MINIREVIEW

Newer Findings on a Unified Perspective of Copper Restriction and Cardiomyopathy[1] (44141)

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Abstract. **The cuproenzymes lysyl oxidase, cytochrome-*c* oxidase, and superoxide dismutase are key factors in understanding the cardiac hypertrophy and cardiomyopathy associated with dietary copper restriction. The role of copper in cardiac lipid and energy metabolism as a consequence of changes in some of these enzyme activities in comparison with what is known about normal cardiac substrate utilization is discussed here. While the decrease in the nuclear encoded subunits of cytochrome-*c* oxidase in hearts from copper-deficient rats is known, new evidence suggests that other factors, such as ATP synthase metabolism may be exerting an influence upon this observation. While this review focuses on newer knowledge about energy and fatty acid metabolism in copper deficiency, the extracellular matrix is considered as well. This complex interplay of extracellular and cellular events in copper restriction is outlined as a model for further studies of this unique model of concentric hypertrophy.**

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Several years ago, a model pertaining to copper deficiency and the subsequent cardiac defects was presented and reviewed in this journal (1). Since that publication, the proposed model has served as a strategic outline for designing experiments to pursue in order to unlock the mechanisms that cause these defects. Novel findings have evolved since that minireview appeared, and these findings are reviewed here. Information on functional as well as mechanism perspectives now allows for greater insight. Our studies demonstrate that marginal copper intake has a deleterious influence upon the heart without overt signs of compromised copper status. The basis of the cardiac defects resulting from copper deficiency is best understood by the role of copper-containing enzymes. Lysyl oxidase, cytochrome-*c* oxidase, and super oxide dismutase, all cupro-enzymes, appear to be factors to consider in validating a mechanism or model. Again we present evidence along these lines, but suggest other additional avenues to consider that were unknown at the time of the initial published review.

Characteristics of the Copper-Deficient Rat Heart

Studies have consistently demonstrated that copper-deficient (Cu^-) rats exhibit a variety of cardiovascular defects, including abnormalities in the connective tissue of blood vessels, ventricular aneurysms, hemothorax, pleural effusion, cardiac hypertrophy, abnormal electrocardiograms, hypercholesterolemia, glucose intolerance, hypotension, and disturbances in fatty acid profiles and norepinephrine metabolism (2–15). Increased mitochondria volume density and mitochondria:myofibril with vacuolated mito-

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chondria and disrupted cristae has been a common observation in hearts from Cu^- rats (16, 17). Our laboratory also demonstrated that anemia was not an initiating factor, nor was its presence necessary for the development of cardiac hypertrophy (16). Reports of anemia causing the hypertrophy were once in vogue as the reason supporting the cardiac hypertrophy with copper deficiency. However, copper deficiency can induce anemia, and, while there is a potential for volume overload, our lab and others have shown that hypertrophy in copper deficiency can occur in the absence of anemia (1, 18). An earlier study reported that cardiac enlargement was greater in copper-deficient pigs than in iron-deficient pigs despite similar hematocrits (19). Cardiac hypertrophy with electrocardiogram anomalies in the absence of anemia has been reported for rats (20). The degree of anemia appears unrelated to the degree of hypertrophy (21–23).

A unique feature of hearts obtained from copper-deficient animals is their morphology. The hypertrophy has been described by our research group as predominantly concentric, where the ventricular and atrial walls are significantly thickened but lumen volumes decrease. This type of hypertrophy is reported in pressure overload or aortic stenosis. In copper-deficiency, however, blood pressure is often decreased. The weakening of the myocardium due to decreased collagen cross-linking *via* decreased lysyl oxidase activity may cause an increased work load, thereby leading to concentric hypertrophy. Other models of hypertrophy have enhanced lumen volumes and thin myocardial walls, but have an expanded circumference. Volume overload as observed in anemia, obesity, etc., often leads to this pattern of hypertrophy (1).

Recently, our group in collaborations with others (24) reported that marginal diet copper fed to rats for 6 months affected cardiac ultrastructure. Compromised basal laminae and mitochondria, and the appearance of glycogen granules and lipid droplets without cardiac hypertrophy were reported. In overt copper deficiency the same observations with hypertrophy have been reported, as reviewed earlier (1). An unpublished study in which marginal copper (2 μg Cu/g diet) was fed to rats for up to 10 months gave similar results in addition to electrocardiogram alterations that are similar to those reported in overt copper deficiency (17). Hunsaker *et al.* (25) reported that the F_1 generation fed the 2–μg Cu/g diet that were from dams fed the same levels of dietary copper from weaning had aortic ultrastructural pathology. These rodent studies are all the more significant in view of the fact that the copper levels fed were designed to simulate what Western diets may contain. Furthermore, the cardiac changes did occur when traditional measures of copper status did not change. Such changes could be clinically significant in humans.

Activity levels also should be considered with respect to nutrient requirements, and this is true for copper as well. When young adult rats were fed copper-deficient diets and were either exercise-trained on a treadmill for 8 weeks or allowed to remain sedentary, several conclusions were made (26). First, after the 8 weeks, rats fed copper-deficient diets were only marginally compromised in copper status when using liver Cu,Zn-superoxide dismutase activity as a copper status measure. Second, rats fed the copper-restricted diets could not continue until Week 8 because they tired easily and refused to run. Thirdly, significant pericapillary and interstitial collagen accumulation and phagocytic activity was noted.

