Regulation of Lysyl Oxidase by Basic Fibroblast Growth Factor in Osteoblastic MC3T3-E1 Cells*

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Lysyl oxidase catalyzes the final known enzymatic step required for collagen and elastin cross-linking. A cross-linked collagenous extracellular matrix is required for bone formation. This study investigated whether lysyl oxidase, like its type I collagen substrate, is down-regulated by basic fibroblast growth factor (bFGF) in osteoblastic MC3T3-E1 cells and determined the degree of post-transcriptional control. Steady-state lysyl oxidase mRNA levels decreased to 30% of control after 24 h of bFGF for 24 h and treatment with 1 nM bFGF for up to 12 h resulted in a modest stimulation of lysyl oxidase gene expression and enzyme activity. At least 50% of the down-regulation of lysyl oxidase was shown to be post-transcriptional. New protein synthesis was not required for the down-regulation by bFGF, but cycloheximide did increase constitutive lysyl oxidase mRNA levels 2.5-fold. We conclude that lysyl oxidase and COL1A1 are regulated similarly by bFGF in these osteoblastic cells, consistent with the in vivo effects of this growth factor on bone collagen metabolism.

Lysyl oxidase catalyzes the oxidative deamination of peptidyl-lysine to peptidyl-α-aminoadipic-δ-semialdehyde in collagen and elastin precursors. Extracellular lysyl oxidase activity is the final enzymatic step required for cross-linking and deposition of elastin and collagen in connective tissues (1, 2). This enzyme is copper-dependent, and decreased dietary copper results in deficient lysyl oxidase activity. Nutritional studies utilizing animals fed either copper-deficient diets or diets containing copper-dependent cross-link dehydro-hydroxylysino-ketohomo-proline demonstrated a decrease in lysyl oxidase activity. Reduced dietary copper results in bone collagen, as well as collagen turnover, translation, and subcellular localization (24–27). Therefore, we investigated whether lysyl oxidase and COL1A1 are regulated similarly by bFGF in these osteoblastic cells, consistent with the in vivo effects of this growth factor on bone collagen metabolism.

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Dose-dependent Regulation of Lysyl Oxidase by bFGF—Pre-confluent MC3T3-E1 cells were cultured in DMEM supplemented with 10% NBS in the absence or presence of bFGF for 24 h. A dose-dependent down-regulation in the steady-state lysyl oxidase and COL1A1 mRNA levels was found (Fig. 1). Down-regulation of lysyl oxidase mRNA to about 40% of control occurred at 1 nM bFGF. COL1A1 was down-regulated to less than 5% of control at 1 nM bFGF. Interestingly, at lower concentrations, bFGF caused a modest up-regulation in steady-state lysyl oxidase mRNA levels but not in COL1A1 (Fig. 1). The same results were observed culturing the cells in serum-free media containing 0.1% BSA (data not shown). Experiments were performed at least 2 times, all with similar results.

**RESULTS**

**Dose-dependent Regulation of Lysyl Oxidase by bFGF**—Pre-confluent MC3T3-E1 cells were cultured in DMEM supplemented with 10% NBS in the absence or presence of bFGF for 24 h. A dose-dependent down-regulation in the steady-state lysyl oxidase and COL1A1 mRNA levels was found (Fig. 1).

**Assay of Lysyl Oxidase Activity**—Lysyl oxidase enzyme activity was measured in the conditioned media and cell culture, using recombiant human [3H]trypsinogen substrate (28). Briefly, cells were cultured as described above. Media samples (0.5 ml) were assayed in quadruplicate in 1 ml of borate buffer (0.1 M NaCl, pH 8.0, and 160,000 cpm of [3H]trypsinogen in the presence and absence of 1 × 10⁻⁴ M BAPN. Reactions were incubated for 90 min at 37 °C followed by distillation under vacuum. Radioactivity in 0.5-ml aliquots of distillate was determined by liquid scintillation spectrometry. Units of enzyme activity were defined as cpm released above the BAPN control. The scintillation counting efficiency was 50%. For normalizing the enzyme activity to cell number, media were collected and assayed for lysyl oxidase enzyme activity, and cells from the same plate were dissociated and counted in a hemocytometer.

