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Fully Processed Lysyl Oxidase Catalyst Translocates from the Extracellular Space into Nuclei of Aortic Smooth-Muscle Cells

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Abstract Lysyl oxidase (LO), a secreted protein, was recently identified within the nuclei of vascular smooth-muscle cells (SMC) and 3T3 fibroblasts. A possible pathway by which LO can enter cell nuclei was explored in the present study. SMC were incubated with purified 32-kDa bovine aorta LO that had been fluorescently labeled with rhodamine (TRITC-LO). TRITC-LO entered the cytosol and then rapidly concentrated within the nuclei of preconfluent cultures of these cells, whereas carbonic anhydrase, a protein of similar molecular weight and similarly labeled, did not enter the cells under these conditions. LO that had been reductively methylated at lysine residues with [^{14}C]HCHO was also taken up into the cytosolic and nuclear compartments. Intracellular uptake and intracellular distribution were not altered by inhibiting LO activity with β-aminopropionitrile. An excess of native LO but not of carbonic anhydrase competitively inhibited the uptake of the isotopically labeled enzyme. Thus, once secreted and proteolytically processed, mature LO can enter the cells and concentrate within nuclei in a manner that appears to be specific and independent of its catalytic activity. J. Cell. Biochem. 79:576–582, 2000.

Key words: lysyl oxidase; aorta; smooth-muscle cells; extracellular space

The oxidation of peptidyl lysine in elastin and collagen to peptidyl α-aminoadipic-δ-semialdehyde by lysyl oxidase (LO) is required for the subsequent spontaneous formation of the lysine-derived crosslinkages that stabilize and insolubilize fibrous forms of elastin and collagen in the extracellular matrix [Kagan, 1986; Smith-Mungo and Kagan, 1998]. This extracellular activity of LO has long been considered to be its sole function in biology. Recent evidence suggests, however, that this enzyme may have additional important roles to play in biology. Thus, LO is potently chemotactic for human monocytes and lymphocytes [Lazarus et al., 1995] and for vascular smooth-muscle cells (SMC) [Li and Kagan, 2000]. Of additional interest is the finding that the cDNA sequence of rat aorta LO is 92% homologous and the protein sequence derived from the cDNA is 98% homologous to rrg, a *ras*-recision gene product, indicating the apparent identity of rrg with LO. The expression of rrg/LO suppressed the oncogenic potential of the *ras* oncogene in transformed mouse fibroblasts [Contente et al., 1990; Kenyon et al., 1991], pointing toward an antiproliferative effect of this catalyst. The observation that the expression of LO is markedly downregulated in a variety of transformed cell lines [Hamalainen et al., 1995] is consistent with these observations. Moreover, it was recently reported that LO is not only secreted by but also occurs within the nuclei of 3T3 fibroblasts and rat aorta SMC and that the nuclear enzyme can exhibit intranuclear catalytic activity [Li et al., 1997]. Earlier studies revealed that bovine aorta LO can oxidize lysine in a variety of basic but not acidic globular proteins, with histone H1 included among the susceptible substrates [Kagan et al., 1984]. Thus, it is possible that the nuclear enzyme may posttranslationally modify one or more

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basic proteins that reside in the nucleus, with consequences for nuclear function.

The route by which LO finds its way into the nucleus has not been discerned as yet. The pathway of nuclear uptake must account for the fact that the enzyme is a secreted protein that is initially synthesized as a 46-kDa preproenzyme containing an N-terminal signal peptide. After signal peptide removal and N-glycosylation within the propeptide domain, the resulting 50-kDa glycosylated proprotein is secreted and then proteolytically processed in the extracellular space by a metalloenzyme to yield the 32-kDa functional catalyst [Trackman et al., 1992; Panchenko et al., 1996]. The present report presents evidence that the fully processed, 32-kDa mature enzyme can be taken up by intact cells and then rapidly enter into and concentrate within the cell nucleus.

MATERIALS AND METHODS

Cell Culture

Neonatal rat aorta SMC, explanted from 2–3-day-old rat pups as described [Oakes et al., 1982], were used in first or second passage. The cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) containing 3.7 g $NaHCO_3$/l, 100 U of penicillin, and 100 μg streptomycin ml^{-1}, 0.1 mM nonessential amino acids,1 mM sodium pyruvate (GIBCO), and 10% fetal bovine serum (FBS).

