Copper, lysyl oxidase, and extracellular matrix protein cross-linking[1–3]

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ABSTRACT Protein-lysine 6-oxidase (lysyl oxidase) is a cuproenzyme that is essential for stabilization of extracellular matrixes, specifically the enzymatic cross-linking of collagen and elastin. A hypothesis is proposed that links dietary copper levels to dynamic and proportional changes in lysyl oxidase activity in connective tissue. Although nutritional copper status does not influence the accumulation of lysyl oxidase as protein or lysyl oxidase steady state messenger RNA concentrations, the direct influence of dietary copper on the functional activity of lysyl oxidase is clear. The hypothesis is based on the possibility that copper efflux and lysyl oxidase secretion from cells may share a common pathway. The change in functional activity is most likely the result of posttranslational processing of lysyl oxidase. Copper is essential for organic cofactor formation in amine oxidases such as lysyl oxidase. Copper-containing amine oxidases have peptidyl 2,4,5 tri(oxo)phenylalanine (TOPA) at their active centers. TOPA is formed by copper-catalyzed oxidation of tyrosine, which takes place as part of Golgi or trans-Golgi processing. For lysyl oxidase, recent evidence (Science 1996;273:1078–84) indicates that as an additional step, a lysyl group at the active center of lysyl oxidase reacts with TOPA or its precursor to form lysyl tyrosylquinone. *Am J Clin Nutr* 1998(suppl);67:996S–1002S.

KEY WORDS Copper, protein-lysine 6-oxidase, lysyl oxidase, collagen, elastin, protein cross-linking, Golgi complex, extracellular matrix

INTRODUCTION

For all animal species in which growth retardation is associated with nutritional copper deficiency, signs of skeletal and vascular tissue anomalies are often reported (1–6). For example, bone defects in response to copper deprivation in ruminants, which were characterized by osteoporotic-like lesions, deformation of joints, and bone fragility, were reported in the 1950s (5). Lesions of arterial vessels were also observed (4, 6). Indeed, severely copper-deprived animals often die of vascular aneurysms or from rupture of a cardiac ventricle. Even subtle or mild nutritional copper deficiency may result in vascular lesions that target the elastic lamina and other structural components of the vascular endothelium (7–10).

Once it was learned that the site of reduced lysyl oxidase activity was a primary site for lesions at the biochemical level, progress toward understanding copper's role advanced quickly. Lysyl oxidase is responsible for the formation of lysine-derived cross-links in connective tissue, particularly in collagen and elastin. Normal cross-linking is essential in providing resistance to elastolysis and collagenolysis by nonspecific proteinases, eg, various proteinases involved in blood coagulation (11). Resistance to proteolysis occurs within a short period of copper repletion in most animals; eg, Tinker et al (12) observed that the deposition of aortic elastin is restored to near normal values after 48–72 h of copper repletion in copper-deficient cockerels.

Effects of copper deprivation are most pronounced in neonates and infants. Offspring of animals fed copper-deficient diets during pregnancy or lactation exhibit abnormalities of the vascular and skeletal system, as well as dilation of the terminal airways in the lungs (13–16). The skin of copper-deficient animals may become friable (3). In egg-laying animals, the egg shell membrane may also be altered, characterized by an abnormal distribution and reduction in the amount of lysine-derived cross-links and abnormal mechanical properties, which may cause distortions in egg shape (17–19). Adequate copper status and functional lysyl oxidase is essential even in early stages of development (14, 15). Embryonic development beyond gastrulation does not occur without stable cross-linking of connective tissue protein (1, 4, 14, 15, 20).