Cell layer lysyl oxidase enzyme activity was measured as follows. The medium was first removed and cells were washed 3 times with phosphate-buffered saline. 1 ml of extraction buffer (0.02 M boric acid, 4 mM urea, 0.15 M NaCl, pH 8.0) plus 0.001% phenylmethylsulfonyl fluoride and 10 μl of aprotonin/10 ml of extraction buffer was added to the plates. The cells were scraped with a rubber policeman and suspended 5 times with a 21-gauge needle and 10 times with an 18-gauge needle. The suspension was transferred to microcentrifuge tubes and spun down at 4 °C for 10 min at full speed. The supernatant was dialyzed overnight against 0.02 M boric acid, pH 8.0, and 0.5-ml samples were assayed in quadruplicate as described above.

RNA Isolation and Northern Analysis—Total RNA was prepared using Tri-Reagent (29). 10 μg of denatured RNA was applied on lane separated by electrophoresis on 1% agarose/formaldehyde gels, and transferred to GeneScreen nylon membranes (30). Membranes were pre-hybridized and then hybridized for 18 h at 42 °C in 50% formamide-containing solutions with a 32P-labeled COL1A1 cDNA (31) or 32P-labeled mouse lysyl oxidase cDNA probe (32) as described previously (33). For normalization, blasts were stripped and rehybridized with a radiolabeled glyceraldehyde 3-phosphate dehydrogenase cDNA (34) or 18 S ribosomal probe (35). Probes were labeled using the random primer method (36). The membranes were washed and subjected to autoradiography at ~80 °C (33). Signals were assessed and normalized by densitometric scanning. Values for standard error were derived from triplicate scans of films. Experiments were performed at least twice.

Analysis of mRNA Decay Rates—Cells were cultured and treated with or without 1 nM bFGF, as described above. Cells were refed with fresh medium containing 20 μg/ml of the RNA polymerase II inhibitor DRB (37). Total RNA was isolated at intervals and was subjected to Northern blotting. Membranes were successively probed with lysyl oxidase, COL1A1 collagen, GAPDH, and 18 S ribosomal RNA probes (see above). Signals were quantitated using scanning laser densitometry, and normalized to the 18 S ribosomal RNA signal. The log of percent RNA remaining against time was plotted, and half-lives were calculated after linear regression analysis (37).

![Fig. 1. Down-regulation of lysyl oxidase and COL1A1 steady-state mRNA levels by bFGF is dose-dependent.](http://www.jbc.org/content/269/13/24121/F1.large.jpg)
activity relative to changes in lysyl oxidase mRNA levels (Table I). bFGF down-regulation of lysyl oxidase and COL1A1 mRNA levels was maintained throughout the experiment (Fig. 2B). Experiments performed under serum-free conditions yielded similar results (data not shown). Thus, bFGF-dependent changes in steady-state lysyl oxidase mRNA levels resulted in corresponding changes in lysyl oxidase enzyme activity.

Analysis of Changes in mRNA Decay Rates—In preparation for analyzing the degree of post-transcriptional down-regulation of lysyl oxidase mRNA by bFGF (see below), a detailed time study was performed. The time at which up-regulation of lysyl oxidase ceased, and down-regulation began was established. We reasoned that the mechanism of down-regulation of lysyl oxidase mRNA might be fully in effect when down-regulation began, given that the half-life of lysyl oxidase mRNA is long (19).

Down-regulation of steady-state lysyl oxidase mRNA levels by 1 nM bFGF began after 12 h and reached maximum effect at 24 h (Fig. 3). Interestingly, down-regulation of steady-state COL1A1 mRNA occurred earlier, to a higher degree, and was maintained for at least 48 h. These results are consistent with the dose-response study (Fig. 1) where COL1A1 was down-regulated at lower concentrations of bFGF compared with lysyl oxidase.