Preparation and Incubation of Fluorescently Labeled Proteins

The rhodamine isothiocyanate derivative of LO or carbonic anhydrase (CA) was prepared according to a published method [Harlow and Lane, 1988]. LO used for this study was purified to homogeneity as the 32-kDa catalyst from bovine aorta, as described [Kagan and Cai, 1995]. Tetramethyl-rhodamine isothiocyanate (TRITC; 50 μg in 50 μl dimethyl sulfoxide) was added in 5-μl aliquots to solutions of LO (175 μg) or carbonic anhydrase (350 μg; Sigma, St. Louis, MO, 10,000 U mg^{-1} protein) in 1 ml of 0.1 M sodium carbonate, pH 9.0. The protein solution was gently stirred at 4°C for 24 h in the dark. Ammonium chloride was added to a final concentration of 50 mM, and the incubation was continued at 4°C for 2 h. Free dye was removed from the protein by chromatography through a 0.79 × 25-cm column of Sephadex G10 in 16 mM potassium phosphate, 0.15 M NaCl, pH 7.8 [phosphate-buffered saline (PBS) buffer]. The eluted pool of fluorescently labeled protein was stored in aliquots at −80°C. The 575 nm/280 nm ratio of light absorbance of each modified protein was 0.6, with an expected value for optimal labeling occurring between 0.3 and 0.7 [Harlow and Lane, 1988].

Vascular SMC were grown on coverslips (five per dish) in 100-mm dishes for 3 days in 10% FBS/ DMEM. The dishes were initially seeded with 1×10^4 or 1×10^6 cells to generate preconfluent or confluent cultures, respectively. The coverslips were then transferred to 12-well plates and incubated with 1.5 ml of 0.3% FBS/ DMEM per well for 30 min. TRITC-labeled protein (2 μg) was added to each well at time zero, individual coverslips were removed at intervals, the coverslips were washed five times with PBS, fixed in 3.7% formaldehyde in PBS for 1 h, washed five times with PBS, and finally mounted in Mowiol [polyvinyl alcohol] (Aldrich Chemical Co., Milwaukee, WI).

Preparation and Cellular Uptake of [^{14}C]LO

Purified bovine aorta LO was labeled by reductive methylation initially reacting the enzyme with [^{14}C]HCHO and then with sodium borohydride (Garre et al., 1992). LO (400 μg) in 16 mM potassium phosphate, pH 7.8, was mixed and incubated with 200 μCi [^{14}C]HCHO (NEN Life Science Products; 58 mCi/mmol) in a final volume of 1 ml at 4°C overnight. LO-HCHO adducts were then reduced by the addition of 5 μl of 1 M sodium borohydride freshly dissolved in 0.1 M NaOH. The mixture was incubated for 30 min and then dialyzed against several changes of 16 mM potassium phosphate, pH 7.8, at 4°C to remove unbound radioactivity. The specific radioactivity of the dialyzed enzyme was 1×10^5 dpm ^{14}C per microgram of LO protein. Aliquots of the modified enzyme were stored at −80°C. In preparation for uptake studies with confluent cells, 1×10^6 cells were seeded on 35 × 10-mm culture dishes and incubated in 10% FBS/ DMEM for 5 days with one media change after the first day of culture. Preconfluent cells were prepared by seeding 0.25×10^6 cells in 35 × 10-mm dishes, which were then cultured at 37°C for 24 h. Each cell preparation was then incubated with 0.3% FBS/DMEM for 30 minutes. [^{14}C]LO (10 μg) was added to the 1-ml volume of tissue culture media in the

