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Hydrogen Peroxide-Mediated, Lysyl Oxidase-Dependent Chemotaxis of Vascular Smooth Muscle Cells

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Abstract Lysyl oxidase (LO), an enzyme secreted by vascular smooth muscle cells (VSMC), initiates the covalent crosslinking of polypeptide chains within collagen and elastin. The present study reveals that purified LO strongly induces directional migration of VSMC in an in vitro assay system. LO-dependent chemotaxis, but not chemokinesis, was abolished by β-aminopropionitrile, an active site inhibitor of LO, or by catalase, as well as by prior heat denaturation. This indicates that the H_2O_2 product of amine oxidation by LO is critical to the expression of its chemotactic activity. The results indicate that the chemotactic response requires direct access between LO and a substrate molecule (or molecules) tightly associated with the VSMC. The addition of LO to VSMC elevated the levels of intracellular H_2O_2, enhanced stress fiber formation, and focal adhesion assembly, is consistent with the induction of the chemotactic response. J. Cell. Biochem. 78:550–557, 2000.

Key words: lysyl oxidase; chemotaxis; hydrogen peroxide; vascular smooth muscle cells

Chemotaxis is defined as the directional locomotion of cells controlled by a gradient of a diffusible chemical. Cells detect chemotactic agents primarily by interactions between these ligands and cell surface receptors. Subsequent activation of signal transduction pathways leads to reorganization of the cytoskeleton, thus facilitating cell movement by promoting adhesion to the matrix at the leading edge of the cell, and coordinated detachment from the matrix at the trailing edge. The chemotactic locomotion of VSMC plays an early and important role in the pathogenesis of atherosclerosis [Ross, 1993].

LO is a copper-dependent amine oxidase expressed and secreted by VSMC and other fibrogenic cells [Smith-Mungo and Kagan, 1998; Kagan et al., 1981]. This catalyst initiates the covalent crosslinking of collagen and elastin by oxidizing peptidyl lysine residues to form peptidyl α-aminoadipic-δ-semialdehyde in these extracellular proteins, thus stabilizing polymeric collagen or elastin as insoluble fibers. Hydrogen peroxide and ammonia are also produced in the LO-catalyzed reaction in amounts stoichiometric with the aldehyde product, as shown:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

Clearly, LO is a critical enzyme for tissue morphogenesis and repair of the cardiovascular, respiratory, skeletal, and other systems of the body. Decreased LO activity is associated with disorders of copper metabolism such as Menke's syndrome, whereas increased LO activity is associated with fibrotic diseases such as atherosclerosis [Smith-Mungo and Kagan, 1998].

In addition to its central role in the crosslinking of collagen and elastin, LO is also a potent chemoattractant for human monocytes [Lazarus et al., 1994]. It is of particular interest that the catalytic function of LO is essential for this activity, although the mediator responsible for this chemotactic response is not known [Lazarus et al., 1994]. In the present study, we report that LO is also potently chemotactic for VSMC and describe evidence that the H_2O_2 product of LO action appears to be the principal mediator of this LO-dependent cell migration. The induction of VSMC chemotaxis by

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purified LO accompanies increased expression of stress fibers and focal adhesion complexes (FA).

MATERIALS AND METHODS

β-aminopropionitrile (BAPN), catalase, collagen type I, Giemsa stain, fibronectin, monoclonal antivinculin antibody, and rhodamine-conjugated rabbit antimouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). BODIPY-phallacidin was obtained from Molecular Probes (Eugene, OR). 2,7-dichlorofluorescin diacetate (DCF-dAc) was obtained from Eastman Kodak Co (Rochester, NY). All tissue culture products were from GIBCO (Grand Island, NY).

Enzyme Purification and Assay

Calf aorta LO (32 kDa) was isolated as described [Bedell-Hogan et al., 1993], yielding a preparation that displayed a single band by sodium dodecylsulfate-polyacrylamide gel electrophoresis [Kagan and Cai, 1995]. The specific activity of the enzyme preparation used in this study was 7.5×10^5 cpm of [^{3}H] H_2O release/mg protein in two h at 37°C, using 1.25×10^5 cpm of [^{3}H] tropoelastin substrate per assay [Bedell-Hogan et al., 1993].