bFGF-dependent changes in lysyl oxidase, collagen, and GAPDH mRNA decay rates were then determined. In the first group of experiments MC3T3-E1 cells were treated without or with 1 nM bFGF for 12 h, and then 20 μM DRB was added to inhibit new mRNA synthesis. Cultures were harvested at intervals over the next 12 h, and RNA was isolated. Parallel cultures not treated with DRB were grown in order to verify bFGF-dependent changes in steady-state mRNA levels. Total RNA was subjected to Northern blot analysis (Fig. 4). Lysyl oxidase mRNA decayed with a t₁/₂ of 10 h in DRB-treated cells. The t₁/₂ for DRB-plus bFGF-treated cells was 6.9 h, a decrease of 31%. Thus, post-transcriptional mechanisms account for about 50% of the lysyl oxidase regulation by 1 nM bFGF, as steady-state mRNA levels were decreased to 63% of controls. Similar figures were found for COL1A1 mRNA (Fig. 4). The control half-life for COL1A1 was 8.2 and 4.3 h for bFGF-treated cultures, accounting for 50% of down-regulation. This degree of post-transcriptional bFGF regulation of COL1A1 is similar to that estimated previously in MC3T3-E1 cells (18). bFGF regulation of GAPDH mRNA was also analyzed. Whereas the bFGF-treated steady-state mRNA levels for GAPDH increased 170%, the stability of GAPDH mRNA decreased to 50% of control. Thus, in contrast to lysyl oxidase and COL1A1, regulation of GAPDH could not be accounted by bFGF-dependent changes in mRNA decay rates. Regulation of GAPDH by bFGF, therefore, likely occurs by a different mechanism (Fig. 4).

We next analyzed the decay of lysyl oxidase mRNA after 21

**TABLE I**

| Time (h) | Control | bFGF | % of control | COL1A1 mRNA |
|----------|---------|------|-------------|-------------|
| 6        | 2,850 ± 802 | 3,468 ± 629 | 121 ± 34 | 160 ± 15 |
| 24       | 7,534 ± 1,284 | 6,450 ± 180 | 85 ± 14 | 12 ± 2.5 |
| 48       | 5,215 ± 1,450 | 4,245 ± 97 | 81 ± 21 | 48 ± 11 |

*Values are mean ± S.E. for samples assayed in quadruplicate and normalized to 10⁶ cells. Data are from one of two replicate experiments.

* a | % changes are based on mean ± S.D. of values obtained from laser densitometric scanning of films developed after different exposure times to radio labeled Northern blots. Data are from one of two replicate experiments.

**TABLE II**

| Time (h) | Control | bFGF | % of control (A) | Lysyl oxidase mRNA (%) of control (B) | Ratio of changes A/B |
|----------|---------|------|------------------|---------------------------------------|---------------------|
| 24       | 5,211 ± 79.4 | 2,502 ± 266 | 47 ± 4.8 | 27 ± 1 | 1.7 |
| 48       | 1,897 ± 175 | 1,033 ± 278 | 54 ± 14 | 33 ± 4 | 1.6 |
| 48 h (refed) | 2,082 ± 507 | 633 ± 184 | 30 ± 8 | 25 ± 6 | 1.2 |
| 72 h (refed) | 4,227 ± 314 | 951 ± 135 | 22 ± 3.2 | 23 ± 7 | 0.9 |

*Values are mean ± S.E. for samples assayed in quadruplicate and normalized to 10⁶ cells. Data are from one of two replicate experiments.

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ho f1n M bFGF pretreatment of MC3T3-E1 cells. This was performed to evaluate whether the post-transcriptional down-regulation of lysyl oxidase was greater after 21 h compared with 12 h of growth factor pretreatment. It is noted that lysyl oxidase steady-state mRNA levels are more stably down-regulated after 21 h of pretreatment (Fig. 3). Interestingly in control cells, no measurable decay of lysyl oxidase mRNA in control cultures was detected (Fig. 5). In bFGF-treated cultures, the half-life was reduced to 10 h. Thus, clear evidence for post-transcriptional lysyl oxidase regulation by bFGF was obtained from cells pretreated for 12 and 21 h (Figs. 4 and 5). The degree of post-transcriptional control appears to be greater after 21 h of bFGF pretreatment and coincides with the maximum and stable down-regulation in lysyl oxidase steady-state mRNA levels.

