Madison, WI) in an LKB Scintillation Counter. Luciferase activity was normalized for transfection efficiency by determining β-galactosidase activity derived from a co-transfected expression vector.

Analysis of MAPK Activity by Western Blot

NRASMC (1×10^6 cells) were incubated in 100-mm dishes in 10% FBS/DMEM overnight, mock transfected in 0.5% FBS/DMEM, and then incubated in the presence or absence of the specified signal transduction inhibitors for 30 min in DMEM in the absence of serum. The cells were then incubated in 10% FBS/DEMEM in the presence or absence of inhibitors as indicated. Cells were then washed twice with ice-cold Puck's saline and lysed in 500 μl of protein extraction buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 0.2% sodium dodecyl sulfate, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl flouride, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor) after 4 and 20 h of incubation of the cells. The extracts were incubated on ice for 15 min and centrifuged at 13,000 rpm at 4°C. Protein concentration was determined using the BCA Reagent (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol using bovine serum albumin as a standard. Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) using 12% cross-linked separating gels [Laemmli, 1970]. Prestained molecular weight markers (New England Biolabs, Beverly, MA) were included on all gels to estimate the apparent molecular weight of the proteins and to monitor protein transfer. In addition, Cruz markers and secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) were used to visualize markers directly on exposed films. Proteins were transferred electrophoretically to nitrocellulose membranes in transfer buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS, 20% methanol) at 100 mA overnight at 4°C. Membranes were then washed in Tris-buffered saline plus 0.1% Tween-20 (TBST) for 5 min, and then stained with Ponceau S to ensure equal protein loading of each sample. Examination of the Ponceau S stained membranes also supported the conclusion that protein degradation did not occur during the extraction and subsequent treatments described. All subsequent washes and incubations were performed at room temperature. Following removal of the staining reagent, the membrane was blocked in TBST containing 5% nonfat dry milk for 1 h. The primary antibody (anti-phosphophorylated MAPK) (New England Biolabs) was diluted 1:2,000 in TBST plus 5% milk and incubated with the membrane for 1 h. Following four 15 min washes in TBST, the horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotech) was diluted 1:2,000 in TBST/milk and incubated with the membrane for 1 h. Following four more 15 min

washes, the proteins were detected using the ECL Reagent (Amersham Pharmacia Biotech) and Kodak X-OMAT film.

RESULTS

Effect of Inhibition of PKC on Steady State Levels of LO mRNA Levels

The potential role of PKC in the regulation of LO expression in NRASMC was investigated. Proliferating NRASMC were incubated for 18 h in 10% FBS/DMEM containing various concentrations of calphostin C, an inhibitor of PKC [Kobayashi et al., 1989]. Total cellular RNA was isolated and subjected to Northern analysis using radiolabeled LO cDNA and 18S rRNA as probes. The effects on the levels of LO mRNA, determined by densitometry relative to densities of 18S RNA bands, are shown in Figure 1. The steady state levels of LO mRNA decreased with increasing levels of the inhibitor in a dose-dependent manner, with reductions varying from a maximum of 80% seen at 500 nM calphostin C to 25% at 62.5 nM calphostin C (Fig. 1; also see inset). There were no apparent cytoxic effects seen in cell cultures incubated with concentrations of calphostin C as high as 250 μM, although slight growth inhibition was observed in the presence of 500 μM calphostin C.

Effect of PKC Inhibitors on LO Promoter Activity

We next investigated whether the down-regulation of LO mRNA by inhibitors of PKC might be attributed to transcriptional or post-transcriptional mechanisms. Accordingly, NRASMC were transfected with a series of LO promoter constructs linked to a luciferase reporter gene. The cells were transfected for 18 h in 10% FBS/DMEM, and then incubated for 24 h with 250 nM calphostin C. Extracts from control cells transfected with −5205, −1740, −639, and −520 bp of LO promoter constructs exhibited significant luciferase activity (Fig. 2). Extracts from cells transfected with the −5205, −1485, or −639 bp constructs were inhibited by approximately 50% by incubation of the cells with 250 nM calphostin C, whereas the cells transfected with the 520-bp construct were largely resistant to this inhibitor.