In view of the findings pertaining to lipid and glycogen accumulation in the myocardium of rodents fed copper-marginal and deficient diets, and the influence of physical activity, some consideration of energy substrate utilization in the heart and potential role of copper nutriture is appropriate.

General Energy Metabolism in Myocardial Tissue

Cardiac myocytes require a tremendous amount of energy for electromechanical activity, ion pumping, and cellular maintenance, energy which is supplied by the breakdown of high-energy phosphate compounds. The high-energy phosphates, primarily ATP and creatine phosphate, are derived from the catabolism of fatty acids, glucose, lactate, pyruvate, acetate, ketone bodies, and, to a lesser degree, amino acids (27). In this sense the heart can be considered an "omnivorous" organ, able to utilize a variety of energy substrates (28).

The rate at which cardiac myocytes utilize different energy substrates is directly related to their relative concentrations in the vascular and interstitial compartments, the degree of mechanical activity of the heart, and the presence of various hormones, either released locally or circulating (29, 30). Although earlier studies overestimated the contribution of fatty acid oxidation to cardiac ATP production to be as high as 70%–100% (31, 32) subsequent investigations estimated the contribution of fatty acids to oxidative metabolism to be on the order of 50%–70% (33–35). The carbohydrate substrates glucose and lactate account for the majority of the remaining oxidative metabolism, with relatively minor contributions from pyruvate, β-hydroxybutyrate, and acetoacetate (36, 37).

Since myocardial activity can only be sustained by anaerobic metabolism for a brief period of time, cardiac myocytes are endowed with relatively large compartment of mitochondria and a rich supply of myoglobin. Mitochondria may make up to one-third of a cardiac myocyte's volume (38, 39), which is roughly three quarters as much space as myofilaments. The inner membrane of mitochondria, the site of oxidative phosphorylation, is space efficiently arranged and is approximately 30 times the surface area of the sarcolemma (38, 39). Further attesting to the fundamental oxidative nature of myocardium is the rich capillary distribution, as a 1-mm^3 region of the left ventricular myocardium of a dog contains approximately 3500 capillaries (40).

As oxidative metabolic generation of ATP prevails in cardiac tissue, the primary substrates will include fatty acid–

derived acetyl coenzyme A (CoA), *via* mitochondrial and peroxisomal β-oxidation, and pyruvate derived from glucose and lactate catabolism (27). Only under extreme conditions, such as ischemia, will anaerobic glycolytic mechanisms increase resulting in dramatic changes in substrate utilization (41).

Endogenous stores of carbohydrate and fatty acids are normally limited in cardiac myocytes. For instance, carbohydrate contribution to total cardiac mass is less than 0.7%, which would also include carbohydrate moieties beyond glycogen stores (42). Meanwhile, canine cardiac tissue contains approximately 50 μmol fatty acid moieties per gram of wet tissue, with the majority (85%–90%) of the fatty acid being incorporated in the phospholipid pool, and the remaining fatty acids comprising the triacylglycerol pool (12%) and cholesterol esters (0.5%) (43). Only a small quantity of fatty acids in cardiac tissue are of the unesterified nature (<0.1%), as the normal dog will contain on the order of 30 nmol/g wet weight (43–45). However, once the fatty acid in the extracellular compartment has been considered, the quantity of unesterified fatty acids in the cytosolic compartment has been estimated at <10 μmol/l (44). As the amount of cytosolic unesterified fatty acids is small and because it is uncertain whether fatty acids associated with membrane phospholipids are available for oxidation and high-energy phosphate production, it seems more likely that the endogenous source of fatty acids as a substrate for β-oxidation is almost entirely reserved for the triacylglycerol pool (46).

Beyond endogenous stores, triacylglycerol is also localized at various sites throughout the heart. Cardiac adipocytes, are visually obvious without magnification in the vicinity of superficial epicardial coronary arteries in dogs and rats, while in humans, adipocytes are abundant in the epicardium (47). Additionally, some triacylglyceride deposition can be observed in the interstitial space (48) as well as a limited amount in endothelial cells (49).

As cardiac myocytes are limited in their ability to store energy substrates in appreciable quantities, they will require a constant perfusion of these molecules by way of coronary circulation. Glucose and lactate circulate independently, while fatty acids are found in the blood either complexed to albumin or esterified in lipoprotein triacylglycerol (47). The uptake of circulating unesterified fatty acids by cardiac tissue is very efficient, with extraction ratios estimated as high as 70% on a single transit of blood (50, 51). The uptake of lactate on a single capillary passage is about 50%, whereas only a minor proportion of glucose is taken up on a single passage (44). The uptake of glucose at a normal workload appears to be hormonally regulated by insulin; however, at higher workloads glucose uptake by cardiac myocytes increases in an insulin-independent manner (28). Oppositely, the extraction of lactate by cardiac myocytes appears to be governed by its concentration in the extracellular compartment (36, 52).

Fatty acids that circulate esterified to glycerol as part of triacylglycerol assembled in lipoproteins, are liberated by the catalytic activity of lipoprotein lipase (53). Phospholipase A_1 activity also exerted by lipoprotein lipase assures exposure of the triacylglycerol-abundant core of chylomicrons and very low density lipoproteins (54). Fatty acids and monoglycerides are the primary products of cardiac lipoprotein lipase activity and are taken up by endothelial and myocardial cells and used for both anabolic and catabolic processes (55).