LYSYL OXIDASE

Protein-lysine 6-oxidase (lysyl oxidase; EC 1.4.3.13) is a copper-containing quinoprotein with a lysyl adduct of tyrosyl quinone at its active center (21). Copper in lysyl oxidase appears to be involved in the transfer of electrons to and from oxygen to facilitate the oxidative deamination of targeted peptidyl lysyl groups in tropocollagen or tropoelastin and to internally catalyze quinone cofactor formation (**Figure 1**). Oxidation of peptidyl lysine results in the formation of peptidyl α-aminoadipic-δ-

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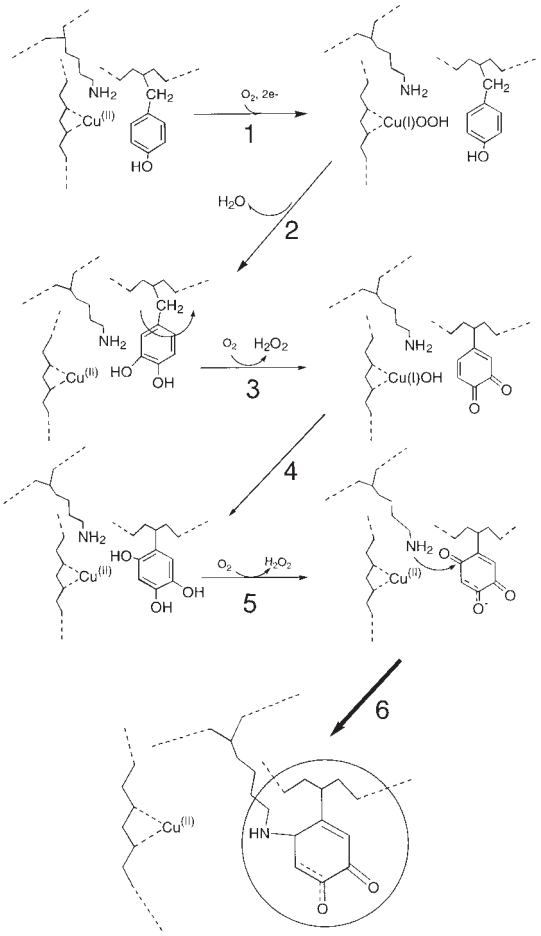

FIGURE 1. Possible mechanism for the generation of lysyl tyrosylquinone. The mechanism is dependent on the copper-catalyzed oxidation of a tyrosyl residue at the catalytic site within lysyl oxidase. Adapted from reference 21.

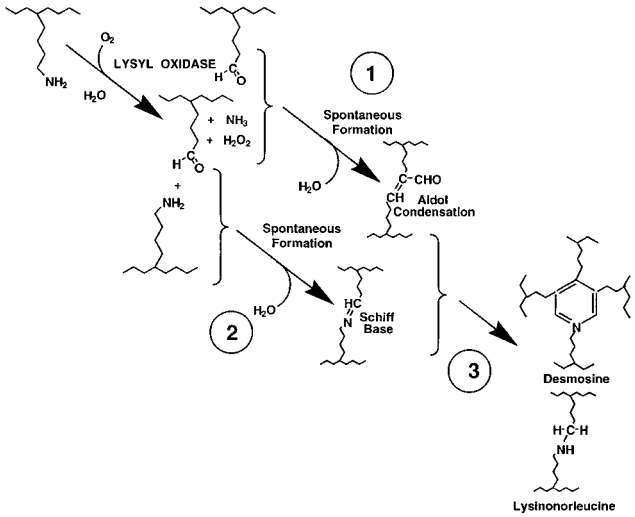

FIGURE 2. Cross-link formation. Lysyl oxidase catalyzes the oxidative deamination of specific lysyl residues in tropocollagen and tropoelastin. The product, peptidyl α-aminoadipic-δ-semialdehyde (allysine), participates in subsequent nonenzymatic condensation reactions. Further condensation of the bifunctional cross-link products, such as the aldol condensation product and dehydrolysinonorleucine (schemes 1 and 2) can result in tri-, tetra-, and even pentafunctional cross-links (scheme 3). The structure of desmosine (found in elastin) is also shown. The final oxidation of the pyridinium ring of desmosine results in the simultaneous reduction of dehydrolysinonorleucine to lysinonorleucine.

semialdehyde. Once formed, this product can spontaneously condense with neighboring aldehydes or with other lysyl groups to form intra- and interchain cross-links (**Figure 2**).