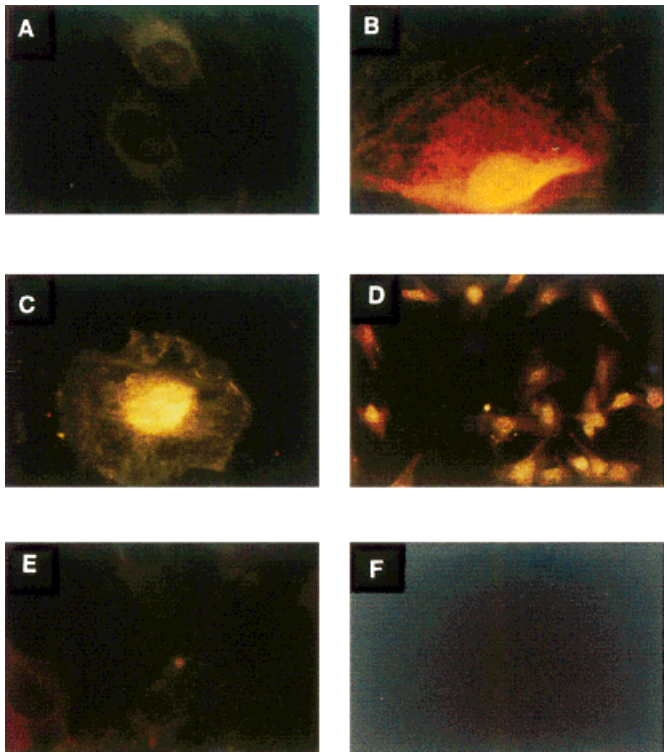

Fig. 1. Uptake of TRITC-LO by vascular SMC. Preconfluent cells were incubated with TRITC-LO for: (**A**) 10 min; (**B**) 60 min; (**C**) and (**D**) 4 h. **E:** Postconfluent cells incubated with TRITC-LO for 4 h. **F:** Preconfluent cells incubated with TRITC-CA for 4 h. (A, B, C, E, and F: ×1,000; D: ×400).

flasks, and the cells were harvested after designated intervals of incubation at 37°C. The cells were washed gently and repeatedly with PBS to remove all measurable, unbound radioactivity. The cells were then incubated in PBS with trypsin for 5 min followed by addition of an equal volume of 10% FBS/DMEM to inactivate the trypsin. The cellular preparation was sedimented by low-speed centrifugation, and the supernatant was removed. The cell preparation was then washed three times with PBS, and cells were processed for nuclear and cytoplasmic separation using a method employing cetylpyridinium chloride as described [Li et al., 1997]. Radioactivity in aliquots of cellular fractions was quantified by liquid scintillation spectrometry.

RESULTS

Uptake of Rhodamine-Coupled LO

Cells were cultured to preconfluency or to confluency, incubated for different intervals of time with TRITC-LO or TRITC-CA and, after removing free TRITC-labeled proteins, were examined for intracellular fluorescence as described in Materials and Methods. As shown in Figure 1, preconfluent cells incubated with TRITC-LO for 10 min (Fig. 1A) displayed slight fluorescence restricted to the cytosolic region. After 1 h of incubation (Fig. 1B), additional TRITC-LO had entered the cell with fluorescence appearing in both the cytosol and the nucleus of the preconfluent cells. Preconfluent cells incubated for 4 h with TRITC-LO predominantly displayed intranuclear staining, with relatively little TRITC-LO remaining in the cytosol (Fig. 1C,D). In contrast, confluent cells incubated with TRITC-LO for 4 h did not appear to have taken up significant quantities of the fluorescently labeled LO enzyme (Fig. 1E). Preconfluent cells were also incubated with TRITC-CA, selecting carbonic anhydrase as a candidate for this study in view of the similarity of its monomeric molecular weight (29 kDa) to that of LO (32 kDa). As shown in Fig. 1F, preconfluent cells incubated with TRITC-CA for 4 h (or earlier, data not shown) did not exhibit intracellular fluorescence, indicating that the labeled CA was not readily taken up into the SMC under the same conditions in which LO entered the cell and concentrated in the nucleus.