The utilization of glucose as a oxidative metabolic substrate in cardiac myocytes begins with glycolysis. Glycolysis is regulated at various steps, the most important of which include hexokinase, phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase, and pyruvate dehydrogenase (34). The last of these enzymes systems would yield acetyl CoA in the mitochondrial compartment. Glucose entry into cardiac myocytes appears to be a carrier-mediated, non–energy-dependent process that can be augmented by increased cardiac work and the presence of elevated levels of insulin, epinephrine, and growth hormone, and by decreased oxidative metabolism such as during anoxia (34). Conversely, efficient fatty acid oxidation will lessen glucose transport into cardiac myocytes. Hexokinase is inhibited by its product, glucose-6-phosphate, which after reversible conversion to fructose-6-phosphate, by phosphoglucose isomerase, is a substrate for PFK. PFK is a major regulator for glycolysis and is impacted by cytosolic pH and allosteric factors. Its activity is stimulated by low-energy phosphates such as AMP, ADP, and inorganic phosphate, and decreased by ATP and citrate (34). The factors that decrease PFK activity are indeed products of fatty acid oxidation. The mitochondrial enzyme complex pyruvate kinase is not only regulated by phosphorylation/dephosphorylation, but is allosterically inhibited by the products of fatty acid oxidation (i.e., NADH and acetyl CoA) and oxidative phosphorylation (ATP) (34).

Fatty acids, derived either from exogenous or endogenous sources interact with a tissue-specific isoform of fatty acid–binding protein (FABP) (56, 57). The heart-type FABP has been reported to be a relatively small protein (15 kDa); however, it makes a significant contribution to cardiac protein mass. Some early estimates of the contribution of cardiac-type FABP to the cytosolic protein mass where as high as 5%–6% (58, 59), although more recent estimates are about half of the earlier reports (60). The primary responsibility of cardiac-type FABP appears to be to facilitate the transport of fatty acids to intracellular sites of metabolic conversion (47).

Before fatty acids can be utilized in oxidative pathways for ATP production, their carboxylic head group must be converted to a reactive thioester in a process referred to as fatty acid activation (61). Fatty acid activation is catalyzed by long-chain, medium-chain, and short-chain acyl-CoA synthetases (47). Activated fatty acids are then available for mitochondrial and peroxisomal β-oxidation. Activated long-chain fatty acids must first be transported into the mi-

tochondrial matrix, *via* a carnitine-dependent shuttle mechanism, prior to engaging in β-oxidation (62, 63). Acyl-CoA esters are first converted into acylcarnitine, by the action of carnitine acyltransferase I located at the inner surface of the mitochondrial inner membrane, and then transported across the inner membrane by carnitine-acylcarnitine translocase (64). Once inside the matrix, acylcarnitine is reconverted to acyl-CoA by carnitine acyltransferase II (64). β-oxidation of acyl-CoA in the mitochondrial matrix yields acetyl CoA, NADH, and $FADH_2$, and integration of acetyl CoA into the tricarboxylic acid cycle yields more NADH and $FADH_2$ (47). The reduced electron transfer complexes ($NAD^+ \rightarrow$ NADH and FAD $\rightarrow$ $FADH_2$) deliver electrons to the respiratory chain located within the mitochondrial inner membrane to support ATP synthesis.

The peroxisomes of cardiac myocytes also possess the ability to oxidize fatty acids; however, their oxidative capacities are limited to long-chain acyl-CoA (47). Furthermore, peroxisomal β-oxidation is somewhat incomplete in that long-chain acyl-CoA are catabolized to acetyl CoA and short-chain acyl-CoA moieties, which need to be further degraded in mitochondria (65).

Cardiac Energy Metabolism during Reduced O_2 Delivery and Hypertrophy

Hallmarks of alterations in normal cardiac function, structure, or perfusion include modifications in energy substrate metabolism. For example, in low-flow ischemic hearts there is a resultant accumulation of cytosolic fatty acids (44). Furthermore, hypoxic and ischemic conditions in humans and experimental animals results in the accumulation of lipid droplets (66, 67). The appearance of lipid droplets is similar to other heart conditions where there are genetic fatty acid defects in metabolism (68). During adaptive cardiac hypertrophy, there is often a trend towards the utilization of less fatty acid and more glucose for oxidative ATP generation (69).

Ischemia resulting in oxygen deficiency in cardiac myocytes causes a significant reduction in oxidative metabolism; however, it still persists as the primary means of residual ATP generation (41). All parameters of mitochondrial function become depressed with ischemia (70), and, in contrast to normal aerobic metabolism, glucose becomes the primary contributor of substrates for the tricarboxylic acid cycle (71). During decreased oxygen delivery to myocardium there is increased glycogenolysis to provide more glucose (34). Furthermore, under conditions of extreme anoxia cardiac glycogen stores may become essentially depleted within 4 min (72). Glucose transport into cardiac myocytes as well as the activity of hexokinase and PFK are increased during decreased oxygen delivery (34). However, in severe anoxia, resulting from ischemia, glycolysis is ultimately reduced primarily due to the inhibitory effect of lactate, both directly upon glyceraldehyde-3-phosphate dehydrogenase, and indirectly by its regulation of the sole remaining means for oxygen-deficient cardiac myocytes to reoxidize NADH through lactate dehydrogenase (73, 74).