Cell Culture and Preparation of Conditioned Media

VSMC were isolated from the aortas of 2–3 day old rat pups as described [Oakes et al., 1982]. Primary cultures were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) with antibiotics [Oakes et al., 1982] at 37°C in a 7.5% CO_2 atmosphere. Cells were used at passages three to six for this study. To obtain growth-arrested cultures, harvested cells were incubated at $3–4 \times 10^3$ cells/cm^2 in 10% FBS/DMEM for 48 h and then in 0.3% FBS/DMEM for 72 h. To prepare conditioned media, growth-arrested cells were cultured in serum-free DMEM in the absence or presence of 10 nM LO or 10 nM LO plus 300 units/ml catalase. After six h of incubation, media were collected and assayed for chemotactic activity.

Chemotaxis Assay

Chemotaxis assays were performed using multiwell cluster plates (Corning Costar Co., Cambridge, MA), as described [Kundra et al., 1995]. Primary cultures were refed with 10% FBS/DMEM 24 h before experiments. Following trypsinization, cells were resuspended in 0.1% bovine serum albumin (BSA)/DMEM. Native BSA is not a substrate for LO [Kagan et al., 1984], and was selected as the protein component of this medium on that basis. Polycarbonate filters (8 μm micropores) in transwells were coated with collagen type I at 100 μg/ml in 0.2 N acetic acid for two days, or with fibronectin (1.33 μg/ml for 15 min) [Kundra et al., 1995]. The results of chemotaxis assays obtained with collagen- or fibronectin-coated filters were essentially the same. Agents tested as chemoattractants, including active or heat-denatured (100°C/15 min) LO in one ml of 0.1% BSA/DMEM, or one ml of conditioned media prepared as described above, were added to the lower compartments of the wells. Cells (1×10^5) in one ml of 0.1% BSA/DMEM were placed in the upper compartments of wells. When included in the experiments, BAPN, an irreversible inhibitor of LO activity [Smith-Mungo and Kagan, 1998], or catalase was added to both the upper and the lower compartments at the indicated concentrations. For the chemokinetic assay, equivalent concentrations of LO were added to both the upper and the lower compartments [Lazarus et al., 1994]. The assembled chambers were incubated for six h, and the microporous filters were then removed for Giemsa staining. The numbers of cells migrating from the upper to the lower surfaces of the membrane filters were counted in a total of 10 fields using a Diaphot Nikon microscope at high magnification.

Measurement of Intracellular Levels of H_2O_2

Intracellular levels of H_2O_2 were assessed by fluorescence activated cell sorting (FACS) analysis using DCF-dAc as a probe, as described [Nishio and Watanabe, 1997]. Growth-arrested VSMC were incubated for 30 min in phosphate buffered saline (PBS) containing 10 mM glucose (PBSG) with different additions as indicated. DCF-dAc dissolved in DMSO was simultaneously added to each culture at a final concentration of 100 μM. After incubation, cells were detached by trypsinization and resuspended in PBS to give a density of 0.5 to 1×10^6 cells/ml. The green emitted fluorescence intensity per sample of 10,000 cells was measured by flow cytometry at the FL1 setting of the FACS instrument (Becton-Dickinson Corp., San Jose, CA).

Fluorescence Microscopy

Growth-arrested VSMC, on coverslips, were treated with or without chemoattractants or inhibitors for six h, fixed in 3.7% formaldehyde/0.2% Triton X-100/PBS, and then briefly incubated with 1% BSA/PBS. Microfilaments (MF) and FA were visualized by incubation of the fixed cells at room temperature for 45 min with a mixture of monoclonal antivinculin to specifically label FA [Burridge and Chrzanowska-Wodnicka, 1996], and BODIPY-phalacidin to identify microfilaments [Li et al., 1994]. Following thorough washing, the coverslips were overlaid with rhodamine-conjugated rabbit antimouse IgG and incubated for an additional 45 min and then were examined under a Nikon fluorescence microscope.

