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Measurement of Medium Lysyl Oxidase Activity in Aorta Smooth Muscle Cells. Effects of Multiple Medium Changes and Inhibition of Protein Synthesis†

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ABSTRACT: Cultures of rabbit aortic smooth muscle (RSM) cells are a valuable model system for studying production and metabolism of connective tissue components. This report describes various assay procedures for lysyl oxidase, the enzyme responsible for deaminating lysine residues to give aldehyde cross-link precursors, in culture medium from these cells. Studies of the medium enzyme from second-passage RSM cells indicate that approximately 40% of the total enzyme activity in the flask of cells is in the medium. The medium enzyme levels are replenished quite rapidly following refeeding, and enzyme levels in the medium appear to be feedback controlled. The mechanism for this control is unknown at present. Multiple refeeding experiments in which the medium was changed every 2–4 h for up to 40 h indicate that these cells are capable of producing large amounts of enzyme and are capable of altering enzyme production and secretion quite rapidly in response to changes in their environment. Protein synthesis inhibitor studies with cycloheximide suggest that the major portion of the enzyme released into the medium following refeeding is newly synthesized although a pool of latent enzyme is also present. As in intact tissue, extraction of the enzyme from the cell layer requires strong denaturing reagents such as 4 M urea. These results suggest that the production of lysyl oxidase is closely regulated and is very responsive to changes in the external environment of the cells. This cell culture system appears to be an excellent one to study the production of lysyl oxidase and its role in connective tissue fibrillogenesis.

Lysyl oxidase (LO), the enzyme responsible for the oxidative deamination of key lysine residues in collagen and elastin prior to cross-link formation, plays a pivotal role in the formation of a stable, insoluble extracellular matrix. In general, this enzyme appears to function predominantly extracellularly and to be bound tightly to some component of the extracellular matrix in vivo (Chvapil et al., 1974). Thus, strong denaturing reagents such as urea are required to solubilize the enzyme prior to in vitro measurement of enzyme activity (Narayanan et al., 1974; Harris et al., 1974; Kagan et al., 1974; Chvapil et al., 1974). There have been few reports of measurement of lysyl oxidase activity in cell culture systems. Layman et al. (1972), studying lysyl oxidase activity in fibroblast cell cultures, have suggested that the enzyme levels are low and require concentration of the cell medium in order to recover measurable amounts of activity. Levine & Heslop (1977) demonstrated increased medium LO activity with increasing culture age in pig aortic endothelial cells. To date, however, there have been no reports on the kinetics of LO production in cell cultures.

This report describes techniques for measuring lysyl oxidase activity in small volumes of unconcentrated medium from rabbit aortic smooth muscle cells and IMR-90 lung fibroblasts. These methods permit serial measurements of enzyme activity over long periods of cell culture which allows one to monitor rapid changes in lysyl oxidase activity in the cell culture medium and, when appropriate, to measure enzyme production in the same cells under a variety of changing environmental conditions. By pulsing the cells with radioactive proline and measuring the amount of hydroxyproline formed, it is also possible to monitor collagen and elastin synthesis concomitantly. It is then possible to correlate the activity of key enzymes involved in the posttranslational processing of collagen and elastin with biosynthesis of these major connective tissue proteins and their incorporation into the extracellular matrix.

Materials and Methods

Cell Culture. Two cell types were used in this series of experiments. Cultures of IMR-90 fibroblasts, an embryonic human lung derived fibroblast strain (Nichols et al., 1977), were obtained from the Institute for Medical Research, Camden, NJ. These cells were subcultured and were used at population doubling levels (PDL) 24–28. These cells were grown in T-75 flasks with 20 mL of modified Eagle's medium (Daniel & Melendez, 1968) containing 2.2 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Ascorbic acid was not included in the growth medium.

Rabbit aortic smooth muscle cells were derived from explants of the aortic arch from weanling rabbits as described previously (Faris et al., 1976). Cells were used during the

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second to fourth week in second passage. These cells were cultured in T-75 flasks with 20 mL of Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959; Smith et al., 1960) containing 3.7 g/L $NaHCO_3$, 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). Except where specified, the medium was changed twice weekly. All cells were cultured in controlled atmosphere incubators under air–5% CO_2 mixtures.