Effectsof Cycloheximide—
Inhibition of protein synthesis can influence growth factor-dependent effects on gene expression by different mechanisms. When regulation is predominantly post-transcriptional, inhibition of protein synthesis by cycloheximide sometimes causes large increases in mRNA levels known as super-induction. This may be due to cycloheximide-dependent loss of labile proteins that function to destabilize the mRNA (38) or protection of the mRNA by bound ribosomes (39). We therefore wished to establish whether bFGF down-regula-

![Fig. 3. Detailed time-dependent regulation of COL1A1 and lysyl oxidase steady-state mRNA levels by 1 nM bFGF. Hatched bars, lysyl oxidase; and black bars, COL1A1. Data are from two pooled experiments. Values are standard error means of triplicate densitometric scans normalized to the 18S rRNA signals.](image)

![Fig. 4. Determination of bFGF post-transcriptional mRNA decay rates of lysyl oxidase, COL1A1, and GAPDH mRNAs by Northern blotting after 12 h of bFGF pretreatment (A–C) and calculation of mRNA decay rates (D–F). A–C, subconfluent MC3T3-E1 cells were treated with 1 nM bFGF for 12 h, and then with 20 μg/ml DRB as described under “Experimental Procedures.” In addition, parallel cultures not treated with DRB were grown in order to establish bFGF-dependent changes in steady-state mRNA levels. All blots were probed with radiolabeled cDNAs for lysyl oxidase, COL1A1, and GAPDH and with a probe for 18S ribosomal RNA. A, Northern blots from bFGF- and DRB-treated cells; B, Northern blots from DRB-treated cells; C, steady-state mRNA changes. For A and B, treatment times refer to hours after DRB addition. Lane 1, 0 h; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 8 h; lane 6, 10 h; lane 7, 12 h. In C, no DRB was added. Lanes 1 and 2, 0 h; lanes 3 and 4, 6 h; lanes 5 and 6, 12 h. D–F, the hybridization signals seen in blots A, B, and C were quantified and normalized to the 18S ribosomal RNA signal after triplicate densitometric scanning. Data are from two experiments were pooled. Error bars are the range of log % mRNA remaining from triplicate densitometric scans. All scanning data from A and B were subjected to linear regression analysis of semi-log plots of the percentage of mRNA remaining versus time, yielding the following decay half-lives: D, lysyl oxidase mRNA decayed with a t1/2 of 10 and 6.9 h in DRB-treated (●) and in DRB plus bFGF-treated (○) cells, respectively; E, COL1A1 mRNA decayed with a t1/2 of 8.2 and 4.3 h in DRB-treated (●) and in DRB plus bFGF-treated (○) cells, respectively; F, GAPDH decayed with a t1/2 of 25.4 and 13.2 h in DRB-treated (●) and in bFGF plus DRB-treated (○) cells, respectively. Correlation coefficients for the regression lines for control and bFGF-treated cells, respectively, were 0.890 and 0.943 for lysyl oxidase, 0.854 and 0.923 for COL1A1, and 0.700 and 0.865 for GAPDH. The amount of regulation accounted for by post-transcriptional mechanisms was calculated dividing the bFGF-dependent change in decay half-life for each transcript by the change in steady-state mRNA isolated from parallel experiment: lysyl oxidase (31%/63% = 50%); COL1A1 (52%/95% = 54%).](image)
Fig. 5. Determination of mRNA decay rate of lysyl oxidase after 21 h of 1 nM bFGF pre-treatment of MC3T3-E1 cells. Sub-confluent MC3T3-E1 cells were treated for 21 h with or without 1 nM bFGF, followed by 20 μg/ml DRB as described under “Experimental Procedures.” Parallel cultures not treated with DRB were grown to establish bFGF-dependent changes in steady-state mRNA levels. Blots were probed for lysyl oxidase and 18S ribosomal RNA. Inset, Northern blots from DRB-treated cells (lanes 1–6), and DRB plus bFGF (lanes 7–12). Times of harvest after DRB-treatment were 2 h (lanes 1 and 7), 4 h (lanes 2 and 8), 6 h (lanes 3 and 9), 8 h (lanes 4 and 10), 10 h (lanes 5 and 11), and 12 h (lanes 6 and 12). Steady-state mRNA levels were determined from parallel cultures not treated with DRB corresponding to zero (lanes 13 and 14) and 12 h of DRB treatment (lanes 15 and 16). The plot of %RNA remaining against time of DRB treatment shows data from two pooled experiments. Error bars represent the range of two experiments, each assessed twice by laser scanning densitometry. All data were subjected to linear regression analysis. Data from zero hours of DRB treatment were not included in the analysis because RNA for this time point from one of the two experiments was degraded. DRB of DRB treatment were not included in the analysis because RNA for data were subjected to linear regression analysis. Data from zero hours experiments, each assessed twice by laser scanning densitometry. All data were subjected to linear regression analysis. Data from zero hours of DRB treatment were not included in the analysis because RNA for this time point from one of the two experiments was degraded. DRB plus 1 nM bFGF-treated (○) cells resulted in a calculated half-life of 10 h for lysyl oxidase mRNA and a correlation coefficient of 0.852. Data from cells not treated with bFGF (□) resulted in a slope of near zero, and thus had no detectable decay of lysyl oxidase mRNA.