These data indicate that the LO promoter in proliferating NRASMC appears to be regulated by PKC and its downstream mediators. The lack of sensitivity of the smallest promoter fragment to calphostin C may reflect the varied response of different PKC isoforms to this inhibitor [Pukac et al., 1998] and/or to the presence of more than one promoter element responding to PKC, which contribute to the regulation of the LO promoter.

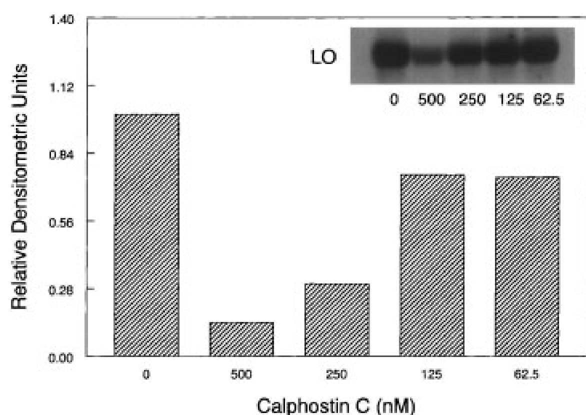

Fig. 1. Effect of calphostin C on LO expression. Proliferating NRASMC in second passage were incubated in 10% FBS/DMEM for 2 days and the medium was then replaced with fresh medium containing 0 (control), 500, 250, 125, or 62.5 nM calphostin C. Cells were harvested after 18 h and RNA isolated and analyzed as described in Materials and Methods. The heights of the bars represent the density of LO mRNA bands seen on Northern blots (shown in inset) corrected for loading variation by comparison to the corresponding densities of the 18S rRNA transcripts in each lane. The data presented are representative of the results of repeated experiments.

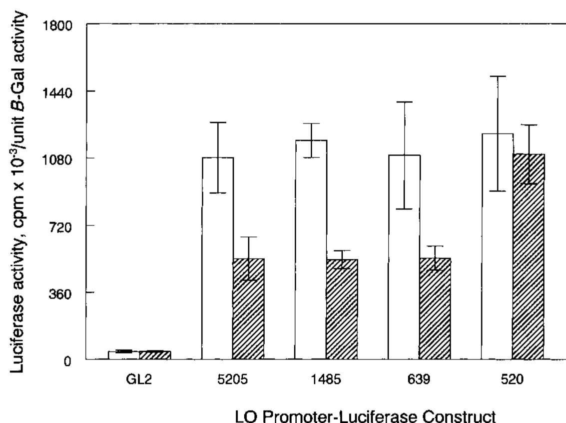

Fig. 2. Effect of calphostin C on LO promoter activity. Cells transfected with the various LO promoter-luciferase reporter constructs in 10% FBS/DMEM for 24 h were cultured for an additional 18 h in 10%FBS/DMEM (Control, open bars) or in 10% FBS/DMEM containing 250 nM calphostin C (hatched bars). Cells were then harvested, lysed, and lysates assayed for luciferase and β-galactosidase activities. The luciferase activity has been normalized to the β-galactosidase activity of the cotransfected β-galactosidase control vector. Each bar represents the normalized activity of samples performed in triplicate. The data presented are representative of the results of repeated experiments.

Effect of Specific Signal Transduction Inhibitors on LO Promoter Activity in NRASMC

Further delineation of the signal transduction cascade involved in the transcriptional regulation of LO expression utilized NRASMC transfected with the −5205 or the −1485 LO promoter construct using the Lipofectamine-PLUS reagent in 10% FBS/DMEM. After 18–20 h, the reagent was removed and the cells incubated with fresh 10% FBS/DMEM containing specific inhibitors of signal transduction components for an additional 24 h. Both the −5205 (Fig. 3) and the −1485 (data not shown) LO promoter fragments behaved similarly in response to each of the inhibitors. As shown in Figure 3, addition of the MEK1 (MAPK kinase) inhibitor, PD98059, resulted in a marked decrease in activity of the −5205 LO promoter fragment relative to control levels as did the addition of apigenin, previously reported to inhibit MAPK activity [Yin et al., 1999]. These results support a role for a PKC-dependent-MEK-MAPK pathway in the regulation of the LO promoter in NRASMC.