Enzyme Assays. (*a*) *Preparation of Substrates.* Assays for both lysyl oxidase and prolyl hydroxylase are tritium-release assays with biologically labeled substrates. Lysyl oxidase substrates were prepared by labeling embryonic chick aortas for 24 h with L-[4,5-$^3H_2$]lysine in the presence of β-aminopropionitrile (BAPN) as described previously (Pinnell & Martin, 1968; Harris et al., 1974). The aortas were homogenized in phosphate-buffered saline and centrifuged, and the insoluble pellet was utilized as substrate (125 000 cpm per assay). Enzyme activity was estimated by vacuum distilling and counting the tritiated water formed. Specificity of the assay system was routinely monitored by inhibition of tritium release in the presence of BAPN. Underhydroxylated procollagen substrate for measurement of prolyl hydroxylase activity was prepared from the medium of embryonic chick calvaria cultured with L-[3,4-$^3H_2$]proline in the presence of α,α′-dipyridyl as described by Gonnerman et al. (1980).

(*b*) *Insoluble Cell Layer.* In cases in which the cell layers were assayed for lysyl oxidase activity, the cell layer from each flask was harvested with the aid of a rubber policeman, homogenized in 3 mL of 4 M urea and 0.01 M K_2HPO_4, pH 7.8, in a ground-glass homogenizer, allowed to sit at 4 °C for 4 h, centrifuged, and dialyzed vs. 0.15 M NaCl and 0.1 M K_2HPO_4, pH 7.8. A 0.2-mL aliquot of this supernatant was then assayed as described for other tissues (Harris et al., 1974).

(*c*) *Medium.* Appropriate aliquots of medium from a single T-75 flask of smooth muscle cells were used to assay the soluble lysyl oxidase activity as described previously (Harris et al., 1974). Incubations in all cases were carried out for 8 h although a linear release of ^3H_2O was obtained for a period of 16 h.

(*d*) *Culture Flasks.* Some assays reported in this paper were carried out in intact cell culture flasks. To each flask at appropriate culture times was added 125 000 cpm of insoluble chick aorta substrate at the time of feeding. At various times, 2 mL of medium was aseptically removed, and tritiated water was separated by vacuum distillation.

Experimental Procedures and Results

General Characterization of Lysyl Oxidase. Initial studies to characterize the nature of lysyl oxidase activity in rabbit smooth muscle cells were conducted by using the whole culture flask procedures described above. At zero time, eight flasks of second-passage rabbit smooth muscle cells were fed with Dulbecco's medium (20 mL) containing 10% fetal bovine serum. Duplicate flasks were given either BAPN (50 μg/mL), α,α′-dipyridyl (0.2 mM), both BAPN and α,α′-dipyridyl, or no inhibitors and appropriate radioactive substrates. At 16, 40, and 88 h, 2 mL of medium was aseptically removed from each flask, and tritiated water was separated by vacuum distillation.

The results from this experiment are given in Table I. These data demonstrate that smooth muscle cells produce measurable amounts of lysyl oxidase. This activity is 80% inhibitable by BAPN, a specific lysyl oxidase inhibitor, for up to 88 h. It should be noted that α,α′-dipyridyl also partially inhibited the long-term release of tritiated water, and a combination of both BAPN and α,α′-dipyridyl completely inhibited the production of tritiated water.

Table I: Appearance of Tritiated Water in Medium from Rabbit Smooth Muscle Cells (Net cpm/Flask)

cell treatment	time after addition of lysyl oxidase substrate[a] (h)		
	16	40	88
none	11080	18720	20860
+BAPN	900	2420	7080
+α,α′-dipyridyl	8900	12160	13500
+BAPN and α,α′-dipyridyl	0	0	0
medium without cells (blank)	2180	4960	9200

[a] Insoluble embryonic chick aorta substrate labeled with L-[4,5-^{3}H]lysine in the presence of β-aminopropionitrile.