During myocardial ischemia, many events take place that depress fatty acid oxidation. The resulting decrease in respiratory chain flux in mitochondria increases the NADH: NAD^+ ratios in mitochondria and the cytosol, and acts to suppress β-oxidation (34, 71), especially the oxidation of β-hydroxy acyl-CoA, which requires NAD^+ (75). Mitochondrial accumulations of long-chain acyl-CoA has been reported as well during ischemic states (76, 77). In fact, accumulation of lipid droplets in hypoxic and ischemic cardiac tissue has been well established in both humans and laboratory animals (66, 67, 78, 79).

In the adaptive hypertrophic state, energy substrate metabolism also appears to be altered. There appears to be a decrease in fatty acid oxidation in the hypertrophic heart when expressed either per gram of cardiac tissue or per unit of cardiac work (33). In one investigation (33), energy substrate utilization was measured in rat hearts which were volume-overloaded by surgical opening of a aortocaval fistula resulting in cardiac enlargement. The overloaded hearts demonstrated decreased mechanical performance and O_2 consumption. $^{14}CO_2$ production from [U-^{14}C]palmitate was also decreased. However, when the hearts were perfused with octanoate, the mechanical performance and O_2 consumption were normalized in the overloaded hearts. Despite normal levels of carnitine in the blood, carnitine levels were depressed in the overloaded rat hearts. CoA levels were not altered in the overloaded rat hearts. Therefore, the reduction in long-chain fatty acid oxidation in mitochondria in the overloaded rat hearts may be related to a reduced ability to translocate activated long-chain fatty acids into the mitochondria for subsequent β-oxidation. Furthermore, it has been speculated that a decreased translocation of activated fatty acids in the cytosolic compartment into mitochondria can lead to increased triacylglycerol synthesis and incorporation into the triacylglycerol pool within those cardiac myocytes (80). The existence of an impaired long-chain fatty acid utilization in mechanically overloaded rat hearts mentioned above (33) confirms the reports of other investigators using different models of experimental cardiac overload (80–82). The work by El Alaoui-Talibi and coworkers (33) suggested that a primary underlining aspect of the decreased long-chain fatty acid utilization observed in mechanically overloaded hearts may be related to altered function of a mitochondrial carnitine palmityl transferase (CPT_0). This would certainly impair the penetration of long-chain fatty acids into mitochondria and thus limit their contribution to cardiac energy production (83).

As glucose utilization as an oxidative substrate increases relative to the reduction in fatty acid oxidation in adaptive cardiac hypertrophy, the question of whether mechanisms associated with glucose utilization are enhanced was addressed. Bishop and Altschuld (84) demonstrated that certain features of glucose metabolism are indeed enhanced in dogs with experimental cardiac hypertro-

phy and congestive heart failure. Progressive pulmonary stenosis was produced, resulting in right ventricular hypertrophy, in one experimental group, while right ventricular hypertrophy and congestive heart failure was produced in another experimental group secondary to infestation with the canine heartworm *Dirofilaria immitis.* There was a significant shift from heart-type lactate dehydrogenase (H-LDH) towards muscle-type lactate dehydrogenase (M-LDH) isozyme. M-LDH is more associated with tissue more accustomed to anaerobic metabolism, such as white skeletal muscle fibers (85). Furthermore, Bishop and Altschuld (84) demonstrated that lactate production was significantly increased, above control levels, in the hypertrophied right ventricles after 30-min anaerobic incubation, further suggesting an increased ability to metabolize glucose in hypertrophied cardiac tissue.

Speculations on Copper-Deficient Cardiac Metabolism

Manifestations of copper deficiency–associated cardiac hypertrophy and myopathy suggest that energy metabolism is indeed altered. Although direct evidence is limited, speculations based upon indirect observations and related models of cardiac hypertrophy and myopathy are feasible and should launch future investigation.

As mentioned earlier, copper restriction in young animals results in a concentric pattern of cardiac hypertrophy (1, 86, 87). Although the exact histological nature of the hypertrophy is forthcoming, it will be assumed till then that the pattern of cardiac growth is consistent with the classic view of hypertrophy, whereby there is an augmentation of the diameter of individual myocardial fibers (88) with little to no change in the ratio of fibers to capillaries (89). However, as the diameter of the myocardial fiber increases there is a concomitant increase in the diffusion distance for oxygen. This may establish a state of relative hypoxia in the central portion of cardiac myofibers, which theoretically could result in an increased reliance on glycolytic metabolism. Normally, anaerobic glycolysis only accounts for about 1% of the ATP produced in cardiac myocytes; however, during hypoxic states that percentage increases relatively (90). Jalili *et al.* (91) measured cardiac LDH activity in copper-deficient rats, fed either a high-fat or low-fat diet, and did not observe a significant difference in relation to copper status or dietary fat level. However, despite the significant shift in isozyme forms of LDH in right ventricular hypertrophy induced by Bishop and Altschuld (84), they too did not observe a significant difference in total cardiac tissue LDH activity in comparison with the control animals. Thus, whether or not there is a shift in isoenzyme forms of LDH as an adaptive response to the concentric hypertrophy developed in copper deficiency remains unresolved and perhaps would be a more descriptive measurement.

