Micro-injection of recombinant lysyl oxidase blocks oncogenic p21-Ha-Ras and progesterone effects on *Xenopus laevis* oocyte maturation

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Abstract Previous evidence suggested an anti-oncogenic role for lysyl oxidase, mainly in ras-transformed cells. Here we prove that recombinant lysyl oxidase is actually able to antagonize p21-Ha-Ras-induced *Xenopus laevis* oocyte maturation. Lysyl oxidase was also effective on progesterone-dependent maturation, indicating a block lying downstream of Ras. Maturation induced by activated 'maturation promoting factor', normally triggered by progesterone, was also inhibited by lysyl oxidase. Finally, lysyl oxidase did not abolish p42^{Erk2} phosphorylation upon maturation triggering, suggesting a block downstream of Erk2. Further investigation showed that lysyl oxidase action depends on protein synthesis and is therefore probably mediated by a newly synthesized protein.
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Key words: Lysyl oxidase; Cross-link; Ras; Progesterone; Germinal vesicle breakdown; *Xenopus laevis*

1. Introduction

Lysyl oxidase (protein 6-oxidase; EC 1.4.3.13) (LOX) [1–3] is a copper-dependent enzyme that converts by oxidative deamination the peptidyl lysine in tropocollagen and tropoelastin into α-aminoadipic-Δ-semialdehydes [4–8]. Since this step is the necessary precursor to cross-linkage formation, LOX is considered the key enzyme in the process of collagen maturation and therefore of extracellular matrix (ECM) stabilization. Primary or secondary alterations of LOX expression and/or activity have been described in several diseases characterized by serious impairment of connective tissues [9–12] or by pathological fibrosis [13–15]. LOX is synthesized as a 50 kDa pre-pro-enzyme that is translocated to the extracellular compartment, where a specific protease releases the active 30 kDa holo-enzyme [16,17], although the pro-peptide does not seem to be necessary for the extracellular translocation [18].

Despite its quite well known physiological activity, the role of LOX has recently been reconsidered on the basis of new experimental evidence, where the enzyme appears to be involved in the oncogenic reversion process. It has been observed that LOX expression and activity are down-regulated in many malignant cell lines derived from human tumors [19], as well as in several oncogenic transformed cell models [20–24]. Surprisingly, in these cellular models, mainly H-Ras transformed, LOX expression returned to the normal levels, no matter how the reversion of the phenotype occurred, whether after treatment with interferon α/β [20,21], azatyrosine [22] or spontaneously [23,24]. LOX involvement in cellular phenotype control has also been stressed in models of cellular differentiation [25]. A recent finding by Harada's group [26] has shed new light on the role of LOX in transformed cell reversion. They proved that IRF-1, a well characterized tumor suppressor induced by interferon, is able to activate the LOX promoter. This evidence provides a reasonable explanation for both flat reversion and LOX expression induced by interferon α/β [20]. Another recent observation suggestive of a role of LOX in tumor suppression showed that the LOX locus lies within the critical region of loss in human myelodysplasia and acute myelogenous leukemia [47].

Nevertheless, there are no hypotheses so far on how LOX could actually function as a tumor suppressor. Its extracellular position suggests a possible indirect action through a modification of the components of the ECM, but an action on some unknown intracellular substrate cannot be ruled out [21]. In fact, it is known that, at least in vitro, LOX can oxidize substrates other than elastin and collagen [27].

In the present report we tried to clarify some of the questions raised above. We took advantage of the *Xenopus laevis* oocyte system. Stage VI *X. laevis* oocytes turned out to be a very useful model to unravel the signal transduction involved in mitogenesis and other oncogene-dependent processes [28,29]. In particular, this model has been largely utilized to study Ras oncogenic effects. Injection of activated p21-Ha-Ras proteins into *X. laevis* oocytes triggers their meiotic maturation within 10–12 h, mimicking the physiological progesterone effect [30]. Therefore, in this model, induction of maturation has been assimilated as equivalent to induction of transformation in the cellular model counterpart [31]. The oocyte cell also allows direct micro-injection of the protein object of study and carries the complete machinery for any needed post-translocation modifications. In our study we tested the hypothesis of a direct interference of LOX in the Ras-dependent pathway, by co-injecting oncogenic p21-Ha-Ras^{val12} and mature LOX proteins into *X. laevis* oocytes. Furthermore, our approach addressed the issue regarding the possibility of an intracellular action of LOX, since in our experiments we used isolated oocytes, ruling out the possibility of an indirect effect through the ECM.