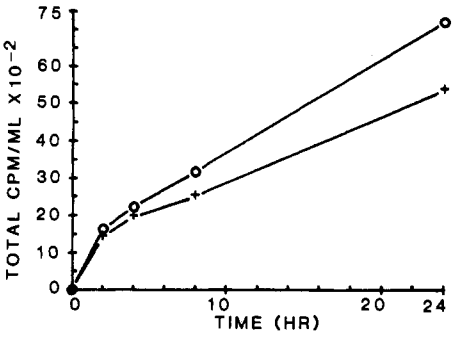

FIGURE 1: Relative contribution of cells and medium to total lysyl oxidase activity. A 10-mL aliquot of medium was removed from smooth muscle cells after 48 h in culture. A 10-mL sample was left on the cells, and insoluble LO substrate was added to both. Counts are net counts per minute/flask minus the background counts from BAPN-inhibited flasks: (O) cells and medium; (+) medium only.

For determination of the contribution of the cell layer to the observed enzyme activity, four flasks of rabbit smooth muscle (RSM) cells and four flasks of IMR-90 fibroblast cells were fed with 20 mL of medium and allowed to incubate for 48 h. After that time, 10 mL of the medium from each of these flasks was removed and placed in sterile flasks with no cells. BAPN (50 μg/mL) was then added to two flasks containing separated medium. Two additional flasks of cells and their corresponding flasks of separated medium were left with no added inhibitors to serve as controls (no treatment). Insoluble aorta lysyl oxidase substrate was added to each of the flasks and incubated for 24 h. A similar set of six flasks of RSM cells was treated as above except that labeled underhydroxylated calvaria medium ([3,4-$^3H_2$]proline), prepared as described above, was added as substrate for prolyl hydroxylase and either BAPN or α,α′-dipyridyl was added to two of the flasks of cells and their corresponding flasks of medium. In addition, eight flasks of confluent IMR-90 cells were set up as above with lysyl oxidase substrate and either BAPN, α,α′-dipyridyl, both of these inhibitors, or no inhibitors were added to the cells and medium. Aliquots of the media from the IMR-90 fibroblasts were taken after 20-h incubation.

Figure 1 demonstrates that the initial enzyme activity that one measures can be largely accounted for by the medium alone. After extended periods, the flasks with intact cells showed higher activity, probably because of replenishment of the medium enzyme levels by the cells. These data suggest that in the smooth muscle cells most of the release of tritiated water can be accounted for by the soluble enzyme present in the medium, since activity in equivalent amounts of medium was similar after 20-h incubation in the presence or absence of cells. Confluent flasks of IMR-90 fibroblasts contained only 10–15% of the activity measurable in smooth muscle cells (data not shown). The addition of underhydroxylated prolyl hy-

Table II: Time Course of Tritiated Water Release following Refeeding of Rabbit Smooth Muscle Cells

zero-time media (cpm/flask)	time after feeding (h)	media net total cpm flask^{-1} h^{-1}	cell layer net total cpm flask^{-1} h^{-1}	total cpm flask^{-1} h^{-1}	% of activity in media
3969 ± 314	3, n = 3	2771 ± 157	7234 ± 628	10005 ± 481	27.7
4356 ± 158	7, n = 3	3614 ± 75	6625 ± 978	9129 ± 908	39.6
3944 ± 598	24, n = 3	4098 ± 404	7540 ± 1139	11638 ± 1514	35.2
3900 ± 1231	54, n = 3	4483 ± 278	8224 ± 822	12717 ± 629	35.2
4269 ± 256	92, n = 2	4609 ± 225	7233 ± 232	11842 ± 457	38.9

droxylase substrate to the cells yielded no significant release of tritiated water (data not shown), suggesting that there is no prolyl hydroxylase activity in the medium and also suggesting that the exogenous collagen substrate does not penetrate the cell.