The possible involvement of upstream activators of PKC in this pathway was also considered. Thus (Fig. 3), the phospholipase C (PLC) inhibitor, U73122, decreased LO promoter activity to a degree comparable to that observed with the MEK and MAPK inhibitors. Conversely, wortmannin, an inhibitor of phosphatidylinositol 3′-kinase (PI 3 kinase), stimulated promoter activity marginally. AG 1433, an inhibitor of the autophosphorylation of the PDGF receptor, significantly reduced promoter activity implicating a role for PDGF in the expression of LO. Addition of farnesyl protein transferase inhibitor I (FPT I), an inhibitor of the post-translational farnesylation of Ras required for its insertion into the cell membrane [Garcia et al., 1993], had no apparent effect on LO promoter activity under these conditions (Fig. 3).

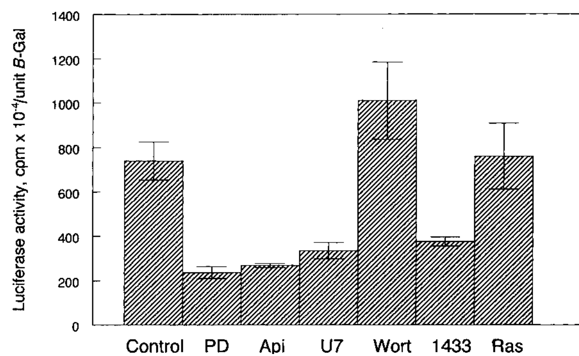

Fig. 3. Effect of signal transduction inhibitors on LO promoter activity. Cells transfected with the LO promoter construct in 10% FBS/DMEM for 24 h were cultured in fresh 10%FBS/DMEM containing signal transduction inhibitors for an additional 18–20 h. Cells were lysed and assayed for luciferase and β-galactosidase activity. The heights of the bars represent the means (± standard deviations) of luciferase activities assayed in triplicate samples and normalized to β-galactosidase activities. Shown is a representative experiment of three different experiments performed with the −1485 LO promoter construct. Control, no inhibitor; PD, 100 μM PD98059; Api, 100 μM apigenin; U7, 1.0 μM U73122; Wort, 100 nM wortmannin; 1433, 100 μM AG 1433; Ras, 200 nM Ras farnesyl transferase inhibitor I.

Assessment of MAPK Activation in NRASMC Treated With Signal Transduction Inhibitors

The results obtained with the signal transduction inhibitors suggest that LO is regulated in SMCs by a PKC-MEK-MAPK pathway. To further test this conclusion, Western blot analyses were performed on extracts of NRASMC treated with these inhibitors to assess their effects on the activation of MAPK. The monoclonal antibody used as a probe in this study selectively recognizes the phosphorylated, but not the nonphosphorylated form of the 42-kDa ERK2 (Fig. 4, lanes 1 and 2, respectively). A significant increase in MAPK activation was observed in extracts prepared from cells incubated in media containing 10% FBS (Fig. 4, lane 4), in comparison to cells incubated with 0% FBS (Fig. 4, lane 3), consistent with the presence of a variety of growth factors and mitogens in serum. Extracts from cells treated with the PKC inhibitor, calphostin C (250 μM), shown here to downregulate LO mRNA and promoter activity, clearly inhibited MAPK activation

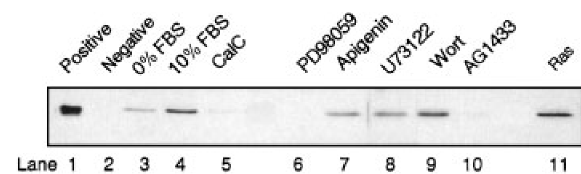

Fig. 4. Effect of signal transduction inhibitors on level of activated MAPK as determined by Western blotting. Cells were treated as described in the legend of Figure 3. Equal quantities (20 μg) of protein of the cell extracts were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Efficiency of the transfer was monitored by Ponceau S staining of the filter following transfer. The blots were probed with antibodies to phosphorylated (activated) MAPK and HRP-conjugated secondary antibody. Levels of activated MAPK were detected using the ECL reagent and autoradiography.