2. Materials and methods

2.1. Recombinant proteins

2.1.1. Lysyl oxidase. LOX and LOX(Δcat) fragments were amplified by reverse transcriptase polymerase chain reaction (RT-PCR)

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(Boehringer, Mannheim, Germany) from human fibroblast primary cell culture. The following primers were used: forward 5′-GGG GAT CCG AGC TCG AGA TGC GGT ACC ACC-3′ and reverse 5′-AGA GAA TTC AGA ACA CCA GGC ACT GAT-3′ for LOX; forward 5′-AAG GAT CCC TAC TAC ATC CAG GCA TCC ACG-3′ and reverse 5′CCG AAT TCG CTT TGT GGC CCT CGG CCA CTC-3′ for LOX(Δcat). The PCR was run in the buffer recommended by the manufacturer, only raising the $MgCl_2$ concentration to 2 mM. The amplification conditions were: for LOX, 1 min denaturation, 1 min annealing at 56°C, 2 min elongation at 72°C for 32 cycles; for LOX(Δcat), 30 s denaturation, 30 s annealing, 1 min elongation at 72°C for 28 cycles. The PCR products were respectively 793 and 444 bp. In particular, LOX encompassed the region 588–1380 of the human LOX cds [48], while LOX(Δcat) corresponded to the region 731–1041. Their identities were first checked by restriction mapping and finally by chain-termination sequencing [32]. Taking advantage of the restriction sites introduced into the PCR primers, *Bam*HI for the forward and *Eco*RI for the reverse primers, the two PCR products were cloned into the pTrc(His)A,B,C prokaryotic expression vector system (InVitrogen, San Diego, CA, USA). In order to obtain the insertion in frame with respect to the vector starting codon ATG, LOX was cloned in the type A vector and LOX(Δcat) in the B vector. The stop codon was included in the PCR fragment for LOX, since we amplified the cDNA portion encoding the full mature protein, while for the LOX(Δcat) fragment, missing the 3′ part of the LOX coding sequence, it was provided by the expression vector, adding 11 amino acids at the COOH terminus. The two recombinants were expressed in *Escherichia coli* JM109 strain upon induction by 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3–5 h. After lysis in 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0 the proteins were purified by affinity chromatography using Ni-NTA silica resin in column or in batch (Qiagen, Hilden, Germany). The non-specific binding was washed out twice by 2 volumes of the same buffer, but at pH 6.3. Finally, the recombinant proteins were collected by elution with washing buffer/0.1 M EDTA and checked for purity on 12% SDS-polyacrylamide gel electrophoresis [33]. The purified proteins were then dialyzed against 20 mM MES, pH 7.0 or PBS, pH 7.2. The average preparation resulted in a 90% pure recombinant protein. Since the two proteins are poorly dyed by the Coomassie blue (Bradford) method [34], their concentrations were estimated by OD_{280}, according to the calculated A_{280} of 1.43 for LOX(Δcat) and 1.95 for LOX.

2.1.2. p21-Ha-Ras^{val12}. The p21-Ha-Ras^{val12} recombinant protein was engineered and purified as previously described [35]. Briefly, after induction by temperature shift to 42°C, the urea extracts (7 M urea, 20 mM MES, pH 7.0) were further purified through a Sephadex G-100 column (90×2.5 cm), using as eluant the same buffer as above. The different fractions were analyzed by SDS-PAGE. The ones containing more than 90% pure p21-Ha-Ras were collected and dialyzed against 20 mM MES, pH 7.0. The protein concentration was estimated by the Bradford method.

2.1.3. Tropoelastin. The bacteria transformed with the plasmid pAS-tropoelastin were kindly provided by Dr. J. Rosenbloom (School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA). The expression and preparation of the recombinant human tropoelastin were performed as described [36,37]. Briefly, an aliquot of an overnight culture of bacteria containing the specific expression vector was diluted 1:50 in 1 liter of L broth containing 50 μg/ml of ampicillin and allowed to grow at 37°C with shaking for about 90 min (OD_{590} = 0.6–0.7). Then the cells were collected and washed three times in lysine-free RPMI medium (RMPI 1640, Select-Amine kit, Life Technologies, NY) and incubated for 10 min with shaking at 37°C. Then the specific protein expression was induced by adding 60 μg/ml of nalidixic acid. After 2 h of further incubation [L-4,5-^{3}H]lysine (1–2 mCi/l) was added and the incubation was continued under the same conditions for 3 more hours. The labeled cells were collected and washed in phosphate buffer and resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA-Na_2, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The cell suspension was then digested with 200 μg/ml of lysozyme, in the presence of 0.05% sodium deoxycholate, at 4°C for 30 min.

The bacteria were then Dounce-homogenized and the resulting pellet was resuspended in 4 ml of 70% formic acid containing 63 mg of CNBr and stirred overnight at room temperature, to get rid off all the methionine-containing bacterial proteins. The solution was diluted with one-half volume of water and incubated uncovered on ice in a hood for 4–5 h to allow the excess HCN to escape. Finally the pellet was discarded and the supernatant dialysed against 0.1 M acetic acid. The purity of recombinant tropoelastin was analyzed by SDS-PAGE and its labeling by autoradiography. Since the degree of tropoelastin was more than 90% and there were no additional labeled proteins, we did not proceed to further HPLC purification as recommended in the original method.