RESULTS

LO-Induced H_2O_2-Dependent Locomotion of VSMC

The presence of varied, low concentrations of purified 32 kDa LO in the lower compartments of the chemotaxis chambers increased the directed migration of VSMC, with an optimum effect of 350 % of the control seen at 10 nM LO (Fig. 1). This profile, displaying a peak response, is consistent with effects seen in other instances of chemotaxis [Kundra et al., 1995]. Subsequent experiments used 10 nM LO as the standard exposure condition with which the effects of various treatment conditions or specific agents on LO-dependent chemotaxis were assessed. As shown in Table 1, the addition of 10 nM LO to both the upper and the lower compartments of the chambers did not induce significant directed cell migration beyond that seen in the LO-free control. Thus, LO predominantly induced a chemotactic rather than a chemokinetic effect on the VSMC [Lazarus et al., 1994]. The LO-dependent response was prevented by inclusion of 100 μM BAPN or 300 units/ml of catalase in the top and bottom chamber compartments, or by prior heat denaturation (100 °C for 15 min) of the native enzyme. Thus, the native structure and the catalytic function of LO, and, apparently, the H_2O_2 product of amine oxidation by LO, seem critical to the expression of chemotactic activity. The lack of chemotaxis by the denatured enzyme emphasizes the requirement for enzyme function and suggests that a sequence element of amino acid residues within LO does not account for the chemotaxis.

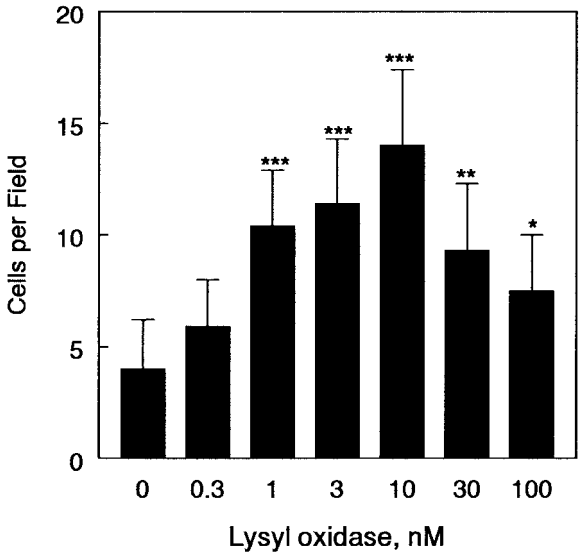

Fig. 1. Chemotactic response of VSMC to different concentrations of native LO. A total of 10^5 cells were plated onto 8 μM porous filters in the upper compartments of the chemotaxis chambers. Native LO was added to the bottom compartments to initiate the chemotaxis assay. The means (± standard deviations) of migrating cells per field were determined from the cell counts of 10 individual fields. These data are representative of three experiments in which each control or variable was assessed in triplicate incubations. Asterisks indicate values significantly different from the control as determined by ANOVA analysis: * $P < 0.05$, * * $P < 0.01$, * * * $P < 0.001$.

Since H_2O_2 appears to be essential to the chemotactic response, efforts were made to determine whether this product resulted from the action of LO on substrates synthesized by the VSMC, or on molecular components of the nonconditioned DMEM medium which might act as substrates of LO. For this purpose, growth-arrested VSMC cultures were incubated for six h in DMEM, or in DMEM supplemented either with 10 nM LO or 10 nM LO plus 300 units/ml catalase. Aliquots of freshly prepared, nonconditioned DMEM were supplemented identically and incubated under cell-free conditions. As shown in Figure 2A, cells exposed to LO-supplemented, conditioned media (bar 3) or to the nonconditioned, LO-supplemented DMEM (bar 8) exhibited enhanced degrees of chemotaxis. This amounted to a 4.1 and 3.3-fold increase, respectively, of basal levels seen with the nonsupplemented conditioned (bar 1) or nonsupplemented, nonconditioned control media (bar 6).