The augmentation in the cardiac mitochondrial compartment during the development of copper deficiency is believed to account for a major portion of the concentric hypertrophy observed (1, 86, 87, 92). However, at this time the signal(s) and precise molecular events leading to the expansion of mitochondrial volume remain speculative. As electron micrographic evaluation of myocardial ultrastructure has revealed that a notable portion of the mitochondria appear swollen and mishapened, with fragmentation of the fine cristae organization, it is difficult to surmise its impact upon oxidative phosphorylation. One potential indication that fatty acid utilization in the copper-deficient heart may be depressed is an accumulation of lipid droplets, as consistently revealed by electron microscopy (1, 86, 87, 92). Interestingly, recent electron micrographic investigation (Fig. 1) of cardiac myocytes from rats fed a marginally adequate copper diet over a 6-month period also presented increased lipid volume density (24). Mitochondria pathology was evident in that the cristae showed signs of disintegration and did not exhibit the usual parallel array. In addition, results of a current investigation by Wildman and Mao has revealed that cardiac lipoprotein lipase activity in cardiac tissue is increased 2-fold in copper-deficient rats. Thus, the increase lipid droplet accumulation observed in copper-deficient rat heart micrographs could be the result of increased translocation of extracellular fatty acids into cardiac myocytes with subsequent resynthesis of triacylglycerol. Furthermore, it is not known whether this possible increased import of fatty acids is merely occurring in excess of normal fatty acid oxidation or occurring concomitant to a reduction in fatty acid oxidation. Future efforts will be focused in this area. In addition to the presence of lipid droplets, phospholipid metabolism has been documented to be altered in hearts from copper-deficient rats in that a decrease in choline phosphotransferase was reported (93) with subsequent decreases in phospholipid phosphorus content. Whether such changes may influence cell membrane function was not reported.

As mentioned previously, hypertrophy is an adaptive response whereby the structural dimensions of cardiac walls and chambers conform to cardiovascular events such as chronic volume overload, mitral or aortic regurgitation, or pressure overload, such as the result of chronic hypertension. In the case of copper deficiency, the concentric pattern of hypertrophy is likened more to a pressure overload scenario (1). In light of its inverse relationship to wall tension, the increase in ventricular wall thickness in the copper-deficient model (86, 87) may be interpreted as an adaptive response to reduce ventricular wall tension and subsequent myocardial O_2 demands for energy metabolism. Chao and co-workers (94) did report that cardiac ATP content of hypertrophic hearts from copper-deficient rats was measured at control levels. This was in spite of despite reduced cytochrome-*c* oxidase (CCO) activity. However, adaptive changes in copper-deficient cardiac myocytes may allow for a reduced energy consumption in hypertrophy, such as upregulation of the more slowly contracting fetal isomyosin V_3, as opposed to V_1 (95, 96). Adaptive changes in cardiac hypertrophy can result in a period of stability; however, they