(Fig. 4, lane 5). As expected, the MEK inhibitor, PD98059, completely inhibited MAPK activation, as did apigenin, although to a lesser degree (Fig. 4, lanes 6 and 7, respectively). The PLC inhibitor, U73122, partially prevented MAPK activation (Fig. 4, lane 8), while wortmannin and the inhibitor of Ras farnesylation had little effect (Fig. 4, lanes 9 and 11, respectively). AG1433 has been reported to inhibit the phosphorylation and function of the PDGF receptor [Bryckaert et al., 1992], and as shown (Fig. 4, lane 10), this agent strongly inhibited MAPK activation in the present study. In toto, these results reveal that MAPK activation was inhibited at the concentrations of the inhibitors that downregulated LO promoter activity, supporting the conclusion that a PKC-MEK-MAPK-dependent signal transduction pathway can regulate LO expression. These data are also consistent with the possibility that the PDGF receptor participates as an upstream activator of this pathway.

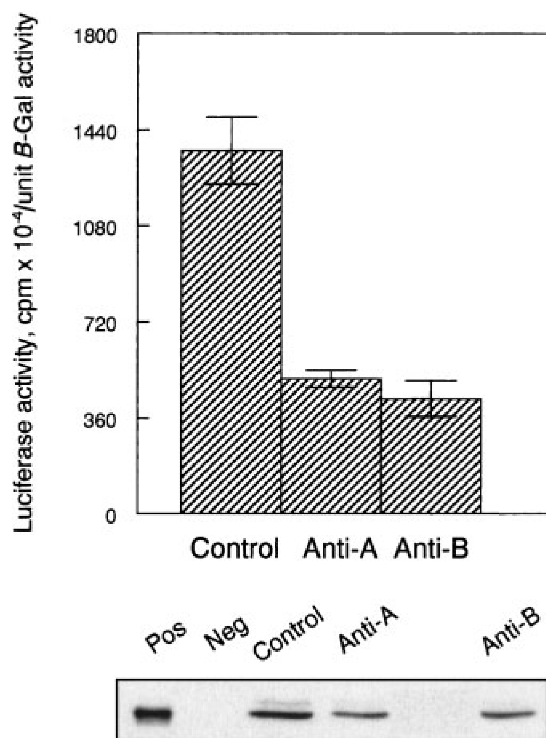

Fig. 5. Effect of anti-PDGF-A and -B antibodies on LO promoter activity and MAPK activation. NRASMC were transfected with the −1485 LO promoter construct in 10% FBS/DMEM for 24 h and then incubated for 24 h with either control media or media pretreated for 30 min with anti-A or anti-B, each at a final concentration of 10 μg/ml. **Top**: Cell lysates were assayed for luciferase and β-galactosidase activity. The heights of the bars represent the means ± standard deviation of luciferase activities assayed in triplicate samples and normalized to β-galactosidase activities. **Bottom**: Western blot of aliquots of the cell probed with antibody to phosphorylated (activated) MAPK. Pos, positive control (phosphorylated ERK2); Neg, negative control (nonphosphorylated ERK2).