2.2. Lysyl oxidase assay

The enzymatic assay was performed using recombinant [L-4,5-^{3}H]lysine labeled tropoelastin (see above) as substrate. The assay was run in 1 ml of incubation buffer, 0.1 M Na-borate, pH 8.0, 0.15 M NaCl and 5–15 μg of recombinant LOX was used as enzyme source. For each assay a sample containing 0.1 mM β-amino-propionitrile (BAPN) was run as blank. The tritiated H_2O_2 released from the reaction was estimated by ultrafiltration in Amicon-10, according to Shackleton [38]. An aliquot from the flow-through was counted with a β-counter. The resulting net cpm (cpm sample−cpm relative BAPN sample) was then expressed as pmol of tropoelastin converted into cross-linked product, according to its specific ^{3}H incorporation (cpm/μg).

2.3. Oocyte maturation and micro-injection

Oocytes were manually excised from *X. laevis* ovaries and selected according to their size and shape, in order to have a homogeneous stage VI population. The micro-injection of the indicated substances was performed as previously described [39]. In all the experiments the oocytes were incubated in Ringer's buffer at a conditioned room temperature of 16–18°C. The maturation was evaluated after 12–18 h by the appearance of a white spot on the animal pole of the treated oocytes. Where needed, the oocytes were fixed in 16% TCA and split open for a visual verification of the nuclear germinal vesicle status.

2.4. Maturation promoting factor (MPF) oocyte extract

MPF was prepared as previously described [40,41]. Within 2 h after maturation induced by 10 μg/ml of progesterone, the oocytes were homogenized in 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 20 mM HEPES, pH 7.5, in a ratio of 20 μl/10 oocytes. The extract was centrifuged at 3000×*g* for 10 min and the supernatants used for micro-injection were indicated.

2.5. p42^{Erk2} Western blot analysis

The oocytes were lysed in lysis buffer (PBS, pH 7.2, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin). Approximately 50 μg of protein for each sample was separated in PAGE on a 10% polyacrylamide gel (Laemmli, 1970) and blotted to a Hybond-Super C nitrocellulose membrane (Amersham, UK). The blots were probed with anti-p42^{Erk2} polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and developed using an alkaline phosphatase conjugated second antibody.

3. Results

3.1. LOX blocks Ras-dependent GVBD: the effect is reverted by a LOX inhibitor

In preliminary experiments we fixed the amounts of p21-Ha-Ras^{val12} protein that would give 60–80% GVBD, since a sub-saturated state of the pathway would allow a better detection of either stimulation or inhibition of the maturation process. In our experiments we used two different recombinants of LOX: one deprived of the Cu^{2+} binding/catalytic domain, LOX(Δcat), and the full active holo-enzyme, LOX. In Fig. 1A a scheme of the generation of the two recombinants is shown. Briefly, the fragments 731–1041 for LOX (Δcat) and 588–1380 for LOX were amplified by PCR from human lysyl oxidase cDNA. LOX, the holo-enzyme fragment corresponded to the mature lysyl oxidase after proteolytic cleavage [16]. The primers that we used contained the restriction sites *Bam*HI at the 5′ end and *Eco*RI at 3′ to make the