The addition of BAPN to the top and bottom compartments fully inhibited the enhanced cell migration induced by the nonconditioned DMEM which had been preincubated with

TABLE I. Effect of Various Agents and Conditions on LO-Induced Chemotaxis of VSMC

Agent or treatment	Cells per field	
	—	+ 10 nM native LO
Control	3.4 ± 2.4	13.6 ± 3.0*
+ BAPN (100 μM)	3.1 ± 1.9	3.4 ± 1.4
+ Catalase (300 units/ml)	2.4 ± 1.6	3.7 ± 1.2
+10 nM heat denatured LO[a]	3.0 ± 1.7	NA[c]
Chemokinesis assay[b]	4.8 ± 2.1	NA[c]

*Significant difference from control ($P < 0.001$).
[a]LO was inactivated at 100°C for 15 min prior to addition to bottom well of Boyden chamber.
[b]10 nM native LO was added to the top and bottom compartments of Boyden chamber.
[c]Not applicable.

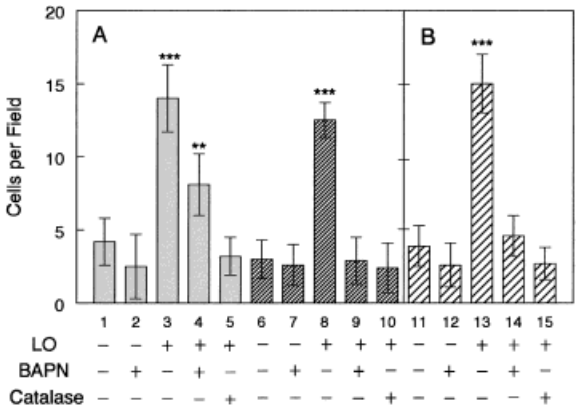

Fig. 2. Chemotactic response of VSMC to LO under different conditions. A total of 10^5 cells were plated onto 8 μM porous filters in the upper compartment. **A**: Medium samples which had been conditioned by VSMC in the presence or absence of either native LO (10 nM) or native LO plus catalase (300 units/ml) (bars 1 through 5), of nonconditioned medium which had been preincubated in the absence of cells but in the presence or absence of LO or LO plus catalase (bars 6 through 10) were added to the lower compartments of the multiwell chambers. **B**: Medium which had been conditioned by cells was then incubated in the absence of cells in the presence or absence of LO or LO plus catalase (bars 11 through 15) and then added to the lower compartments of the multiwell chambers. When present, BAPN (100 μM, final concentration) was added to both the upper and the lower compartments at the start of the chemotaxis assay. As indicated below the graph, (−) = not added, (+) = added. Notes on reproducibility and statistical treatment are as indicated in the legend of Figure 1.

added LO (compare bars 9 and 8). However, addition of BAPN to the LO-supplemented, conditioned medium (bar 4) reduced the enhanced degree of chemotaxis of this sample only partially: it remained 2-fold higher than that of the nonsupplemented, conditioned control (bar 1), or 3-fold higher relative to the LO-free, conditioned control medium tested in the presence of BAPN (bar 2). In contrast, the addition of catalase to the LO-supplemented, conditioned medium (bar 5), or to the LO-supplemented nonconditioned medium (bar 10) fully reduced the chemotactic activity of both samples to basal levels, again demonstrating a key role of H_2O_2 in this LO-dependent phenomenon.

BAPN, present during the chemotaxis assay but not during the conditioning period, only partially reduced (while catalase completely reduced) the chemotactic response to the LO-supplemented, conditioned medium. Therefore it seems reasonable to propose that the enhanced chemotactic response to this medium was due to the expression of the activity of LO during the period of conditioning of the medium by the cells. The residual, catalase-sensitive chemotactic response seen in the presence of BAPN can be attributed to the presence of H_2O_2 formed prior to the addition of BAPN, due to LO action during the conditioning period. The presence of either BAPN or catalase during the chemotaxis assay fully inhibited the enhanced chemotactic response due to LO in the nonconditioned medium. Therefore, it appears that the preincubation of LO with the nonconditioned medium did not yield significant quantities of H_2O_2 capable of stimulating chemotaxis. The enhanced chemotaxis seen with this medium can be reasonably and completely attributed to the requirement for the catalytic expression during the chemotaxis assay of the LO originally added to the nonconditioned medium. Since catalase eliminated that response as well, the enhanced chemotactic response is due to the H_2O_2 product of LO action formed during the chemotaxis assay period. Thus, these data indicate that chemotactic stimulants do not result from the action of LO on intrinsic components of the noncondi-

tioned DMEM but rather from the action of LO on substrate(s) derived from the VSMC.