Important features of lysyl oxidase synthesis and processing are shown in **Figure 3**. In many respects, the metabolism of the enzyme is as complex as the metabolism of its principle substrates, collagen and elastin. Trackman et al (27, 28) and Mariani et al (29) were the first to characterize lysyl oxidase complementary DNA (cDNA) from rat and human cell lines. Rat lysyl oxidase cDNA recognizes 4.5- and 5.8-kb messenger RNA (mRNA), which initially encode a 46–48-kDa protein product, prolysyl oxidase. This proform is processed further by the addition of carbohydrate (22, 30), addition of copper (26), and eventual formation of the quinone cofactor (20). Recently, Kagan et al (23, 31) and Cronshaw et al (24) showed that a metalloproteinase is also important to the processing of the secreted form of lysyl oxidase. This metalloproteinase is localized on the cell surface and appears to be the same proteinase that cleaves the c-terminal propeptide from type I-procollagen. However, Kagan et al (31) showed that lysyl oxidase synthesized from cDNA constructs in cultured Chinese hamster ovary cells does not require the propeptidyl region for efficient secretion and activity.

Within the primary structure of lysyl oxidase, there are also binding domains for cytokines, eg, transforming growth factor β and cell-surface adhesion proteins (32). The functional activity of lysyl oxidase is influenced by factors that influence structural conformation of substrates, hormones, and environmental agents (1, 9, 10, 25, 33–50). Lysyl oxidase has the highest affinity for collagens (precipitated in the form of fibrils) and coacervated forms of elastin (49). However, simple monoamines and diamines or lysine-rich proteins, such as histones, may serve as substrates (48). Environmental conditions and hormones associated with fibrotic responses (eg, ozone, aerosolized cadmium, and elevated transforming growth factor β concentrations due to local trauma) result in increased lysyl oxidase activity (9, 10, 34–50). Reduced food intake and refeeding also can alter lysyl oxidase activity severalfold (9, 10, 36). Lysyl oxidase is inhibited by diamines, heparin, amino nitrites, semicarbazides, hydrazines, and some alkylation agents (33, 40, 48). Of the inhibitors, β-aminopropionitrile is commonly used to inhibit lysyl activity (40–47).

There is still much to learn about the relation between lysyl oxidase expression and regulation of cross-link formation. In some tissues, lysyl oxidase is not the rate-limiting step for cross-link formation. In dense connective tissue, lysyl oxidase is present at high concentrations, ie, from ≈50 to >300 μg enzyme/g tissue (**Figures 4** and **5**). This amount is more than sufficient for the daily deposition of new elastin and collagen on the basis of catalytic parameters (25, 34). Consequently, it may be speculated that in addition to catalyzing cross-link formation, lysyl oxidase may play structural or other novel regulatory roles in extracellular matrix (ECM) assembly (9, 10). Although in cell culture, measurable amounts of lysyl oxidase are released into the media (eg, as much as 50% of the total activity), in tissues the