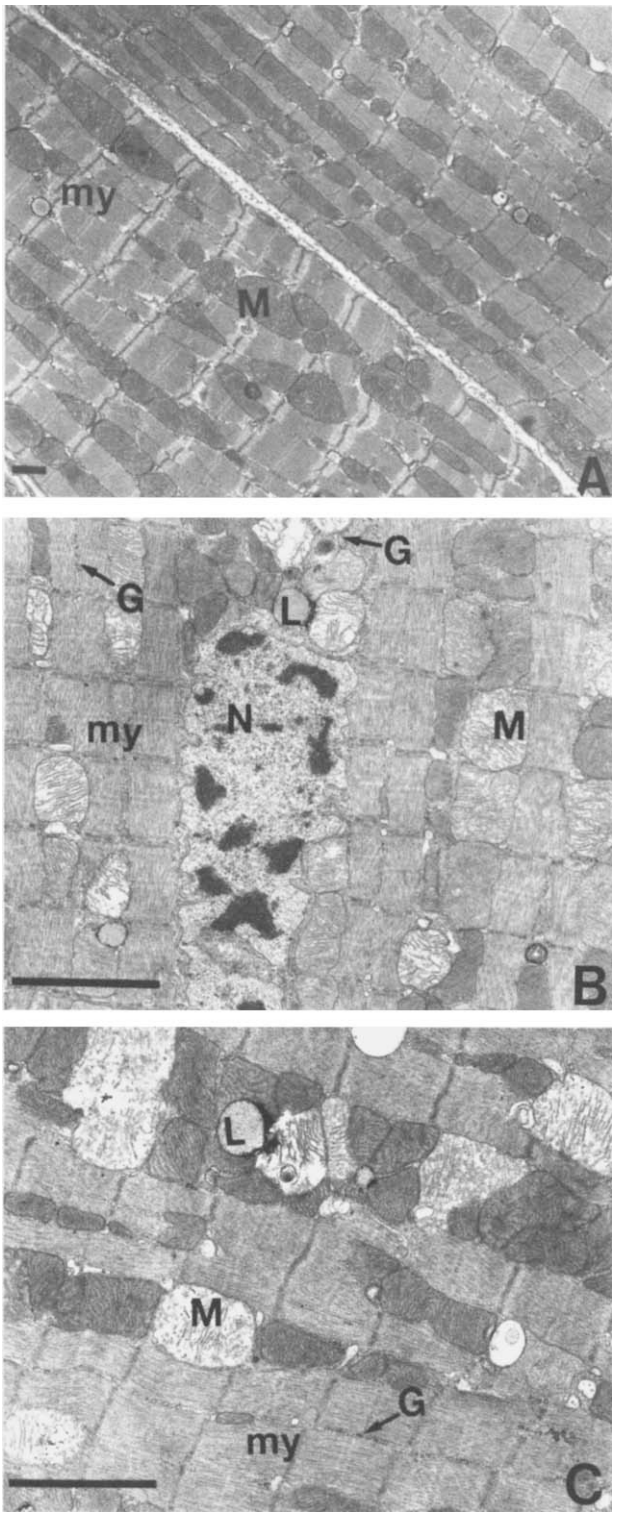

Figure 1. Electron micrographs of heart sections from rats fed copper-adequate (A) or copper-marginal diets (B and C). Note the presence of lipid droplets (L) and glycogen granules (G) in marginal animals. The architecture of the mitochondria (M) appears compromised among marginal-copper-fed rats (B and C) in that the cristae are not in the usual parallel arrays and vacuoles are apparent. my, myofibrils; N, nucleus. Bar, 1 μm.

may ultimately lead to a decrease in cardiac performance and ultimate failure. Wildman and co-workers (87) did report a decreased left ventricular $+dP/dt_{max}$ in copper-deficient rats. This measurement is an indicator of left ventricular systolic performance and is affected by preload and myocardial contractility. Since the rats in this study were not anemic, nor did they present tachycardia, and myocardial ATP content has been reported to be at control levels elsewhere (94), the predominant influencing factor would seem to be a reduction in sarcomere contractility. A reduction in cardiac contractility may be partly explained by the reported shift from V_1 to V_3 isomyosin protein in copper-deficient rat hearts (95, 96). The V_3 isomysin protein decreases the rate of cross-bridge cycling, thereby reducing myocardial contractility (97). However, the tension generated during systole is increased thereby facilitating the ejection of an overloaded heart. The net effect of a shift towards the "slow-type" V_3 isomyosin protein is a decreased inotropic effect; however, it improves the mechanical efficiency of the heart and spares energy (98).

One point that must be considered when comparing the energetics of myocardium in the state of hypertrophy and normal hearts is energy apportionment. This is to say that a heart in state of hypertrophy has to dedicate a certain portion of its generated ATP to anabolic processes such as sarcomere development, mitochondrial proliferation, and biomembrane expansion. This allotment of energy must hold high priority as the heart tries to adapt to a given physiological situation such as pressure or volume overload. This may perhaps render the enlarging heart somewhat energy-poor for other activities such as contraction and relaxation events.

At this time it is difficult to predict confidently the metabolic activities in copper-deficient myocardial tissue. Whether there is a shift towards the utilization of more glucose for oxidative and anaerobic energy metabolism remains undetermined. If this is indeed the case, it would occur in an animal whereby there is a decrease in whole-body respiratory quotient (RQ), indicating greater fatty acid oxidation (99). Also, if cardiac myocytes from copper-deficient animals do indeed use less fat as an energy source, the nature of the reduction needs to be established. Future efforts need to focus on cardiac lipoprotein lipase, carnitine-involved translocation of fatty acids into mitochondria, activation of fatty acids, competitive re-esterification events for triacylglycerol formation, β-oxidation, as well as enhanced mechanisms for glucose utilization.

Cytochrome-*c* Oxidase and ATP Synthase

Copper deficiency decreases cytochrome-*c* oxidase activity, and one study demonstrated that repletion normalized the activity (100). The structure of CCO has recently been published (101). The rat CCO enzyme is composed of three larger mitochondrial encoded subunits and about 10 nuclear encoded subunits. The mitochondrial encoded subunits possess the catalytic activity and the nuclear ones the regulatory

activity (102). The classical interpretation of decreased CCO activity in copper-deficiency has been the role of copper has as a co-factor for the mitochondrial encoded subunits I and II (103). However, we have reported that the nuclear encoded subunits are markedly decreased in copper deficiency while the mitochondrial encoded subunits appear unaffected (104, 105). Unpublished data from our laboratory demonstrated that the CCO nuclear encoded subunits were decreased after 3 weeks of copper restriction and occurred at the same time that the hypertrophy became evident. The decrease in the CCO nuclear encoded subunits is not simply the result of, or secondary to, the pathology, but of copper deficiency *per se.* This observation comes from three conducted experiments. As reviewed above, in rats fed the Cu^- diet in combination with the strong antioxidant dimethyl sulfoxide, mitochondria volume density remained elevated, but the degree of organelle disruption appeared less extensive than in copper-deficient rats without an antioxidant (106). However, the nuclear encoded CCO subunits and the δ subunits of ATP synthase were still depressed (105). A close examination of the mitochondria revealed that the copper-deficient rats, whether pair or *ad libitum* fed, or administered DMSO concurrently with the copper-deficient diet, all had irregular and fragmented cristae. This would be expected, since these enzymes are part of the inner mitochondrial membrane. A second experiment examined the CCO subunits in a genetic heart disease rat strain (SHHF). Results demonstrated no apparent differences in rat hearts that were hypertrophied compared with controls (105). Finally, using an iron-deficient (Fe^-) rat, which causes cardiac hypertrophy, but an eccentric type, as a check for a nutritional model, we failed to observe any change in the CCO subunits in Fe^- rats compared with controls, despite the presence of both hypertrophy and anemia in the animals (106). These studies strongly suggest that the decrease in the nuclear encoded subunits of CCO is due to copper deficiency *per se.*