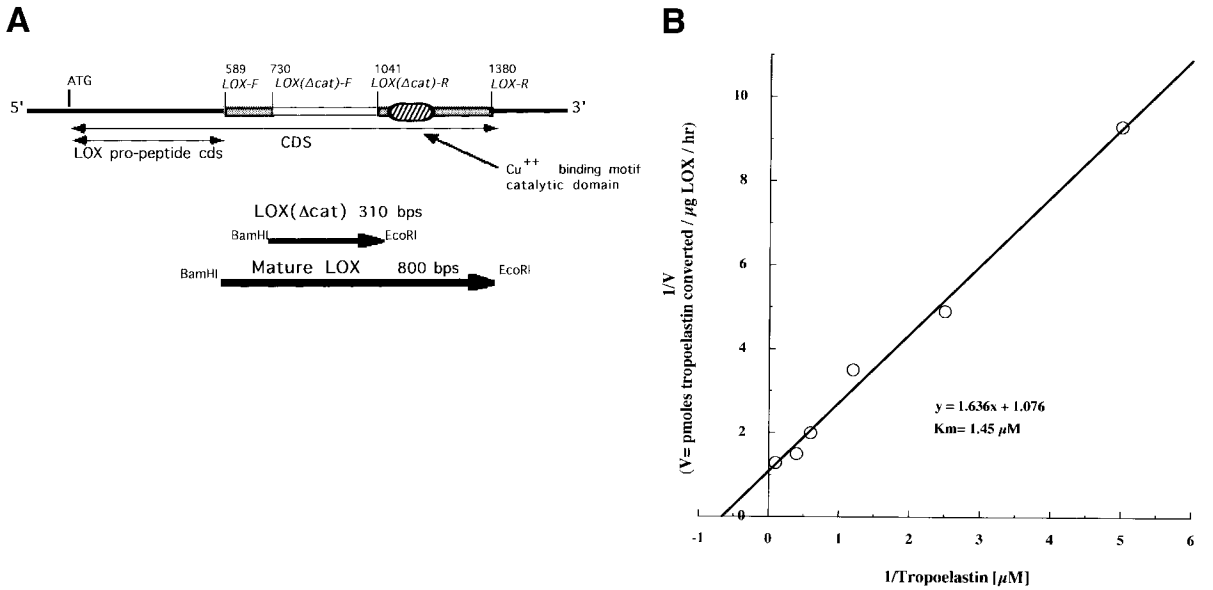

Fig. 1. A: Generation of recombinant lysyl oxidase fragments. The two lysyl oxidase fragments were obtained by PCR amplification from human fibroblast total RNA. The PCR primers contained at their 5′ end the *Bam*HI and *Eco*RI restriction sites needed to subclone the amplified fragments into the specific pTrc(His)A or B prokaryotic expression vector. More details are specified in Section 2. B: Kinetic analysis of the enzymatic activity of recombinant lysyl oxidase. Lineweaver-Burke plot. 10 μg of recombinant LOX was used for the assay, while the substrate, the recombinant tropoelastin, labeled with [L-4,5-^{3}H]lysine, was used at the indicated concentrations. The enzymatic activity was calculated from the release of tritiated H_2O_2 and converted into pmol of tropoelastin converted per μg of LOX per hour. A $K_m = 1.45$ μM calculated from the equation, $1/V = K_m/V_{max}\ 1/S + 1/V_{max}$, describes the linear interpolation of the experimental results. Further details about the assay are described in Section 2.

resulting PCR products compatible with a directional cloning into the prokaryotic expression vector pTrc(His)A,B,C. More details are given in Section 2. In Fig. 1B a typical enzyme kinetic of the recombinant LOX is shown. The apparent K_m, 1.45 μM, calculated from the Lineweaver-Burk plot, is consistent with the previously published analysis performed on the purified LOX [6,27,36]. When LOX was co-injected with p21-Ha-Ras^{val12}, the GVBD was strongly inhibited in a dose-dependent fashion, which already accounts for a specific action (Fig. 2). The inhibition was quite complete for p21/LOX ≤ 1. On the other hand, LOX alone did not affect the quiescent status of the oocyte, while LOX(Δcat) only slightly affected p21-Ha-Ras^{val12}-induced oocyte maturation, as shown in Table 1a. Surprisingly, instead, we noticed that LOX(Δcat) interfered significantly with LOX effects. Furthermore, we tested if LOX effects were affected by BAPN, a recognized highly specific LOX inhibitor [42,43]. In Fig. 3 we show that while BAPN did not affect Ras-dependent GVBD, it completely abolished LOX effects. BAPN was effective both by micro-injection and by addition to the oocyte incubation medium, being able to diffuse through the cellular membrane (data not shown). These results provide further confirmation of the specificity of LOX inhibition of Ras-dependent GVBD.

3.2. *LOX inhibits GVBD triggered by Ras-independent pathways*

The above findings did not assess a LOX action specific for Ras-dependent meiotic maturation. Thus, the inhibition could involve some step common to different GVBD triggering pathways. To address this issue we studied LOX effects on progesterone-dependent GVBD, which follows a pathway different from that triggered by Ras [29]. As expected, in our experimental system, progesterone was able to induce close to 100% oocyte maturation at concentrations ranging between 2 and 10 μg/ml (data not shown). As in the Ras-dependent process, LOX was able to inhibit progesterone action in a dose-dependent way (Fig. 4). Furthermore, Fig. 4 shows that, as in Ras experiments, the LOX effect was specific, since it was reversed by co-injection of BAPN. In Table 1b we also show that LOX(Δcat) is not able to reproduce LOX effects, ruling out any non-specific effect. However, in our experimental conditions we could not obtain more than 60–70% inhibition of progesterone-induced GVBD, even with concentrations of LOX much higher than those used for p21-Ha-ras

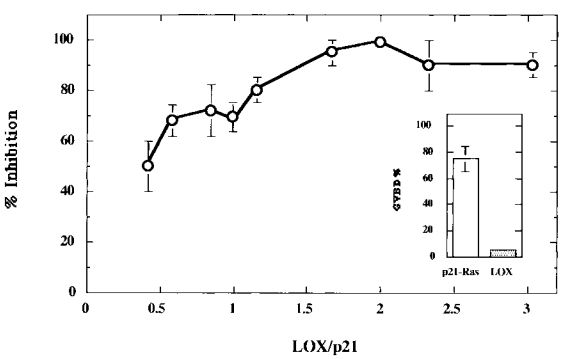

Fig. 2. Dose-dependent inhibition of p21-Ha-Ras^{val12} (p21)-induced GVBD by recombinant LOX. Different molar ratios of p21 and LOX recombinant proteins were injected into *X. laevis* stage VI oocytes. The GVBD was evaluated by the appearance of depigmentation in the animal pole of the oocytes or by physical examination of the disappearance of the germinal vesicle, upon dissection of the oocytes after TCA fixation. In the insert is shown the relative percent GVBD induced by 0.5 pmol of p21-Ras or 0.3 pmol of recombinant LOX alone. The details are specified in Section 2. Each point represents the average of at least three independent experiments ± S.E.M.