Identification of PDGF as a Serum Factor Regulating LO Promoter Activity

As noted, the inhibition of LO promoter activity by AG1433 points toward a role for PDGF in the regulation of the expression of LO. Indeed, PDGF is known to activate the MAPK signaling cascade [Bornfeldt et al., 1994; Graf et al., 1997; Nelson et al., 1998; Pukac et al., 1998] and has been reported to upregulate LO mRNA in VSMC [Green et al., 1995]. To address this issue within the context of the present study, medium containing 10% FBS was preincubated for 30 min in the absence or presence of neutralizing antibodies directed against specific peptide sequences within PDGF isoforms A and B, respectively. Anti-A antibody is expected to bind to PDGF subtypes AA and AB, while the anti-B antibody will bind to PDGF AB and PDGF BB. NRASMC transfected with the −1485 LO promoter constructs were then incubated in this medium for 24 h after which cell extracts were prepared and assayed for luciferase activities. As shown (Fig. 5), the presence of the anti-A and anti-B antibodies in the culture medium reduced the promoter activity of the −1485 LO fragment by 64 and 68%, respectively, relative to the control. These experiments were also performed with the −5205 LO promoter fragment with identical results obtained (data not shown). Thus, it appears that multiple isoforms of PDGF in the fetal calf serum are capable of activating the PDGF receptor and its downstream signaling cascade leading to regulation of LO expression. Incubation of cells with medium containing 10% FBS and the neutralizing antibodies also inhibited MAPK activation (Fig. 5, bottom panel). Clearly, PDGF can activate the MAPK pathway in these cells since sequestering the PDGF in FBS as complexes with specific antibodies significantly decreases serum-induced MAPK activation.

Effects of Specific PKC Inhibitors on LO Promoter Activity in NRASMC

To date, at least 12 different isoforms of PKC have been identified in mammalian tissues that are classified into three different groups. These include the calcium-activated enzymes (α-, β-, and γ-PKC), the calcium insensitive enzymes

(δ-, ϵ-, θ-, η-, and μ-PKC), and the atypical enzymes (ζ-, λ-, and τ-PKC), which are not activated by calcium or phorbol esters [Puceat and Vassort, 1996]. The results obtained in the present study with the PKC inhibitor, calphostin C, suggest that the isoforms regulating LO in NRASMC are the calcium-activated α-, β-, and/or γ-PKC species. To further test this hypothesis, several inhibitors were assayed, which have been reported to affect selected PKC isoforms. Go6976 and RO-31-8220 inhibit α- and β-PKC, as well as selected calcium-insensitive isoforms [Martiny-Baron et al., 1993; McKenna and Hanson, 1993]. Bisindolylmaleimide I inhibits β I-, δ-, and ϵ-PKC [Toullec et al., 1991], while hispidin appears to be specific for β-PKC [Gonindard et al., 1997]. The effects of these agents were assessed in NRASMC transfected with the −5205 LO promoter fragment and then cultured in 10% FBS/DMEM (Fig. 6). The concentrations of inhibitors used in this study, indicated in the legend of Figure 6, were selected from the results of preliminary experiments revealing the concentrations of each giving the maximum inhibition without apparent cytotoxicity (data not shown). Once again, LO promoter activity was inhibited by treatment with calphostin C (Fig. 6). Phorbol myristate acetate can also inhibit PKC activity under selected conditions [Lee and Severson, 1994], and as shown, this ester also effectively reduced LO promoter activity. Ro-31-8220 and Bisindolylmaleimide I, inhibitors of the calcium-independent PKCs, had little or no significant effect on promoter activity. In contrast, hispidin and GO6976 decreased the promoter activity by 73 and 55%, respectively. Hispidin appeared to be the most potent inhibitor of LO promoter activity among these agents under the conditions used here. These results clearly implicate β-PKC in the regulation of LO expression, although the possible participation of other calcium-sensitive isoforms such as α- and γ-PKC are not excluded by these results.

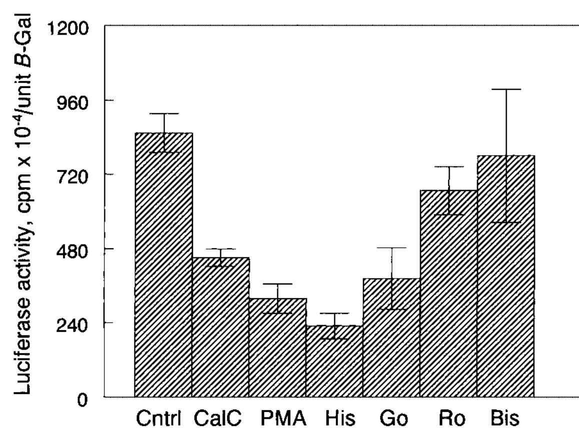

Fig. 6. Effect of PKC inhibitors on LO promoter. NRASMC were transfected with the −5205 LO promoter construct in 10% FBS/DMEM for 24 h and then incubated either with control media or media containing PKC inhibitors for an additional 18–20 h. Cell lysates were prepared and cells were assayed for luciferase and β-galactosidase activity. Each bar in the graph represents the luciferase activity normalized to β-galactosidase activity for triplicate samples. Cntrl, control; CalC, calphostin C; PMA; phorbol myristate acetate; His, hispidin; Go, GO; Ro, RO; Bis, bisindolemaleimide.