An additional probe was prepared with which to assess intracellular uptake of LO. For that purpose, LO was reductively aminated with [^{14}C]HCHO and $NaBH_4$ to generate isoto-

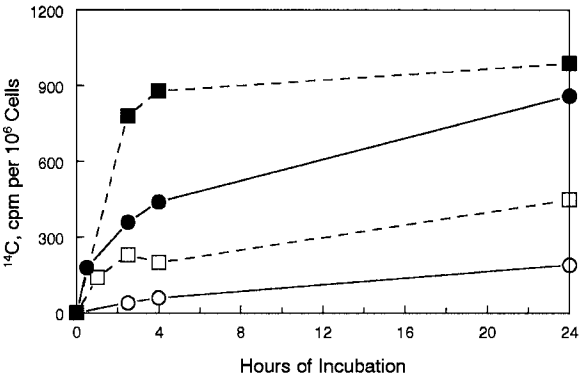

Fig. 2. Uptake of [^{14}C]LO by vascular SMC. Closed squares: preconfluent cells, cytosol; closed circles: preconfluent cells, nuclei. Open squares: postconfluent cells, cytosol; open circles: postconfluent cells, nuclei.

pically labeled N-epsilon, N′-epsilon-dimethyllysine ([^{14}C]DMLO) residues within the protein. The isotopically labeled enzyme was incubated for varying periods with confluent and preconfluent SMC, and the distribution of isotope in subcellular fractions was then determined as described. As shown in Figure 2, [^{14}C]DMLO entered and accumulated within the preconfluent cells more rapidly and to a greater degree than in the confluent cells, consistent with the results obtained with TRITC-LO (Fig. 1). Although the accumulation of [^{14}C]DMLO is greater in the cytosol than in the nuclei of the preconfluent cells at and before 4 h of incubation, the nucleus continues to accumulate this enzyme between 4 and 24 h while the concentration in the cytosol remains reasonably constant, consistent with the kinetically gated passage of extracellular LO to the cytosol and then the nucleus. The ratio of the significantly smaller amounts of accumulation in the cytosol and nuclei of confluent cells is relatively unchanged during this period (Fig. 2).

The relatively minor intracellular accumulation in the confluent cultures likely represents the uptake of enzyme by the small fraction of these cells present in the preconfluent growth condition that were seen at the periphery of the cultures (not shown). Moreover, the confluent cultures of these cells characteristically accumulate significant amounts of extracellular matrix, contrasting in this regard with the minimal matrix content of preconfluent cultures. Indeed, it is likely that the significantly smaller uptake of LO by confluent cultures reflects the strong tendency of LO to bind to extracellular fibers of collagen and elastin, which would then strongly compete for cellular uptake of the added enzyme by these cells. Although the [^{14}C]DMLO usefully enhances the options for following the intracellular uptake of LO, there are apparent differences between the kinetics of uptake of TRITC-LO and of [^{14}C]DMLO. Various possibilities can be suggested to account for these differences. For example, because of the different volumes of the nuclei and the cytosol, the concentrations of exogenously added LO appearing in these two compartments may be considerably different, whereas the total amounts of nuclear and cytosolic LO may not differ as dramatically. The quantitation of [^{14}C]DMLO content was based on the total amount in each compartment. In contrast, the visual impression of TRITC-LO content is likely to be influenced by the concentration of the fluorescently labeled enzyme, expected to be greater in the smaller nuclear volume even under conditions where equivalent levels of TRITC-LO might exist in both compartments. It is also possible that interactions of the rhodamine constituent of TRITC-LO with other molecules in the cytosol and nucleus may differentially affect the visible fluorescence yield of the enzyme derivative. It should also be noted that significant chemical differences exist between the substituent groups covalently added to native LO on modification with tetramethyl-rhodamine isothiocyanate or by the combined treatment with formaldehyde and sodium borohydride, with the former adding large aromatic substituents and the latter simply adding methyl groups to the ϵ-amino groups of peptidyl lysine. Such differences could influence the distribution of enzyme between nucleus and cytosol. Nevertheless, the data of Figures 1 and 2 are consistent with the conclusion that significant quantities of extracellular LO enter the cell and accumulate within the nuclei of preconfluent cells. This conclusion is also consistent with our previous report that Western blot analyses of the 32-kDa endogenous, intracellular enzyme occurred predominantly within the nuclei and not in the cytosol of vascular SMC [Li et al., 1997].