An experiment was designed to discriminate between the possibilities that such substrate(s) may be secreted into the medium where they become oxidized in the freely soluble state by LO, or that the substrate(s) critical to the chemotactic response remain associated with the cell layer. For that purpose, growth-arrested VSMC were incubated in DMEM for six h in the absence of added LO. The conditioned medium was decanted and freed of cells by sedimentation. This conditioned medium was then supplemented with LO (10 nM) or with LO plus catalase (300 units/ml), incubated at 37 °C for six h, and then tested for its chemotactic activity in the lower compartment of the chemotaxis chambers in the presence or absence of 100 μM BAPN. As shown in Figure 2B, BAPN that was present only during the chemotaxis assay period abolished the bulk of the enhanced chemotactic activity induced by the conditioned medium preincubated with LO (compare bars 13 and 14). The presence of catalase, along with LO, during the preincubation of the conditioned medium prevented the enhanced chemotactic response seen with the medium which had been preincubated with LO alone (compare bars 13 and 15). These results argue that H_2O_2 was not generated by the preincubation of LO with the cell-free conditioned medium, and thus that LO substrates secreted by VSMC and remaining soluble in the medium do not account for the chemotactic response to LO.

As noted, inclusion of added LO in medium while it is in contact with the cells results in partial BAPN sensitivity (compare bar 4 to bars 1 and 2, Fig. 2A), while the addition of BAPN to conditioned medium incubated with added LO under cell-free conditions completely prevents the chemotaxis response (bars 13 and 14, Fig. 2B). In toto, these results indicate that the chemotactic response stems from the action of LO on substrates adhering or intrinsic to the VSMC which do not become soluble in the medium. This conclusion would also pertain to the conditions used when active LO is exposed to migrating extensions of test cells that become accessible to the added enzyme only during the chemotaxis assay. Moreover, the H_2O_2 product of this activity of LO is the effective mediator of the chemotactic response.

Intracellular Levels of H_2O_2

Since H_2O_2 is membrane permeable [Ohno and Gallin, 1985], it was of interest to assess whether intracellular levels of this peroxide were affected by incubation of cells with LO. For that purpose, H_2O_2 levels within VSMC, incubated under various conditions, were determined by FACS analysis using DCF-dAc as an intracellular peroxide probe, as described [Nishio and Watanabe, 1997]. Exposure of cells to 10 nM LO for 30 min increased the intracellular fluorescence intensity to 324 ±19% (bar 2) of the control (100 ± 13%, bar 1) (Fig. 3). The elevation of intracellular H_2O_2 over the control value was completely prevented by the inclusion of 100 μM BAPN (bar 3) or 300 units/ml catalase (bar 4) in the incubation with LO. Intracellular H_2O_2 levels were significantly reduced (68%) by 300 units/ml catalase (bar 6) and slightly reduced (29%) by 100 μM BAPN (bar 5) in cells treated with these reagents individually in the absence of added LO, in comparison to the control (bar 1).

LO-Induced H_2O_2-Dependent Microfilament Reorganization and Focal Adhesion Assembly

Since structural changes in MF and FA are involved in cell migration [Burridge and Chrzanowska-Wodnicka, 1996], we assessed for LO-dependent effects on microfilament organization and FA assembly in VSMC exposed to this catalyst. Growth-arrested VSMC on coverslips were incubated for six h in the absence or presence of LO, LO plus BAPN, LO plus catalase, BAPN, or catalase at the indicated concentrations. As shown in Figure 4A, growth-arrested VSMC contain numerous thin, short actin fragments in the cytoplasm, many of which occur as aggregates of needle-like structures. Relatively few elongated, cable-like actin filaments (stress fibers) were seen at the cell periphery. FA, identified by their reactivity with antivinculin, appeared as small, punctate deposits that were often localized at the ends of microfilaments, particularly at sites of intersecting actin fragments (Fig. 4B). Cells which had been preincubated with 10 nM LO exhibited markedly increased amounts of thick, elongated actin cables. These fibrous deposits of actin occurred as large bundles distributed along the long axis of the cell and spanned across the cytoplasm (Fig. 4C). Preincubation with LO also appeared to enhance FA assembly, as evidenced by the increased occurrence of sites reactive to antivinculin, with