Despite the reduction in activity that Rusinko and Prohaska (107) and our lab (94) have demonstrated for cardiac CCO activity, no significant decrease in ATP levels or energy charge have been reported. Fields *et al.* (108) did observe decreased liver ATP levels in copper-deficient rats fed fructose in comparison with their glucose- and cornstarch-fed counterparts. Kopp *et al.* (109) did report decreases in ATP level of copper-deficient rat hearts using NMR techniques. However, their rats could have been in failure, whereas ours may not have been since our studies were shorter in duration than theirs. A lack of change in ATP level in our study and that of Rusinko and Prohaska (107) is intriguing since we also have data that suggest a peptide of 16-kDa molecular weight that is decreased in the Cu^- rat heart to have a high degree of homology to the δ subunit (nuclear encoded) of ATP synthase (104), as determined by peptide sequencing. These findings suggest one of two possibilities: (i) the decrease in CCO and/or ATP synthase activity is not enough to impair ATP production; that there is much more of these enzymes present than needed to maintain normal energy metabolism; or (ii) the heart has adapted in some fashion either to conserve energy or to produce ATP anaerobically, or by another mechanism such as enhanced ATP production from phosphocreatine, which is elevated in hearts of copper-deficient rats (90), as reviewed above. However, it takes ATP to synthesize phosphocreatine *via* creatine kinase, thereby appearing to negate any advantage.

Molecular Control Mechanisms of Fatty Acid Oxidation in the Heart

Mitochondrial defects are likely to impair fatty acid oxidation, but peroxisomes may also oxidize fatty acids, though mainly the longer chain fatty acids. Using electron microcopy, we have not been able to detect anything unusual regarding the structure of these organelles in comparison with our observations with mitochondria. Much of the pathology observed in hearts from copper-deficient rats appears to resemble some of the pathologies observed from human hearts of subjects with inborn errors of fatty acid metabolism (68) that result in diminished fatty acid oxidation.

Medium-chain acyl-CoA dehydrogenase (MCAD) is a nuclear encoded mitochondrial enzyme that catalyzes the first key rate-limiting step in fatty acid oxidation. This enzyme is a common inherited defect in human inborn errors of metabolism (68). MCAD reacts upon medium-chain acyl-CoA thioesters that can arise from a variety of sources such as those medium chain fatty acids that enter the mitochondria by diffusion, products of mitochondria β-oxidation of saturated and unsaturated long-chain fatty acids, and products of peroxisomal β-oxidation of long-chain and very long chain fatty acids (110). The enzyme is highly regulated in tissues such as the heart and parallels mitochondrial oxidation rates (111). The enhanced expression of MCAD mRNA parallels that of the TCA cycle enzyme malate dehydrogenase (MDH) mRNA levels. Both enzymes have increased mRNA levels with enhanced mitochondrial β-oxidation and, conversely, decreased mRNA levels in response to declines in fatty acid oxidation (110, 112).

Two factors appear to regulate MCAD at the gene level. The MCAD gene promoter has a region containing retinoic acid receptors or retinoid response regulatory element. There are several known receptors, designated RARα, RARβ, RARγ, and RXRα. Another transcriptional factor to be considered is the peroxisome proliferator activated receptor (PPAR) which is RXR's heterodimeric partner. These two transcriptional elements are within the steroid/thyroid superfamily of nuclear hormone receptors that regulates MCAD (110, 111). The retinoid response regulatory element appears to regulate transcription of the MCAD gene in response to a variety of hormonal and metabolic stimuli (112). For instance, fasting is known to enhance the transcriptional activity of RXR (110).

The inner mitochondrial membrane enzyme carnitine

palmitoyl-transferase I (CPT-I) catalyzes the CoA-carnitine exchange needed for the mitochondria import of long-chain fatty acids. Inhibition of this enzyme results in enhanced MCAD mRNA levels that are mediated by peroxisome proliferator activated receptor (PPAR). Apparently, the inhibition of long-chain fatty acid mitochondrial entry induces PPAR, which activates MCAD transcripts. Given the disruption of the mitochondrial membranes, CPT-I could be compromised leading to decreased long-chain fatty acid oxidation and enhanced medium-chain fatty acid oxidation. Based upon the findings of Gulick *et al.* (110) it is possible that the expression of nuclear genes encoding mitochondrial enzymes is induced (autoregulatory phenomena) in copper deficiency as with CPT-I inhibition. However, based on our preliminary findings, copper-deficient rats ($n = 5$) have decreased expression of MCAD mRNA levels relative to copper-adequate rats ($n = 4$), but this could be secondary to the presence of hypertrophy. Our early work with the levels of neutral and polar fatty acids of copper-deficient hearts supports that oxidation of longer-chain fatty acids could be decreased, since Ovecka *et al.* (6) reported that cardiac long-chain fatty acids increased in the triglycerides of copper-deficient rats. It is possible that all transcripts involved with oxidation are downregulated in favor of enhanced anaerobic metabolism.

The Role for ATP Synthase?