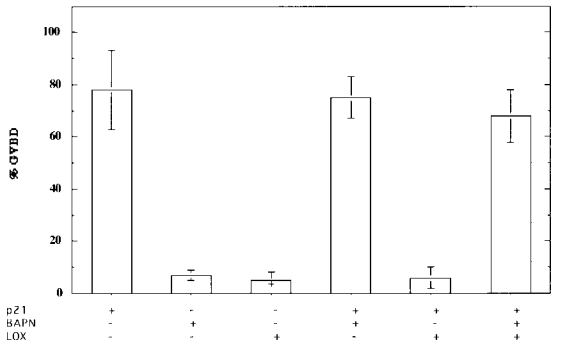

Fig. 3. Reversion of LOX inhibition of GVBD by BAPN. About 0.3 pmol of recombinant p21-Ha-Ras^{val12} was injected alone or with equimolar amounts of LOX or LOX+BAPN as indicated. Where present, 5 nl of 5 mM BAPN solution, in order to have an intra-oocyte concentration of ~0.01 mM, assuming an average oocyte volume of 500 nl. The figure shows the average results of at least two independent experiments ± S.E.M.

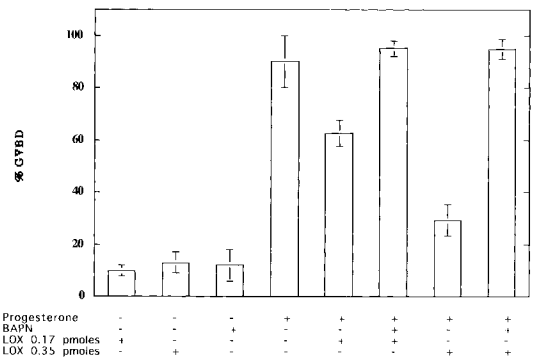

Fig. 4. LOX inhibition of progesterone-dependent GVBD. *X. laevis* oocyte maturation was monitored after 12–18 h in the presence of the indicated substances. When present, progesterone was used at a concentration of 10 μg/ml. More details are given in Section 2. The figure shows the average results of at least three independent experiments ± S.E.M.

experiments. Unfortunately we were not able to micro-inject higher doses of recombinant LOX, because of its instability at concentrations above 0.6–0.8 mg/ml. So it remains unclear if the incomplete inhibition of the progesterone-dependent GVBD process is indicative of a partial or a 'side effect' of LOX, as compared with the more radical effects observed on the Ras-dependent process. This point will be the object of more extended speculation in Section 4. In order to define the step inhibited by LOX, we turned our attention to the MPF, which is a complex constituted by p34^{cdc2} and cyclin B [44]. This complex is activated upon progesterone treatment [41,45]. In Fig. 5 we show that the micro-injection of activated MPF from freshly progesterone matured oocytes [40] was able to antagonize LOX effects on Ras-dependent GVBD. On the other hand, LOX still blocked oocyte maturation when the process was triggered by the same MPF preparation alone. All together these data suggest a block lying downstream of MPF. Going further down the maturation pathway we tested the influence of LOX on the activation status of p42^{Erk2} upon Ras or progesterone triggering. The anti-p42^{Erk2} Western blot shown in Fig. 6 demonstrates that LOX micro-injection in both cases did not affect the activation. The retardation shift due to the p42^{Erk2} phosphorylation is indeed maintained in the presence of LOX. These findings are compatible with a LOX-dependent block downstream of Erk2. Considering that the Ras and progesterone pathways are known to merge at Erk2 level, our results provide a rationale for the LOX inhibitory effect on both triggering factors.

Table 1
Influence of LOX(Δcat) fragment on LOX inhibition of Ras or progesterone-dependent GVBD

Addition	% GVBD
a	
p21-ras	72 ± 10
+LOX	3 ± 0.6
+LOX(Δcat)	60 ± 6
+LOX+LOX(Δcat)	32 ± 9
b	
Progesterone	93 ± 5
+LOX	52 ± 7
+LOX(Δcat)	88 ± 6
+LOX+LOX(Δcat)	65 ± 7

GVBD was induced by (a) sub-saturating amounts of p21-Ha-Ras^{val12} (0.3 pmol) or (b) 10 μg/ml of progesterone. Where indicated, equimolar amounts of LOX(Δcat) or/and LOX were co-injected. The table shows the average results of at least three independent experiments ± S.E.M.