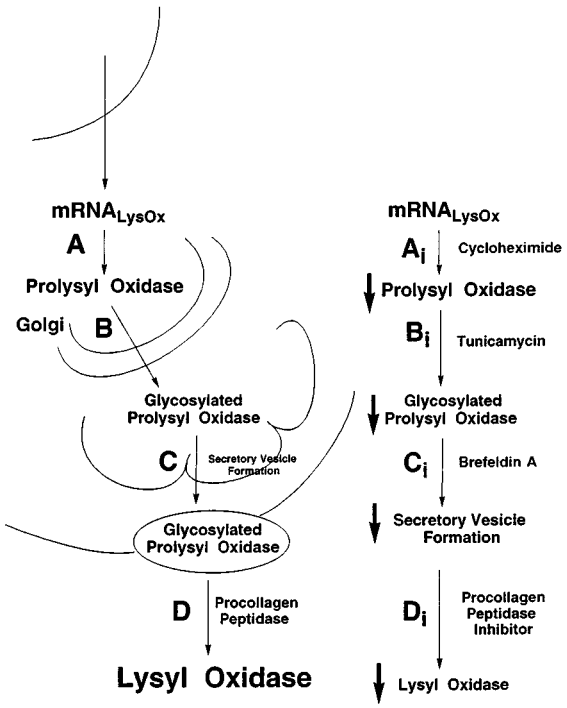

FIGURE 3. Processing of prolysyl oxidase to lysyl oxidase (LysOx). Lysyl oxidase messenger RNA is translated to produce prolysyl oxidase (A). Prolysyl oxidase is glycosylated (22) in the Golgi complex (B) and vectorally transported into vesicles (C) for its eventual secretion. At the cell surface, prolysyl oxidase has been suggested to be cleaved to lysyl oxidase by the same peptidase that cleaves the *c*-terminal peptide from the major forms of fibrillar procollagens and perhaps other proteinases (23–25). Copper appears to be added at a processing step in the trans-Golgi complex (26). The secretion and production of prolysyl oxidase is inhibited by cycloheximide (A_i). Interference of secretory vesicle formation, eg, by brefeldin A (C_i), inhibits the extracellular secretion of lysyl oxidase. In contrast, inhibition of lysyl oxidase glycosylation [eg, by tunicamycin (B_i)] or inhibition of the prolysyl oxidase (same as procollagen) peptidase (D_i) does not significantly alter the net production and secretion of prolysyl oxidase into the extracellular matrix.

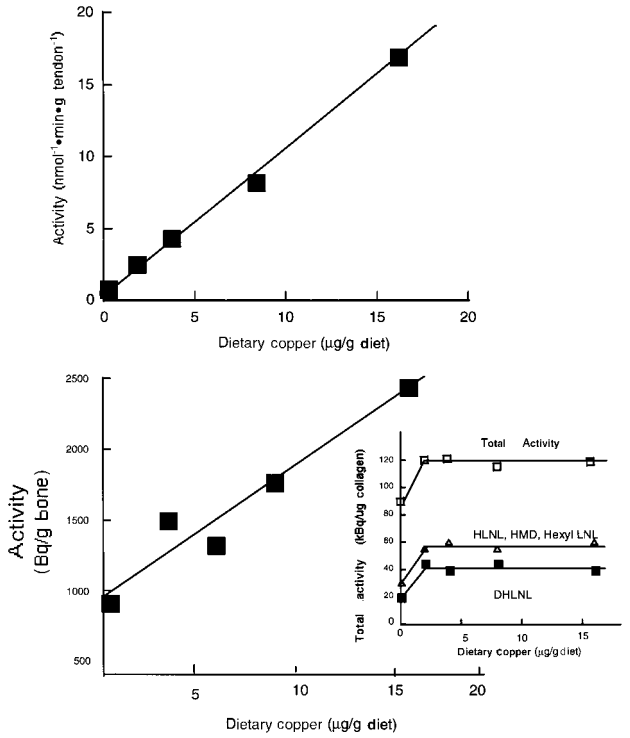

FIGURE 4. Relation of lysyl oxidase functional activity in bone and tendon extracts to the concentration of copper in the diet. The inset panel contains data for the relative amounts of reducible cross-links in bone [measured after $NaBH_4(^3H)$ reduction (*see* reference 3 for details)]. The amount of cross-links in bone is reduced at dietary copper amounts of ≤2 μg/g diet. HLNL, hydroxylysinonorleucine; HMD, hydroxymerodesmosine; Hexyl LNL, hexosyl derivatives (galactose and glucose derived) of lysinonorleucine; and DHLNL, dihydroxylysinonorleucine.

bulk of the enzyme is tightly bound to its substrates within the ECM. Lysyl oxidase is only solubilized by extraction into denaturants or detergents, such as 4–6 mol urea/L (25, 33, 34). Purification of the enzyme is difficult because of its association with ECM proteins and inactivation due to self-aggregation (25, 33). Furthermore, various molecular weights of lysyl oxidase have been observed, which range from ≈30 kDa, the predominant active subunit of lysyl oxidase, to > 100 kDa, which most likely represents lysyl oxidase in aggregated form or interconnected with other proteins (10, 25, 34).