In view of the catalytic potential of LO, it was of interest to assess whether the functional active site was essential to the cytosolic and nuclear uptake of the enzyme. In vitro assays

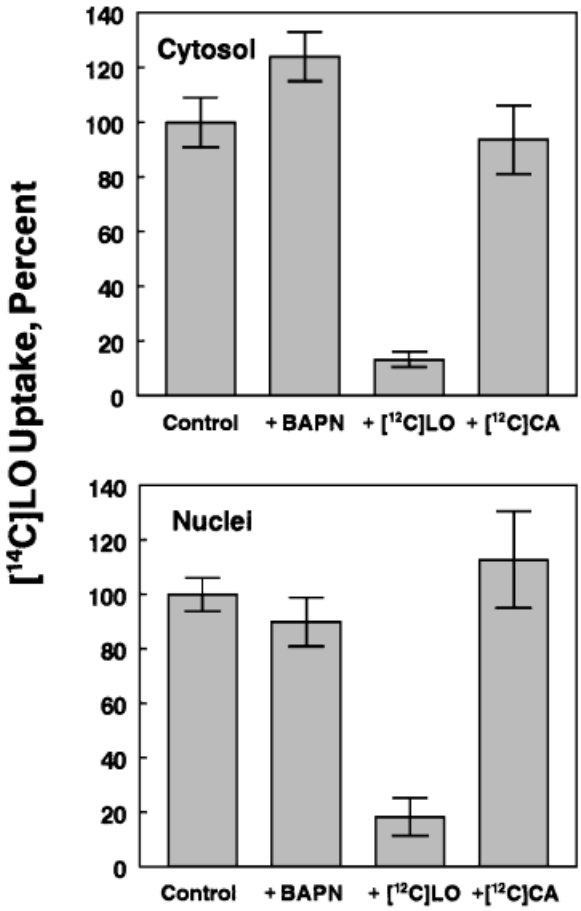

Fig. 3. Effect of BAPN, [^{12}C]LO, or [^{12}C]CA on the uptake of [^{14}C]LO by vascular SMC. Top: uptake into cytosol. Bottom: uptake into nuclei.

of the catalytic activities of both the TRITC-LO using [^{3}H]tropoelastin [Bedell-Hogan et al., 1993] and [^{14}C]DMLO using 1,5-diaminopentane as the oxidizable substrate [Trackman et al., 1981] revealed that both of these derivatives retained the bulk (95 ± 7%) of the specific catalytic activity of the native enzyme. Thus, SMC were incubated for 4 h with [^{14}C]DMLO at 37°C as before in the presence and absence of 100 μM β-aminopropionitrile (BAPN), an active site-directed inactivator of LO. After incubation, the medium was removed, and the cell layer was washed with PBS and then digested with trypsin to suspend the cells and to remove any externally bound [^{14}C]DMLO before disruption of the cell membranes. The cells were then extracted and the cytosolic and nuclear fractions were prepared. As shown in Figure 3, the uptake of the enzyme into the cytosolic and nuclear fractions in the presence of BAPN was not significantly different from the distribution seen in the control cells incubated without BAPN. Thus, the catalytic function does not appear to be essential to the uptake seen.

To assess the specificity of the intracellular uptake of [^{14}C]DMLO, SMC were incubated for 4 h at 37°C with 10 μg of the isotopically labeled enzyme in the presence and absence of either 75 μg of native LO or 100 μg of native carbonic anhydrase. As shown in Figure 3, native LO markedly reduced the appearance of the labeled enzyme in both the cytosol (Fig. 3, top) and nuclear compartments (Fig. 3, bottom) and thus successfully competed for the cellular uptake of [^{14}C]DMLO. In contrast, native carbonic anhydrase did not significantly alter the uptake of [^{14}C]DMLO into either subcellular fraction (Fig. 3).