3.3. *LOX effects are mediated by protein synthesis*

It is known that Ras induction of oocyte maturation is independent of protein synthesis [46], while progesterone needs newly synthesized protein to achieve its effect [41]. In our model we show that LOX also needed active protein synthesis to inhibit the GVBD process. In fact, as shown in Table 2, cycloheximide was able to relieve completely LOX inhibition in Ras-induced GVBD. This finding proves that LOX acts indirectly, inducing a protein factor(s), which would be actually responsible for the above described effects.

4. Discussion

Our study shows that mature 30 kDa lysyl oxidase protein is actually able to antagonize oncogenic Ras-dependent meiotic maturation of *X. laevis* oocytes. This finding assumes

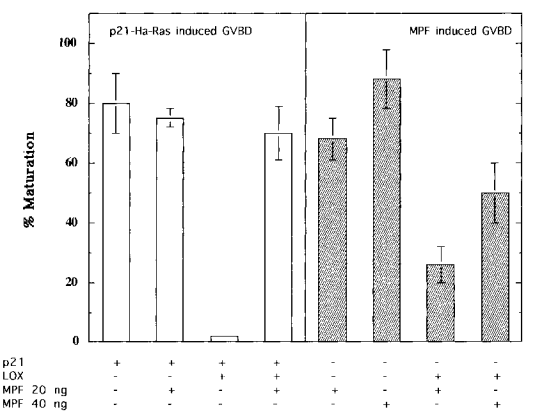

Fig. 5. Reciprocal effects of MPF and LOX in GVBD induction. GVBD was induced by 0.3–0.5 pmol of p21-Ha-Ras^{val12} or by the indicated amounts of MPF extract, alone or in the presence of 0.35 pmol of LOX. Percent GVBD was assessed as specified in Table 1 and in Section 2. The figure shows the average results of at least three independent experiments ± S.E.M.

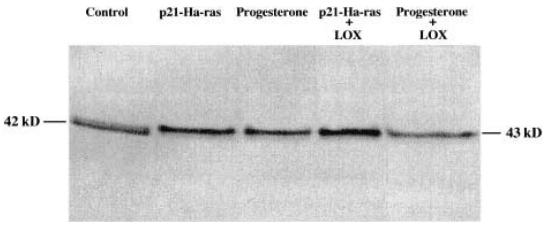

Fig. 6. Anti-p42^{Erk2} and anti-phosphotyrosine Western blots of oocyte extracts treated with progesterone alone or progesterone plus LOX. The oocytes were incubated with Ringer buffer (control), progesterone 10 μg/ml or micro-injected with 0.3 pmol of p21-Ha-Ras^{val12} and/or with 0.35 pmol of LOX where indicated. After the usual incubation time needed to observe the induced GVBD (8–12 h), the oocytes were lysed in lysis buffer and processed for 12% PAGE/Western blotting as described in Section 2. The blots were developed using alkaline phosphatase-coupled color forming reaction.

particular interest considering that a role has been claimed for LOX in tumor suppression [19–24,26]. Our results show that LOX abolished Ras-dependent GVBD in a dose-dependent way (Fig. 2). Its effect is specific as we demonstrated that co-treatment with BAPN, a strong inhibitor of LOX catalytic activity, was able to abolish it (Fig. 3). Furthermore, micro-injection of a LOX fragment deprived of catalytic activity, LOX(Δcat), failed to inhibit Ras-dependent GVBD (Table 1). This also proved that the observed results could not be explained by any interference of $(His)_6$ tag at the NH_2 terminus of recombinant LOX. We also noticed a partial relief of LOX inhibition when LOX(Δcat) and LOX were co-injected with p21-Ha-Ras^{val12}. This can be explained by a competition between the two protein constructs for a common ligand (or substrate). Thus, the consequent subtraction of the physiological ligand by LOX(Δcat), unable to process it, would result in the observed impairment of active LOX action. Obviously, other models can be compatible with this finding, although it does not change the fact that the observed inhibition of Ras effects is strictly dependent on the presence of a catalytically active LOX.