In cell culture, the expression of lysyl oxidase is most active when proliferation stops or slows, eg, at cell confluency. Moreover, it has been observed that lysyl oxidase activity and collagen synthesis are markedly reduced in malignantly transformed human cell lines (50–56). Thus, a link between lysyl oxidase activity, ECM regulation, and tumorigenesis has evolved. The role of lysyl oxidase in abnormal cell proliferation may also involve cytosolic forms of the enzyme (53, 56). For example, the normal expression of the *ras* recision gene (*rrg*) in mouse 3T3 cells is down-regulated by the oncogene c-H-*ras*, which is used to induce the transformation of 3T3 cells into tumorigenic cells, eg, RS485 cells. High levels of *rrg* expression are restored by interferon-induced reversion of RS485 cells. The importance of the *rrg* gene to the reversion has been shown to be related to the persistence of revertants to develop the transformed phenotype if cells are stably transfected with *rrg* cDNA. The cDNA sequence for *rrg* is > 85% identical to that for the extracellular form of lysyl oxidase (55, 56).

DIETARY COPPER AND LYSYL OXIDASE PRODUCTION

Our interest in lysyl oxidase stems from earlier observations that lysyl oxidase activity in growing animals appears to be influenced directly by the amount of dietary copper over a wide physiologic range of intakes (3). In 1982, Opsahl et al (3) reported that lysyl oxidase activity varies by as much as five- to sixfold in response to dietary copper, ranging from 0 added Cu to 25 μg Cu/g diet (Figure 4). This observation has intrigued us because there is no compelling reason why an excess of dietary copper per se should increase lysyl oxidase activity above what might be needed for normal cross-linking (3, 25, 34). For example, for optimal growth in chickens the copper requirement is 5–10 μg Cu/g diet (3). Decreased collagen cross-linking does not occur until the copper intake is substantially < 1 μg Cu/g diet. Copper deficiency also has little or no effect on lysyl oxidase protein or lysyl oxidase mRNA concentrations in tissues (34; Figure 5).

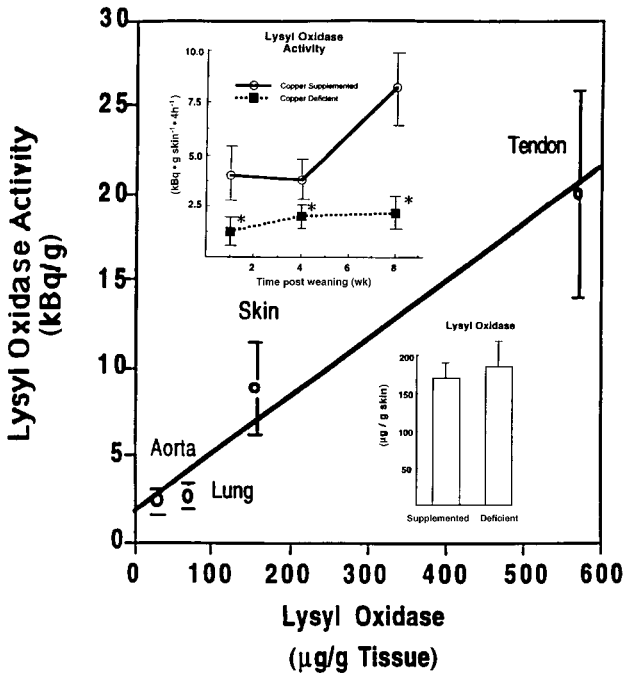

FIGURE 5. Amount of lysyl oxidase in rat connective tissues. The inset panel gives data for rat skin. Activity decreased in rats fed copper-deficient diets, but there was no change in lysyl oxidase protein. Moreover, over a wide range of copper intakes in rats, we detected no changes in the steady state concentrations of messenger RNA lysyl oxidase (for details *see* references 25 and 34).