Distribution of Enzyme between Cell Layer and Medium. Fourteen flasks of smooth muscle cells were used to determine the relative amount of enzyme extractable from the cell layer and the time course of lysyl oxidase appearance in the cell medium after the addition of fresh medium. Media from each flask were collected after incubation with cells for 3 days. Activity in these media was assayed and presented as base-line values. Following the addition of fresh media containing 10% FBS, three flasks were harvested at 3, 7, 24, and 54 h, and two flasks were harvested after 92 h. Media were decanted in a sterile manner and stored at 4 °C until all were collected and were then assayed. Cell layers were harvested by scraping into 3 mL of 4 M urea, 0.01 M K_2HPO_4, and 0.15 M NaCl and were then homogenized in a ground-glass homogenizer and dialyzed vs. 0.1 M K_2HPO_4 and 0.15 M NaCl, pH 7.8, prior to assay. Because of the difference in the length of incubation time for the assay of the cell layer (8 h) and media (16 h), the activity is presented as total counts per minute of tritium released per flask per hour of assay incubation. These data demonstrate that the medium levels of lysyl oxidase are replenished very rapidly with little or no effect on the cell layer (Table II). In addition, the levels of enzyme in the medium appear to rapidly reach a steady state, suggesting some feedback control of enzyme production. In smooth muscle cells, medium enzyme consistently accounted for approximately 40% of the total enzyme activity in the flask. In contrast, in IMR-90 cells, approximately 90% of the total activity was in the medium (data not shown).

Effect of Frequent Replenishment of Medium on Lysyl Oxidase Activity. Experiments in which the cells were refed repeatedly over a 30-h period indicate that the smooth muscle cells have a remarkable ability to secrete enzyme (Figure 2). Medium from cells refed with 10 mL of medium every 4 h had 50% less activity in the last 4-h period than cells maintained for 24 h without changing the medium. The total amount of activity secreted into the medium over the 24-h period was several fold greater in flasks in which the medium was changed frequently. Cells that were refed with 20 mL of medium secreted twice as much enzyme into the medium at the early time points as cells refed with 10 mL of medium. The concentration of enzyme in the 20-mL refeeding group fell off more rapidly with time than did that of the group refed with 10 mL. It is also interesting to note that the activity of enzyme after 24 h without refeeding in the 10-mL group was comparable to that in the zero-time medium that had been exposed to the cells for 96 h (data not shown).

Effect of Inhibition of Protein Synthesis on Medium Lysyl Oxidase Activity. In order to determine if the rapid replenishment of medium lysyl oxidase after refeeding is dependent on new protein synthesis or represented release or activation of previous synthesized enzyme, the following experiment was

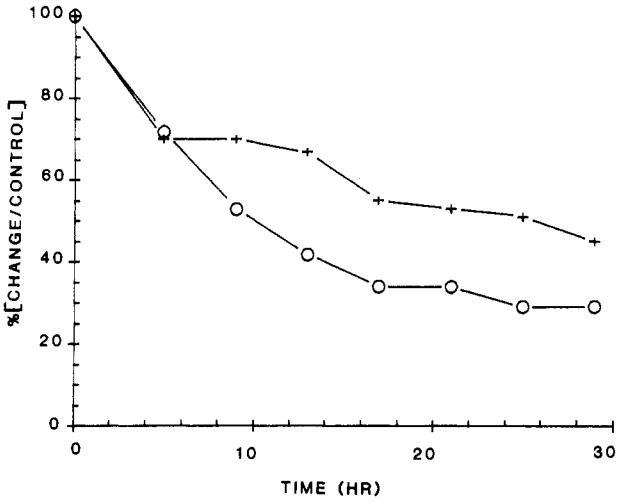

FIGURE 2: Effect of multiple medium changes on lysyl oxidase activity. Each data point represents activity accumulated in the previous 4 h following complete medium change with either 10 or 20 mL of medium. The data are expressed as the percent of activity in medium that had been exposed to cells for 96 h immediately prior to the start of the refeeding experiment: (+) 10 mL of medium; (O) 20 mL of medium.