Fig. 6. Effect of cycloheximide on lysyl oxidase, COL1A1, and GAPDH mRNA levels. MC3T3-E1 cells were preincubated without (control) or with 1 nM bFGF for 12 h, and then treated with or without 5 μg/ml cycloheximide. After 8 h, total RNA was isolated and Northern blot analysis was performed. A, lane 1, control; lane 2, bFGF; lane 3, control plus cycloheximide; lane 4, bFGF plus cycloheximide. This experiment was performed twice with similar results.

These time- and dose-dependent effects of bFGF on lysyl oxidase and COL1A1 gene expression may be related to time-dependent effects of bFGF on collagen synthesis observed in fetal rat calvaria organ cultures (17, 40, 41). In these studies, treatment of organ cultures with bFGF for 24 h resulted in lowered collagen synthesis. In contrast, treatment with bFGF for 24 h followed by culturing in the absence of growth factor for an additional 24 or 48 h resulted in increased collagen synthesis. The stimulatory effects on collagen synthesis were principally due to the mitogenic effect of bFGF on osteoblasts in the organ cultures (17), although direct effects on collagen gene expression were not excluded. Similarly, in vivo studies of chick embryos treated with bFGF resulted in decreased osteogenesis in the short term but stimulated bone formation in the long term, after bFGF application was discontinued. It was suggested that in addition to increasing the numbers of osteogenic cells, bFGF treatment might also ultimately increase the osteogenic potential of osteoblastic cells compared with embryos not treated with bFGF (42). Time-dependent effects on lysyl oxidase and collagen gene expression could be physiologically important, since exposure of osteoblasts to bFGF in vivo resulting from injury is likely to be acute. A more complete understanding of the effects of bFGF on bone formation will require varying the duration of bFGF exposure of osteoblastic cells and determining the subsequent changes in cell number, lysyl oxidase activity, collagen accumulation, and ultimately mineralization in vitro.

The observed bFGF-dependent increase in steady-state GAPDH mRNA levels is probably related to changes in the proliferative state of MC3T3-E1 cells. Meyer-Siegler and coworkers (43) found that the expression of the GAPDH gene was dependent on the proliferative state of several human cell lines. Mitogenic effects of bFGF on MC3T3-E1 cells are well established (40). bFGF influences differentiation of osteoblasts by inhibiting the expression of osteoblastic markers, including alkaline phosphatase and type I collagen (41). bFGF also induces the expression of collagenase and plasminogen activator, leading to matrix degradation (44, 45). Thus, the down-regulation of lysyl oxidase expression by bFGF is consistent with induction of a proliferative matrix-resorbing phenotype.

The results presented show that lysyl oxidase expression is regulated by bFGF in osteoblastic MC3T3-E1 cells. Steady-state lysyl oxidase mRNA levels decreased after 24 h of 1 and 10 nM bFGF treatment, similar to changes found in COL1A1 mRNA. Lowered lysyl oxidase mRNA levels resulted in diminished production of new lysyl oxidase enzyme activity.