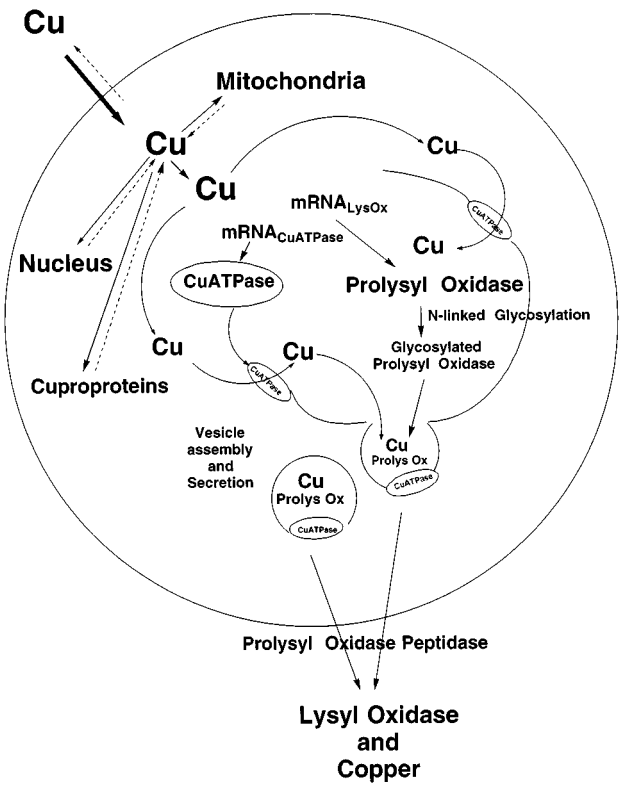

FIGURE 6. Overview of cellular copper metabolism. Copper interacts with lysyl oxidase at steps that require protein synthesis and vesicle assembly and secretion. For example, cycloheximide and brefeldin A inhibit copper secretion as lysyl oxidase (26). Because copper is retained in cells and is not incorporated into lysyl oxidase in conditions such as Menkes syndrome (58), the association of Cu-adenosinetriphosphatase (Cu-ATPase) with the same vesicle that transports lysyl oxidase is highlighted. Recent work (26) also suggests that glycosylation and prolysyl oxidase (Prolys Ox) cleavage are not required for the secretion of copper bound to lysyl oxidase.

LYSYL OXIDASE, CU-ATPase, AND EXPRESSION OF LYSYL OXIDASE FUNCTIONAL ACTIVITY

On the basis of the observations noted in the previous section, a hypothesis has evolved that links increased dietary copper intakes to a direct increase in lysyl oxidase activity. This hypothesis invokes a mechanism that requires a copper concentration–dependent stimulation of lysyl oxidase activity. Two recent developments contributed to the formulation of this hypothesis.

The first was the recognition that two genetic diseases—Menkes syndrome and occipital horn syndrome—possibly a form of X-linked cutis laxa, are related to defects in the gene important to Cu-adenosinetriphosphatase (Cu-ATPase) gene expression (*see* 57 for review). The uptake of copper by many cells appears to be relatively permissive; thus, cellular copper homeostasis may be regulated by cellular copper efflux pathways. As described in several articles in this supplement, Cu-ATPase activity is an important component of the copper efflux pathway. Exit of copper from connective tissue cells occurs by transport involving secretory vesicles, with the eventual release of copper into extracellular space. We propose that lysyl oxidase with or without copper may be transported by the same secretory vesicles or interacts with copper at some point during the egress pathway designed to transport copper out of cells. Such a mechanism may explain why there appears to be a copper concentration–dependent increase in lysyl oxidase activity, which is closely related to dietary copper intake (**Figure 6**).