done. Thirty flasks of smooth muscle cells 3 weeks into second passage were refed and allowed to incubate 24 h. At this time, the medium was changed in all flasks, and ten flasks were fed with medium containing 20 μg/mL cycloheximide. After 4 h, the medium was again changed, and ten additional flasks were treated with cycloheximide, and cycloheximide was again added to the preincubated ten flasks. Ten flasks of cells were refed with cycloheximide-free control medium. In addition, all media this time contained 5 μCi/mL L-[^{14}C]proline. At 2, 4, 8, and 22 h, the cells from two flasks from each group were harvested. Aliquots of 1 mL of the medium were assayed for lysyl oxidase. The remainder of the medium and the cell layers were dialyzed vs. distilled water, lyophilized, and resuspended, and [^{14}C]proline incorporation into nondialyzable material was estimated by counting an aliquot of the dialyzed material in a liquid scintillation counter. At the end of the 22-h incubation period, the medium in the remaining two flasks from each group was poured off, and fresh medium containing L-[^{14}C]proline but without cycloheximide was added. These cells were then allowed to incubate 24 h, and the cell layers and media were harvested. At this time, aliquots of the cell layer homogenate were extracted with urea, and lysyl oxidase activity was estimated in this extract as described above. Medium lysyl oxidase activity was also measured.

The effect of the addition of cycloheximide to cells on the release of lysyl oxidase into the medium is shown in Table III. Cells receiving cycloheximide had reduced levels of medium lysyl oxidase as early as 2 h after addition of the inhibitor. Medium levels in these cells remained relatively constant over the 24-h culture period while levels in the control flasks increased. An estimate of protein synthesis from incorporation of radiolabeled proline into nondialyzable protein demonstrated

Table III: Effect of Cycloheximide on Appearance of Lysyl Oxidase in Culture Medium (cpm/Flask)

	time (h)						
	zero time	2	4	8	18	24	48[a]	cell layer
control	30330 ± 3240[b]	28125	31275	31975	39000	43135 ± 2945	19375 ± 554	21075 ± 2115
cycloheximide added at zero time	30580 ± 2344	22704	27537	26200	25690	26005	7220	12225 ± 2376
cycloheximide preincubated 4 h	27990 ± 2750	17600	19070	20480	20870	21680	5540	10794 ± 1408

[a] Medium changed and cycloheximide removed 24 h previously in treated groups. Controls were not exposed to cycloheximide at any time. [b] ±standard deviation.

that protein synthesis in the inhibited cells was less than 2% of that in the controls as early as 2 h after addition of cycloheximide (data not shown). Recovery experiments in which the cells were refed medium without cycloheximide demonstrated that the cells that had been treated with the inhibitor were viable and capable of resuming protein synthesis although at a reduced level compared to that of controls. At the end of the 24-h recovery period, cell layer levels of lysyl oxidase were significantly depleted (50% of control levels).

Discussion

These data suggest that smooth muscle cells secrete a measurable amount of lysyl oxidase into the culture medium. This activity is 80% inhibitable by BAPN for up to 88 h. It should be noted that α,α'-dipyridyl also inhibits the long-term release of tritiated water, and a combination of both BAPN and α,α'-dipyridyl completely inhibits the production of tritiated water. There are at least two possibilities for the effect of α,α'-dipyridyl and the additive effect of α,α'-dipyridyl and BAPN. First, since the substrate that one is adding is not a pure elastin, part of the tritiated water formation, i.e., that which is not inhibited by BAPN, could come from lysine hydroxylation in collagen. This is unlikely since no prolyl hydroxylase activity is demonstrable in the medium, and lysyl hydroxylase, like prolyl hydroxylase, is an intracellular enzyme. Second, the α,α'-dipyridyl may have an effect via chelation of Cu^{2+} in the medium.

The most surprising portion of this study is the demonstration that these cells respond to refeeding by releasing large amounts of enzyme very quickly. In addition, the enzyme levels in the medium reach a given level and do not increase further, suggesting that the synthesis or secretion of the enzyme is feedback controlled. The mechanism of this control is not known at this time.

Studies to determine if the rapid release of enzyme is dependent on new protein synthesis suggest that there is a relatively large pool of enzyme available for secretion from the cells or that there is enzyme bound to some component of the extracellular matrix that can be released into the medium.

At this time, it is not known if the production of enzyme is coupled to synthesis of connective tissue matrix components. Studies to determine the relationship between synthesis of enzyme and collagen or elastin are now under way.

These studies demonstrate that lysyl oxidase production in these cells is a very active system. This in vitro cell culture system provides an exciting system to study the effects of different culture conditions on the production and turnover of the enzyme. In addition, it provides a meaningful system to correlate enzyme activities with biosynthesis and processing of connective tissue components.

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