Activation of Selected LO Promoter Fragments by Serum

The results presently obtained with the anti-PDGF antibodies (Fig. 5) indicate that the presence of this growth factor in serum activates the expression of the LO promoter. This does not preclude the possibility that other serum factors may also activate this promoter. To assess the effect of serum on the LO promoter, a series of LO-promoter deletion constructs were transfected into NRASMC. The cells were then cultured for 18 h in medium containing 0.5% serum followed by incubation of the cells for 4 h in medium containing either 0 or 10% FBS. Substantial increases were observed in the luciferase activities of the −1485 (2.7-fold) and −639 (2.1-fold) promoter fragments in response to the culture medium containing 10% serum (Fig. 7). The stimulating effect of 10% serum was diminished in the case of the − 520 promoter fragment, while the −308 fragment displayed much lower basal activity and essentially no serum effect. Surprisingly, the largest fragment responded only minimally to the presence of 10% serum. Thus, it appears that there are elements in the LO promoter between approximately −1485 and −520 bases, which are activated by factors present in serum, while silencing elements may exist upstream of −1485. PDGF clearly plays a role in this serum induction, as including the neutralizing antibodies in the 4-h serum treatment as described above abrogates the serum response, as do the inhibitors of PKC, MEK, and MAPK (data not shown).

DISCUSSION

Evidence is provided in this report for the regulation of LO in VSMC by a PKC-MEK-MAPK pathway. We demonstrate that the PKC

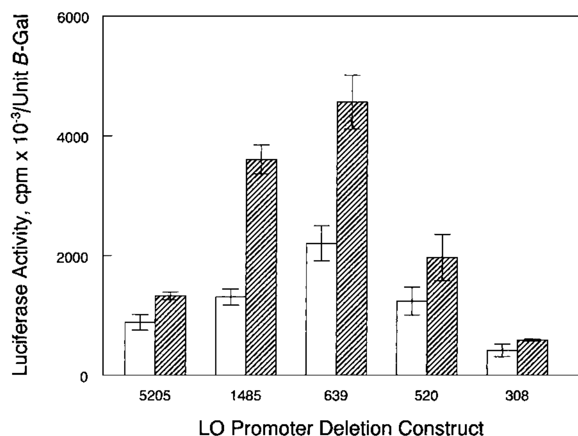

Fig. 7. Effect of serum on LO promoter constructs. NRASMC were transfected with the LO promoter-luciferase reporter constructs in 0.5% FBS/DMEM for 24 h. Cells were then incubated with media containing either 0 or 10% FBS for 4 h. Cells lysates were assayed for luciferase and β-galactosidase activity. The heights of the bars represent the means ± standard deviation of the values for luciferase activities assayed in triplicate samples and normalized to β-galactosidase activities. The results of the experiment shown are typical of four experiments of the same design.

inhibitor, calphostin C, decreased steady-state levels of LO mRNA and markedly decreased transcriptional activity of LO promoter/reporter constructs. The LO promoter activity was also inhibited by treatment with PMA under conditions which downregulate PKC. Various other inhibitors targeting enzymes within the MEK-MAPK cascade also reduced LO transcriptional activity. The efficacy of the various inhibitors was assessed by their ability to decrease MAPK activation, which was determined by Western blotting using antibodies specific for the activated (phosphorylated) form of MAPK. We further provide evidence that the PDGF component of serum is a principal source of the serum-dependent stimulation of LO promoter activity seen in the present study.