The inhibition of GVBD by LOX was effective also when triggered by other factors, like progesterone or MPF, a component in the kinase cascade leading to oocyte maturation (Figs. 4 and 5). It is worth noting the paradoxical results we obtained in the experiment with activated MPF. When p21-Ha-Ras was the trigger of GVBD, co-injection of MPF antagonized the LOX inhibiting effect, while when the MPF triggered GVBD, LOX was still able to achieve its inhibition. The two apparently contrasting results can be reconciled, if we assume that LOX blocks a factor downstream of MPF. In this case, when p21-Ha-Ras has already activated the maturing pathway, the addition of MPF does not improve the final effect, but it does increase the amount of that putative factor in its activated state. As a result, being in constant amount during the experiment, LOX might have been unable to contrast the excess of target. In the condition when MPF alone triggers GVBD, the amount of activated factor/LOX could fall in a stoichiometric ratio to allow the observed inhibition. In effect MPF induced a percent GVBD similar to or lower than that achieved with p21-Ha-Ras, which would correspond, in the above model, to a comparable if not lesser amount of activated LOX target. Since it is known that the Ras and progesterone maturing pathways merge at the Erk2 level, we investigated a possible influence of LOX on p42^{Erk2} activation. Western blot analysis (Fig. 6) clearly showed that LOX-treated oocytes still undergo p42^{Erk2} phosphorylation upon progesterone triggering. Together our data definitely account for a LOX-dependent block downstream of Ras and closer to the late events preceding the maturation. The inhibited step appears to be downstream of MPF, where probably Ras, progesterone and other maturing signals merge into the final common effect. Although so far no difference of Ras and progesterone-dependent maturing signals downstream of MAP kinases has been proposed, the observed LOX partial inhibition (30–60%) of progesterone/MPF-dependent oocyte maturation might mean that there are some. Also LOX might affect some main meiotic mechanism that mainly inhibits the Ras pathway, but at the same time activates negative feedbacks to block co-targeting signals, like the progesterone-triggered ones. Nevertheless a much simpler explanation, as already previously mentioned, is that we could not achieve a more effective concentration of recombinant LOX for intrinsic solubility reasons. In support to this hypothesis the experiment in Fig. 4 showed that the inhibition of LOX of progesterone-triggered maturation is dose-responsive in the tested range of concentrations. Furthermore, we proved that LOX effects toward Ras-induced GVBD are abolished in the presence of cycloheximide (CHX), an inhibitor of protein synthesis. This strongly suggests that LOX-dependent inhibition is mediated by a protein produced during the duration of the experiment. Thus, LOX must activate some specific transcription factor, driving the production of a protein, actually responsible for GVBD inhibition. In agreement with the previous results, this putative factor should block the maturation pathway at a point that lies downstream of Ras and MPF and Erk2.

Finally, our study brings new evidence on a possible LOX tumor suppressor role. We showed that, although such an activity might not directly affect a Ras-dependent biochemical step, LOX effects result in an inhibition of a Ras-triggered process. Furthermore, our data suggest that LOX effects are mediated by a de novo synthesized protein, that blocks the oocyte at a late step of its maturation pathway. Although we identified a 50 kDa endogenous LOX form in *X. laevis* oocytes, using antibodies raised against our recombinant LOX (data not shown), we have no proof that this mechanism is actually effective in oocyte maturation. Nevertheless our model suggests a possible explanation for LOX inhibition of other Ras-induced effects, like mitogenesis and transformation in cellular models.

Table 2
Cycloheximide effects on LOX inhibition of GVBD

Addition	% GVBD
p21-Ha-Ras	80 ± 10
+CHX	80 ± 5
+LOX	2 ± 0.3
+CHX+LOX	56 ± 7

GVBD was induced by micro-injection of 0.3–0.5 pmol of p21-Ha-Ras^{val12} with or without equimolar amounts of LOX. Where indicated, the *X. laevis* oocytes were treated with 2 μM cycloheximide (CHX). GVBD evaluation was performed as described in Section 2. The table shows the average results of two independent experiments ± S.E.M.