DISCUSSION

The present study relied on the use of two different derivatives of LO to assess the potential of the enzyme to enter the intracellular compartment. Although each of the enzyme probes used here were generated by chemical modification of the native enzyme, enzyme activity was retained by both modified preparations, consistent with the likelihood that the native conformation of the enzyme was largely retained in these derivatives. Thus, the uptake of these enzyme derivatives appears to be reliably representative of the ability of native LO to enter the intracellular domain and then the nucleus. Therefore, although both the 50-kDa proenzyme and the fully processed, 32-kDa form of LO occur in the extracellular space, the present results indicate that the fully processed, functional catalyst derived from the extracellular space is a reasonable candidate for the source of the enzyme found in the cell nucleus. These findings are consistent with the previous demonstration by Western blot analysis of nuclear extracts that the 32-kDa LO species is found endogenously within the nuclei of SMC and 3T3 fibroblasts.

There are several precedents for extracellular proteins such as LO to find their way into cell nuclei. Among these examples, interleukin 1 and fibroblast growth factor-2 have been well documented [Bouche et al., 1987; Curtis et al., 1990], although the precise functions of these proteins within nuclei remain to be established. Other secreted products that, like LO,

have extracellular roles, such as the HIV-Tat protein [Frankel and Pabo, 1988; Westendorp et al., 1995] and homeoprotein Antennapedia [Bloch-Gallego et al., 1993] activate gene expression either within the cells that produce them or in neighboring cells after nuclear uptake of the extracellular forms. Although the exact mechanism of reentry of such secretory proteins into the cells is unknown, it has been speculated that they are initially concentrated in the pericellular environment by interacting with low-affinity binding sites, thus increasing their accessibility to the cell membrane through which they can pass by receptor-dependent or receptor-independent endocytotic mechanisms. HIV-Tat protein [Frankel and Pabo, 1988; Westendorp et al., 1995] and fibroblast growth factor-2 [Klagsbrun and Baird, 1991; Wiedlocha et al., 1994] can bind to pericellular heparan sulfate, whereas the homeoprotein Annapedia can bind to polysialic acid [Frankel and Pabo, 1988; Westendorp et al., 1995]. LO is known to show affinity to extracellular components, including its elastin and collagen substrates, which occur in proximity to the cell membrane during early stages of their formation [Kagan et al., 1986]. It is of interest in this regard that, once bound to collagen fibers, LO slowly dissociates from this matrix as free enzyme [Cronlund et al., 1985]. Such a transient binding relationship with pericellular collagen fibrils might then allow the same population of enzyme molecules to sequentially carry out their extracellular, crosslinking function and then their putative, intracellular function. LO also has affinity for tyrosine-rich (sulfated) acidic matrix protein [Forbes et al., 1994], although the significance of this interaction requires further analysis.

The biosynthetic pathway by which mature LO appears in the extracellular space initiates with the synthesis of the 46-kDa preproprotein from LO mRNA. Cleavage of the N-terminal signal peptide is followed by the addition of N-glycosyl units within the propeptide domain of the resulting proprotein [Trackman et al., 1992]. It is likely that the lysine tyrosylquinone cofactor is generated autocatalytically from its peptidyl lysine and tyrosine progenitors within the sequence destined to become the active site domain. This posttranslational modification may occur before secretion of the proenzyme [Curtis et al., 1990]. After secretion of the N-glycosylated, 50-kDa proenzyme, full catalytic potential is generated by a secreted metalloproteinase by cleavage between the two aspartic acid residues at a -GlyAspAsp- site within the proenzyme to remove the propeptide and yield the 32-kDa functional catalyst [Panchenko et al., 1996]. The present results suggest that a secretion–proteolytic activation-reuptake paradigm could account for the appearance of the processed enzyme in the nucleus. The possibility that the proenzyme may also find its way to the nucleus and then undergo processing within that organelle to the mature catalyst remains viable.

The clear affinity of the enzyme for the nucleus is consistent with the occurrence of several potential nuclear localization signals within the primary structure of LO, as previously noted [Li et al., 1997]. Indeed, studies in progress reveal that the oligopeptide sequences derived from the mature enzyme and expressed as a fusion protein with β-galactosidase result in the marked accumulation of β-galactosidase activity within the nuclei of transfected cells [Risitano and Kagan, unpublished data]. Although the role of nuclear LO remains to be discerned, the presence of numerous, lysine-containing, basic proteins within cell nuclei indicates that potential substrates are likely to exist within this cellular organelle.

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