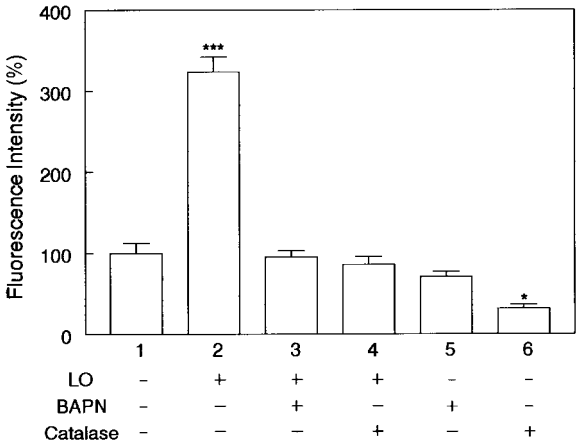

Fig. 3. LO-induced elevated levels of intracellular H_2O_2. Growth-arrested VSMC were washed with cold PBS and incubated with 100 μ M DCF-dAc in PBSG for 30 min in the absence (bar 1) or presence of 10 nM LO (bar 2), 10 nM LO plus 100 μ M BAPN (bar 3), 10 nM LO plus 300 units/ml catalase (bar 4), 100 μ M BAPN (bar 5), or 300 units/ml catalase (bar 6). The fluorescence intensities of aliquots containing 10,000 cells each were analyzed by FACS scan flow cytometry. The data are expressed as the mean (± standard deviations) of three experiments each determined with duplicate dishes. $*P < 0.05$, $***P < 0.001$ vs. control.

patches of FA predominating at the polar ends of the long axis of the cells (Fig. 4D). Coincubation of cells with LO and either 100 μM BAPN (Fig. 4E and 4F) or 300 units/ml of catalase (Fig. 4G and 4H) significantly inhibited the formation of stress fibers induced by LO (Fig. 4E and 4G), with a particularly notable reduction of the elongated actin cables spanning the cytoplasm. FA deposits were also reduced, along with the decrease of stress fibers in these cells (Fig. 4F and 4H). Cells on coverslips were also incubated in the absence of added LO with 100 μM BAPN (Fig. 4I and 4J) or 300 units/ml catalase (Fig. 4K and 4L) to evaluate possible effects of endogenous LO activity on microfilament organization and FA assembly. As shown, both inhibition of endogenous LO by BAPN and depletion of H_2O_2 by catalase appeared to block actin polymerization, resulting in the occurrence of actin fragments in the cytoplasm (Fig. 4I and 4K) which were significantly shorter than those of the control cells not exposed to these reagents (Fig. 4A). Elongated actin cables persisted at the cell periphery where FA were also predominantly distributed (Fig. 4J and 4L). These results indicate that functional LO in VSMC can contribute to the regulation of stress fiber formation and FA assembly in these cells.