Previous reports have revealed that the expression of LO can be accompanied by changes in the growth state of SMCs. More specifically, TGF-β elevates LO expression as it inhibits VSMC proliferation [Gacheru et al., 1997], while increases in intracellular levels of cAMP, an effector which can reduce VSMC proliferation, also resulted in increased LO expression [Ravid et al., 1999]. In the present study, we observe that inhibition of PKC, a manipulation which has been reported to inhibit VSMC proliferation [Newby et al., 1995], decreases LO expression, in contrast to the correlation noted with TGF-β [Gacheru et al., 1997]. Furthermore, inhibition of the downstream MAPK pathway also decreases LO expression supporting the view that LO is promoted by this mitogen activated pathway. These experiments were carried out in the presence of 10% FBS, which contains a complex mixture of growth factors, cytokines, and mitogens that activate a multitude of pathways. Clearly, the ultimate level of LO expression observed will depend upon the balance between and amounts of these positive and negative regulatory factors.

We have observed that the activities of the various LO-luciferase promoter constructs examined in this study are differentially responsive to variations in the serum content of the growth medium. The largest promoter fragment (−5205) shows relatively little activity in the absence of serum and marginal induction in 10% serum. In contrast, the fragments between −1485 and −520 are intrinsically more active in the absence of serum, and their activities are induced to significantly higher levels in the presence of 10% serum. It is possible that a silencer or another *cis* acting element that is downregulated by factors in serum exists in the region between −5205 and −1485. Additionally, the deletion of the region between −520 and −308 largely abrogates the serum response. We have observed two elements within this region at −493 to −484 and at −448 to −439 that closely resemble the canonical c-fos serum response element [Ramirez et al., 1997]. Further studies are required to determine whether these sequences function as serum response elements in the LO promoter.

PDGF is a prominent growth factor in serum, which stimulates SMC proliferation. Moreover, highly-purified PDGF has previously been shown to induce LO expression in VSMC [Green et al., 1995]. As shown in the present study, the PDGF component of serum appears to be a major source of the stimulating effect of serum on LO expression since sequestering PDGF in serum samples with anti-PDGF prior to addition of the serum to cells markedly reduced the level of LO expression. In unpublished studies, we have directly tested the effect of PDGF on LO promoter activity by treating SMCs transfected with a reporter construct of the −1485 LO promoter fragment with the three isoforms of human recombinant PDGF in serum-free medium. These studies revealed that PDGF AB (25 ng/ml) and PDGF BB (5 ng/ml), but not PDGF AA (25 ng/ml), stimulated LO promoter

activity to the level observed with 10% serum (data not shown). This is consistent with previous reports that VSMC express significantly more type B than type A PDGF receptors. Both PDGF AB and BB would be expected to bind to these type B receptors and promote signal transduction including activation of the downstream MAPK pathway [Heldin et al., 1998; Widmann et al., 1999]. Collectively, these data indicate that specific PDGF species within serum are capable of stimulating LO promoter activity. The actual promoter elements and corresponding transcription factors involved in this induction remain to be identified. In this regard, the cAMP response element binding protein (CREB) has been shown to be activated by PDGF via a Raf-MEK pathway [Seternes et al., 1999]. Although, we have previously demonstrated the presence of a CREB element at position −100 to −93 of the human LO promoter [Ravid et al., 1999], this is within a region of the promoter that responds only minimally to serum. However, another potential CREB site is located at position −352 to −345 within this promoter, which may prove to be responsive to the treatment of these cells with PDGF.

The ERK-MAPK pathway has been implicated in the regulation of other extracellular matrix (ECM) macromolecules and in the phenotypic alteration of VSMC induced by specific components of the ECM. For example, type I collagen gene expression is enhanced through a MAPK/ERK-dependent pathway by angiotensin II in mice [Tharaux et al., 2000]. Induction of vascular fibrosis by bradykinin involves the MAPK-dependent activation of the α 2(I) collagen gene [Douillet et al., 2000]. TGF-β and TGF each stimulate fibronectin production through the MAPK pathway [Kaiura et al., 1999]. Of further interest, fibronectin promotes the transformation of VSMC from the contractile to the synthetic phenotype via the ERK-MAPK pathway [Qin et al., 2000]. Clearly, the sensitivity of the LO gene to activation by this pathway emphasizes the central importance of this signal transduction mechanism in fibrogenesis.

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