In contrast, 0.01–0.1 nM bFGF caused an increase in lysyl oxidase mRNA levels but not in COL1A1 mRNA. Short-term treatment of MC3T3-E1 cells for 6 h with 1 nM bFGF caused increased steady state levels of both COL1A1 and lysyl oxidase mRNAs. This short-term treatment caused a corresponding increase in the culture medium lysyl oxidase enzyme activity.

DISCUSSION

The observed bFGF-dependent increase in steady-state GAPDH mRNA levels is probably related to changes in the proliferative state of MC3T3-E1 cells. Meyer-Siegler and coworkers (43) found that the expression of the GAPDH gene was dependent on the proliferative state of several human cell lines. Mitogenic effects of bFGF on MC3T3-E1 cells are well established (40). bFGF influences differentiation of osteoblasts by inhibiting the expression of osteoblastic markers, including alkaline phosphatase and type I collagen (41). bFGF also induces the expression of collagenase and plasminogen activator, leading to matrix degradation (44, 45). Thus, the down-regulation of lysyl oxidase expression by bFGF is consistent with induction of a proliferative matrix-resorbing phenotype.

Post-transcriptional control of gene expression has been demonstrated for several extracellular matrix genes including elastin (46) and COL1A1 (47). Transcript stability is believed to be regulated principally by sequences located in the 5′- and 3′-untranslated regions and the length of the polyadenyl tail (39). Due to the presence of long 5′- and 3′-untranslated regions in lysyl oxidase, we wished to establish the degree of post-transcriptional mechanisms in the bFGF-mediated effects. Our analyses indicate that changes in mRNA decay rates were responsible for at least 50% of lysyl oxidase mRNA regulation.
by bFGF. The remaining bFGF down-regulation of lysyl oxidase is likely to be transcriptional, as has been shown for COL1A1 (18). Comparison of the complete 3.5 kilobase pairs of rat 3′-untranslated region of rat lysyl oxidase (19) to that of human (48) reveals sequence conservation with an identity of 72.6% (not shown). It will be of interest to establish whether conserved regions of the 3′-untranslated region of lysyl oxidase mRNA regulate its stability in response to growth factors and cytokines, as may be true for COL1A1 (49, 50).

We tested whether labile protein factors might be necessary for the bFGF-mediated effect on lysyl oxidase and COL1A1 expression. Cycloheximide caused increased levels of lysyl oxidase messages, but no changes were found in COL1A1 and GAPDH mRNAs. Furthermore, labile bFGF-induced protein factors appear not to mediate down-regulation of COL1A1 and lysyl oxidase mRNA levels. This effect indicates that the constitutive expression of lysyl oxidase mRNA is influenced by labile factors in MC3T3-E1 cells. As unstable proteins appear not to directly mediate bFGF-dependent regulation of lysyl oxidase, we speculate that activation of stable protein factors by post-translational modification such as phosphorylation/dephosphorylation pathways may result in bFGF-dependent down-regulation of lysyl oxidase steady-state mRNA levels.

The essential role of lysyl oxidase in collagen cross-linking and accumulation suggests that its regulation by growth factors may have biological importance in osteogenesis. Interestingly, type I collagen synthesis and accumulation have been shown to be uncoupled in mineralizing MC3T3-E1 cells (11, 51). In these studies, it was shown that the peak of collagen synthesis did not correspond to the peak of collagen accumulation, as these cells formed a mineralized extracellular matrix. Although lysyl oxidase steady-state mRNA levels were reported not to increase during the period of increased collagen accumulation (51), measurements of lysyl oxidase enzyme activity were not presented. It is notable that lysyl oxidase has a complex biosynthetic pathway (52). The proenzyme is secreted as a 50-kDa glycoprotein and undergoes extracellular processing to form the 32-kDa product known to be active. As the studies reported here now demonstrate, changes in lysyl oxidase activity may not correspond to changes in steady-state mRNA levels at a given time due to residual pools of active enzyme. Similarly, we have recently shown that intracellular or extracellular pools of potentially activable proenzyme may accumulate under specific conditions (53). These precursor molecules may become activated as osteoblastic cells undergo phenotypic changes in the absence of increased lysyl oxidase steady-state mRNA levels. We believe that critical analysis of the possible role of lysyl oxidase activity in controlling collagen accumulation, and ultimately bone mineralization, are now required.
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