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References

[1] Trackman, P.C., Pratt, A.M., Wolansky, A., Tang, S.S., Offner, G.D., Troxler, R.F. and Kagan, H.M. (1990) Biochemistry 29, 4863–4870.
[2] Svinarich, D.M., Twomey, T.A., Macauley, S.P., Krebs, C.J., Yang, T.P. and Krawetz, S.A. (1992) J. Biol. Chem. 267, 14382–14387.
[3] Hämäläinen, E.R., Kemppainen, R., Pihlajaniemi, T. and Kivirikko, K.I. (1993) Genomics 17, 544–548.
[4] Pinnel, S.R. and Martin, G.R. (1968) Proc. Natl. Acad. Sci. USA 61, 708–716.
[5] Siegel, R.C. (1974) Proc. Natl. Acad. Sci. USA 71, 4826–4830.
[6] Siegel, R. (1979) Int. Rev. Connect. Tissue Res. 8, 73–118.
[7] Kagan, H.M. and Trackman, P.C. (1991) Am. J. Respir. Cell Mol. Biol. 5, 296–310.
[8] Reiser, K.M., McCornick, R. and Rucker, R.B. (1992) FASEB J. 6, 2439–2449.
[9] Starcher, B., Madaras, J., Fisk, D., Perry, E. and Hill, C. (1978) J. Nutr. 108, 1229–1233.
[10] Royce, P.M., Camakaris, J. and Danks, D.M. (1980) Biochem. J. 192, 576–586.
[11] Kuivaniemi, H., Peltonen, L. and Kivirikko, K.I. (1985) Am. J. Hum. Genet. 37, 798–808.
[12] Romero-Chapman, N., Lee, J., Tinker, D., Uriu-Hare, J.Y., Keen, C.L. and Rucker, R.R. (1991) Biochem. J. 257, 657–662.
[13] Murawaki, Y., Kusakabe, Y. and Hirayama, C. (1991) Hepatology 14, 1167–1173.
[14] Sommer, P. et al. (1993) Lab. Invest. 69, 460–470.
[15] Di Donato, A., Jivotenko, E., Di Duca, M., Ghiggeri, G., Acinni, R., Campolo, J., Ginevri, F. and Gusmano, R. (1997) Nephron 76, 192–200.
[16] Cronshaw, A.D., Fothergill-Gilmore, L.A. and Hulmes, D.J. (1995) Biochem. J. 306, 279–284.
[17] Panchenko, M.V., Stetler-Stevenson, W.G., Trubetskoy, O.V., Gacheru, S.N. and Kagan, H.M. (1996) J. Biol. Chem. 271, 7113–7119.
[18] Kagan, H. and Cai, P. (1995) Methods Enzymol. 258, 122–132.
[19] Kuivaniemi, H., Korhonen, R.M., Vaheri, A. and Kivirikko, K.I. (1986) FEBS Lett. 195, 261–264.
[20] Contente, S., Kenyon, K., Rimoldi, D. and Friedman, R.M. (1990) Science 249, 796–798.
[21] Kenyon, K., Contente, S., Trackman, P.C., Tang, J., Kagan, H.M. and Friedman, R.M. (1991) Science 253, 802.
[22] Krzyzosiak, W.J., Shindo-Okada, N., Teshima, H., Nakajima, K. and Nishimura, S. (1992) Proc. Natl. Acad. Sci. USA 89, 4879–4883.
[23] Oberhuber, H., Seliger, B. and Schafer, R. (1995) Mol. Carcinogen. 12, 198–204.
[24] Hajnal, A., Klemenz, R. and Schäfer, R. (1993) Cancer Res. 53, 4670–4675.
[25] Dimaculangan, D.D., Chawla, A., Boak, A., Kagan, H.M. and Lazar, M.A. (1994) Differentiation 58, 47–52.
[26] Tan, R.S., Taniguchi, T. and Harada, H. (1996) Cancer Res. 56, 2417–2421.
[27] Kagan, H.M., Williams, M.A., Williamson, P.R. and Anderson, J.M. (1984) J. Biol. Chem. 259, 11203–11207.
[28] Smith, L.D. (1989) Development 107, 685–699.
[29] Maller, J.L. (1990) Biochemistry 29, 3157–3166.
[30] Birchmeier, C., Broeck, D. and Wigler, M. (1985) Cell 43, 615–621.
[31] Feramisco, J.R., Gross, M., Kamata, T., Rosenberg, M. and Sweet, R.M. (1984) Cell 38, 109–117.
[32] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463.
[33] Laemmli, U.K. (1970) Nature 227, 680–685.
[34] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[35] Lacal, J.C. and Aaronson, S.A. (1986) Proc. Natl. Acad. Sci. USA 83, 5400–5404.
[36] Bedell-Hogan, D., Trackman, P., Abrams, W., Rosenbloom, J. and Kagan, H.M. (1993) J. Biol. Chem. 268, 10345–10350.
[37] Indik, Z., Abrams, W.R., Kucich, U., Gibson, C.W., Mecham, R.P. and Rosenbloom, J. (1990) Arch. Biochem. Biophys. 280, 80–86.
[38] Shackleton, D.R. and Hulmes, D.J.S. (1990) Anal. Biochem. 185, 359–362.
[39] Lacal, J.C. (1990) Mol. Cell. Biol. 10, 333–340.
[40] Campa, M.J., Glickman, F.J., Yamamoto, K. and Chang, K.-J. (1992) Proc. Natl. Acad. Sci. USA 89, 7654–7658.
[41] Carnero, A., Jimenez, B. and Lacal, J.C. (1994) J. Cell. Biochem. 55, 465–476.
[42] Riley, D.J., Kerr, J.S., Berg, R.A., Iamu, B.D., Pietra, G.G., Edelman, N.H. and Prockop, D. (1982) Am. Rev. Respir. Dis. 125, 67–73.
[43] Levene, C.I. and Carrington, M.J. (1985) Biochem. J. 232, 293–296.
[44] Ferrel, J.E., Wu, M., Gerhart, J. and Martin, G.S. (1991) Mol. Cell. Biol. 11, 1965–1971.
[45] Nebreda, A.R., Porras, A. and Santos, E. (1993) Oncogene 8, 467–477.
[46] Allende, C.C., Hinrichs, M.V., Santos, E. and Allende, J.E. (1988) FEBS Lett. 234, 426–430.
[47] Fairman, J., Kottickal, L. and Lalitha, N. (1995) Am. Soc. Biochem. Mol. Biol., San Francisco, CA, 21–25 May, abstr. A1281, No. 147.
[48] Hämäläinen, E.R., Jones, T.A., Sheer, D., Taskinen, K., Pihlajaniemi, T. and Kivirikko, K.I. (1991) Genomics 11, 508–516.