A second advance relates to the connection between copper and formation of quinone cofactors in amine oxidases (21, 59–63). It is now certain that copper aids in initiating the oxidation of tyrosyl residues to form TOPA or a related compound (63). The α,β-unsaturated quinone structure of dopaquinone would be expected to promote the reaction at the C-2 position (59–63) to produce TOPA. Recent evidence suggests that this reaction occurs within the nascent polypeptide chain of copper-containing amine oxidases, such as lysyl oxidase, when only copper is added to initiate the reaction (21, 59–63; Figure 1). For many of the copper amine oxidases there are also data suggesting that copper resides near the C-2 position of the TOPA quinone. Consequently, it is conceivable that binding of hydrogen peroxides to copper in the unmodified protein would support the initial hydroxylation of peptidyl tyrosine to peptidyl dopa. For lysyl oxidase, subsequent steps involve the formation of a peptidyl lysyl adduct, which ultimately results in lysyl tyrosyl quinone (Figure 1).

Lysyl oxidase has a high avidity for copper, 5–7 mol Cu/mol lysyl oxidase, although only 1 mol Cu/mol enzyme is a requisite for functional activity (64). Because of its high concentration in some tissues, lysyl oxidase may represent a significant component in the conduit for copper from cells involved in ECM production. In genetic diseases of connective tissue, such as Menkes syndrome and cutis laxa, copper accumulates in connective tissue cells and is not allowed to enter into secretory vesicles because of abnormal Cu-ATPase expression (57). In severe cases of Menkes syndrome, lysyl oxidase activity can be as low as 10% of normal

(58, 65). Such low activity can clearly account for the severe connective tissue pathology associated with the disease. If the formation of lysyl tyrosyl quinone occurs in secretory vesicles, a linkage can be made between the copper concentration in vesicles and enzymatic potential, ie, lysyl tyrosyl quinone formation. What remains to be shown is whether a subtle change in plasma copper (induced by diet) is reflected by more dynamic changes in vesicular copper concentrations.

In this regard, we have begun to examine the uptake of copper by fibroblasts and the export of lysyl oxidase–bound copper from fibroblasts. Figures 3 and 6 outline the general pathways that are observed in fibroblasts for the secretion of ^{67}Cu bound to lysyl oxidase from fibroblasts.

Recently, we reported that virtually all of the copper associated with protein in the media of fibroblast cultures appears bound to lysyl oxidase (26). Little copper in the medium (bound to protein) is secreted when cycloheximide, an inhibitor of protein synthesis, was added to culture (Figure 3). This finding corroborates the previous finding by Harris et al (66, 67) that protein synthesis is important to the incorporation of copper into lysyl oxidase. In contrast, when tunicamycin was added to inhibit *N*-linked glycosylation, it had no effect on copper secretion as protein. This observation is similar to that of Sato and Gitlin (68), who showed that glycosylation is not a requisite for copper incorporation into ceruloplasmin. Moreover, the inhibition of propeptide cleavage from the ≈50-kDa form of lysyl oxidase to generate the ≈30-kDa form of lysyl oxidase does not appear to affect the amount of copper as lysyl oxidase in the media. This agrees with the observation by Kagan et al (31) that cleavage of prolysyl oxidase is not an essential feature for the release of lysyl oxidase into the culture medium of cultured cells. However, an inhibitor of secretory vesicle assembly, brefeldin A, inhibits the secretion of copper as lysyl oxidase. Complete experimental details of these studies were published previously (26). Such observations are important because they underscore that factors known to influence the secretion of lysyl oxidase from cells may also influence the efflux of copper from cells in a similar manner.

In summary, there is a relation between lysyl oxidase activity and dietary copper intake. Increases in the copper concentration in secretory vesicles are hypothesized to be important to quinone cofactor formation. For lysyl oxidase, such a mechanism could potentially link several observations and help to explain why the activity of the enzyme is sensitive to changes in copper intake when there is no change in the constitutive expression of the enzyme.

The lysyl oxidase gene in humans is found on chromosome 5; however, the Cu-ATPase gene is X-chromosome linked. The latter may help to explain why a defect in an X-linked gene may have such profound effects on ECM components encoded by autosomal genes. The complex interconnections among lysyl oxidase activity, TOPA formation, dietary copper, Cu-ATPase, and secretory vesicle formation may explain why such a diverse set of factors seems to influence the functions of this important enzyme. If the proposed mechanism is established, it will provide a novel description of enzyme activation that is directly linked to dietary nutrient intake.

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