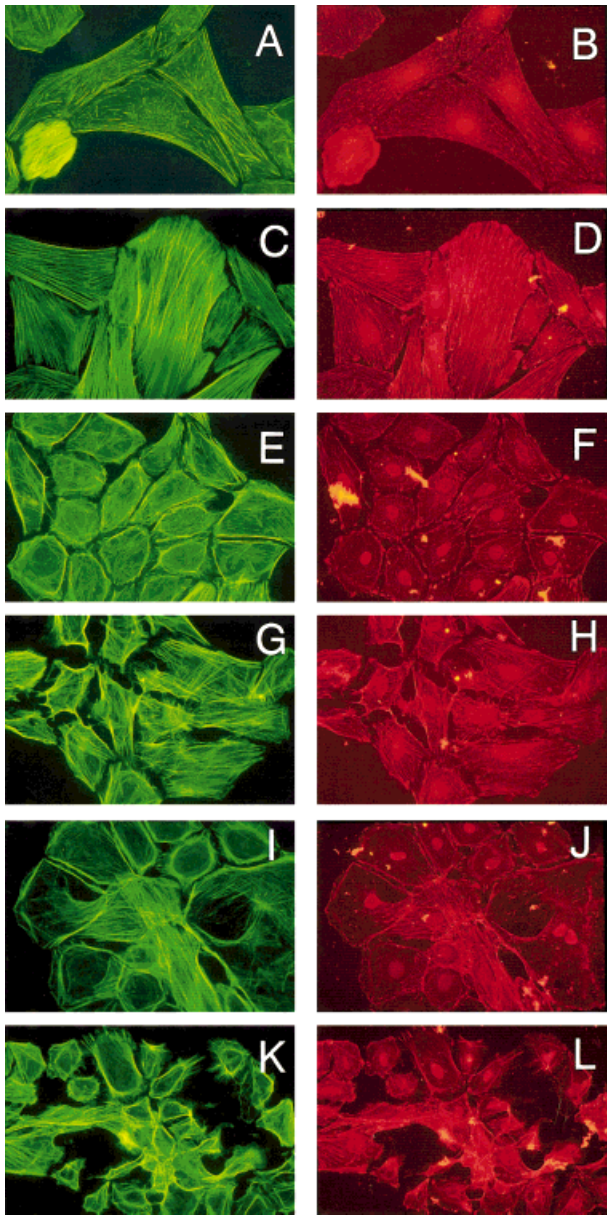

Fig. 4. Reorganization of MF and FA induced by LO. Immunocytochemical analyses. Growth arrested VSMC on coverslips were incubated for six h in the absence or presence of LO or other additives and processed for fluorescence microscopy to visualize MF and FA. The photomicrographs at identical magnifications show MF **(A, C, E, G, I, K)** and FA **(B, D, F, H, J, L)** of control cells (A, B) and cells treated with 10 nM LO (C, D), 10 nM LO plus 100 μM BAPN (E, F), 10 nM LO plus 300 units/ml catalase (G, H), 100 μM BAPN (I, J) or 300 units/ml catalase (K, L).

DISCUSSION

The present studies reveal that LO, a secreted product of VSMC, can induce a prominent chemotactic response in these cells. Clearly, this phenomenon requires catalysis by

the functional active site, as indicated by the prevention of chemotaxis by treatment of the enzyme by BAPN, a mechanism-based inactivator of this catalyst. The chemotactic response also does not appear to be due significantly to random, enhanced chemokinetic motion resulting from exposure to LO.

The prevention of the induced chemotaxis by the addition of catalase, under the varied conditions described, indicates that it is the H_2O_2 product of LO action which is the critical, LO-dependent mediator of the chemotactic response of the cells. The prevention of chemotaxis by BAPN is also consistent with this conclusion, since this inhibitor prevents substrate oxidation and thus H_2O_2 formation by LO. Moreover, the elevation of intracellular H_2O_2 upon exposure of cells to added LO provides additional evidence for the production of this peroxide during exposure of the cells to LO. The moderate decrease in the chemotactic response from the maximum seen at 10 nM LO that occurs at significantly higher enzyme concentrations (30 and 100 nM; see Fig. 1) is likely due to the production of excess H_2O_2 at these increased enzyme levels. Although H_2O_2 at low concentrations can play essential roles in cellular homeostasis, the decrease of chemotaxis at higher LO levels is consistent with the observation that increased levels of this reactive oxygen species induce apoptosis in VSMC [Li et al., 1997] and actin fragmentation in Chinese hamster CCL39 cells [Huot et al., 1996].

The efforts employed here to identify the source of the substrate(s) involved indicate that they are not derived from the nonconditioned DMEM medium, nor does chemotaxis result from the action of LO on secreted macromolecules that remain freely soluble in the medium conditioned by the VSMC. Indeed, LO-dependent, enhanced chemotaxis occurred only under conditions in which catalytically functional LO had direct access to the VSMC. This happened either by its addition to the medium of cells in culture, or by its access to cellular projections of VSMC extending through the membrane of the chemotaxis chambers during the chemotaxis assay. While the specific identity of substrate(s) whose oxidation by LO initiates the chemotactic response remains to be established, the candidate substrates are not necessarily restricted to collagen and elastin associated with the cells. Lysyl oxidase can oxidize a number of basic (pI $>$ 8), globular proteins in assays in vitro [Kagan et al., 1984]. This indicates that other matrix proteins, and/or protein constituents of the plasma membranes of the cells, must also be considered as sources of the reducing equivalents transferred to oxygen by LO to produce the chemotactic stimulant, H_2O_2.

The LO substrate appears to be either tightly bound to the cell surface or to be an intrinsic membrane protein accessible to LO action. The formation of the chemotactic H_2O_2 product generated by LO at the cell surface would be expected to favor the rapid penetration of H_2O_2 into the cell at the site of production, since H_2O_2 is freely permeable through the plasma membrane [Ohno, 1984]. Indeed, intracellular levels of H_2O_2 were markedly elevated upon cell exposure to added LO [Fig. 3]. It is well established that intracellular H_2O_2 can act as a second messenger [Hancock, 1997] to induce changes in cytoskeletal components that result in directed cell migration [Sundaresan et al., 1995]. In that regard, the present study revealed that marked alterations occur in MF organization and FA formation in response to the exposure of the VSMC to LO. MF polymerization and FA assembly provide the driving force for chemotactic movement of cells [Burridge and Chrzanowska-Wodnicka, 1996], consistent with the response to LO seen here. Since these morphological changes were inhibited by BAPN and catalase, the H_2O_2 product of LO action is implicated in these phenotypic changes. Hydrogen peroxide can stimulate actin assembly in P388D1 cells, a macrophage cell line, presumably by increasing the release of actin from monomer-sequestering proteins such as profilin, thus enhancing F-actin formation [Omann et al., 1994]. H_2O_2 derived from intra- or extracellular origin can also induce intracellular protein phosphorylation [Zent et al., 1999], among the substrates of which is focal adhesion kinase [Sonoda et al., 1999], which is expected to play a key role in the chemotactic response. Thus, the H_2O_2 produced by LO may stimulate the changes in the content and architecture of filamentous and adhesive cell proteins. It may do this either by a chemical triggering of filament formation and FA assembly, and/or by modulation of genes involved in the expression of these proteins.

In view of the present results, it is of some interest that Nelson et al. [1988] reported, approximately a decade ago, that BAPN (an agent selected because of its inhibition of collagen fiber production) inhibited fibroblast mi-

gration into fibrin clots covering chick tendons in organ culture. This system was used as an in vitro model of wound environments. The effect of BAPN was attributed to the possibility that collagen crosslink formation may be important for cell attachment and migration. While this hypothesis remains viable, the present results indicate that inhibition of the production of H_2O_2 by LO is likely to have been critical to the results obtained in this model system. Indeed, we have noted that in addition to VSMC, 3T3 fibroblasts are chemotactically attracted to exogenous LO, and that this chemotactic response is prevented by catalase or BAPN [Li and Kagan, 1999, unpublished studies].

Implications for the development and chronic nature of atherosclerotic lesions flow from the present study. As atherosclerosis progresses, medial smooth muscle cells migrate to the subendothelial intimal space where they proliferate, secrete, and crosslink abundant quantities of the elastin and collagen substrates. The crosslinking of these vascular proteins is initiated by the action of the LO, which is also secreted by these smooth muscle cells. It is likely that the initial migration of contractile, medial smooth muscle cells, in the earliest stages of lesion formation, occurs in response to chemotactic agents derived from inflammatory cells, which infiltrate the subendothelial space from the circulation. It also seems reasonable that the LO secreted by the activated smooth muscle cells within the developing intimal lesion may stimulate the continuing migration of additional medial cells into the lesion, by generating intimal H_2O_2 upon its oxidation of collagen and elastin substrates. This would contribute to the chronic nature of atherosclerotic disease. Indeed, the oxidation of lysine in collagen and elastin by LO in wound repair may be an important source of chemotactic H_2O_2 in many instances of inflammation and fibrogenesis.

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