A perplexing observation is the diminished level of a subunit with homology to the ATP synthase δ subunit. There is no known role of copper for this enzyme. However, the role of ATP synthase in this model may be more significant than previously believed. Divalent ions have been demonstrated to be required for the activity of several of the F1 ATPase subunits, specifically the δ subunits, which facilitates stability in binding of the α and β subunits to the γ and ϵ subunits (113). More important however, is the finding that the presence of a natural protein inhibitor of ATPase, a 95-kDa regulatory peptide of ATP synthase, requires the presence of both Cu and Zn (114). Decreased activity of this peptide in the absence of either metal allows ATP hydrolysis to proceed unchecked even when there is a diminished electron gradient present. Another report suggested that Cu itself had a weak but positive influence upon the activity of the ability of F1 ATPase to stimulate ATP hydrolysis (115).

The apparent decreased stability of the mitochondrial membrane in hearts from copper-deficient animals (16, 17, 23, 116) might be what leads to decreased binding of these protein subunits and enhanced breakdown. Cytochrome-*c* oxidase and ATP synthase are in close proximity to each other on the inner mitochondrial membrane. However, it is not certain why the mitochondrial encoded subunits are not affected as are the nuclear encoded subunits of CCO. Keyhani and Keyhani (117) reported that yeast cells grown in copper-deficient media also were reported to have decreased nuclear encoded subunits. They suggested that copper was needed for the movement of the newly synthesized subunits into the mitochondria for assembly. Their work suggested that in copper deficiency, the nuclear encoded subunits of CCO were synthesized but the assembly was impaired. CCO nuclear encoded subunits are synthesized on free ribosomes as a precursor. For subunit IV in the rat, the apparent molecular weight of the precursor form is estimated at 18–19.5 kDa, and the mature subunit is 16.5 kDa (118). These precursors appear to depend on a chaperonin protein, which is essentially the heat-shock protein 70 (hsp70) (103, 118, 119). We have conducted experiments with Cu^- and Cu^+ rat hearts and have not noted any differences in the levels of Hsp 70 or Hsp 90 proteins, suggesting that the copper deficiency does not alter the presence of this important transport proteins (106) in the hearts. This is somewhat in contrast to the findings of Matz *et al.* (120), who reported decreased mRNA levels for HSP 70 in aorta and mRNA levels for HSP 60 in atria of copper-deficient rats. The chaperonin protein binds to a receptor on the mitochondria and the subunits enter the mitochondria through a pore complex. The import of the cytoplasmic-derived proteins also requires an electrochemical potential across the inner membrane of the mitochondria. The process is ATP dependent. Blockage of electron transport with KCN or actimycin and blockage of F_1F_0-ATP synthase activity with oligomycin results in high matrix ATP and thus a low electrochemical gradient across the mitochondrial membrane. When this occurs, protein import is blocked as in the case of the β subunit of the F_1 ATP synthase complex. Uncoupling of electron transport will also lead to a decreased uptake of cytoplasmically synthesized proteins that are imported to the mitochondria (103, 118). Our studies (94) suggest that some uncoupling occurs in cardiac mitochondria from hearts of Cu^- rats in that respiratory control ratio is depressed compared with copper-adequate rats. The decrease in CCO nuclear encoded subunits could be due in part to this aspect. A finding by Matz *et al.* (121) in isolated mitochondria from hearts of Cu^- rats demonstrated that during state 3 respiration (in the presence of added ADP) oligomycin inhibited respiration less than in Cu^+ controls. This finding is revealing in that it suggests that the immediate defect is due to ATP synthase itself and not CCO. Oligomycin binding to the F_0 ATPase complex is for some reason defective in Cu^- rats. The same mitochondria, however, maintained their respiration properties in the presence of oligomycin. The addition of 2,4-dinitrophenol to reverse the oligomycin effect caused greater increase in respiration in Cu^- rats compared with controls. This finding is also revealing since it suggests that the electron transport system functions without impairment in copper deficiency, suggesting CCO is not impaired. This is not too surprising in reference to CCO in that it has been known that the mitochondrial encoded subunits can function without the presence of the nuclear encoded ones (103).

The increase in mitochondria is the first sign of copper deficiency in the heart, and this variable best correlates with

previously reported (16, 146) that in both Cu^- rats and pigs valvular pathology has been clearly documented (sparse connective tissue, disruption of the zones of the valves and thickening of valves). More significant is whether upon copper repletion the compromised valves can be repaired and normal function returned. Since heart valves have both collagen and elastin, and the latter has a half-life approximately the life of the entire organism (147, 148), repletion may not reverse the damage.

Figure 2 presents a revised hypothetical model for the sequence of events and possible factors leading to heart failure in copper deficiency. The model is based on the role copper exerts upon three major heart components: (i) antioxidant systems, (ii) connective tissue, and (iii) energy metabolism. In essence, the known roles copper has upon the activities of the cuproenzymes Cu,Zn-SOD, lysyl oxidase, and cytochrome-*c* oxidase collectively forms the basis of the present model. The model suggests that the interplay of these three enzyme components leads to cardiomyopathy under conditions of compromised copper status. Our research to date has demonstrated many of the changes noted in this model. For instance, we now know there is a shift to the V_3 isomyosin form in the Cu^- rat heart; fibronectin levels are increased and laminin levels decreased in the Cu^- rat heart; and fibronectin and Types I collagen mRNA expression is increased. Cardiac valves are compromised in terms of function. The changes in CCO nuclear encoded subunits without concomitant change in transcripts have been delineated above in great detail. What remains is to determine the sequence of events, potential signaling mechanisms, and reversibility and age specific aspects of copper deficiency.

Future Research Directions

While this review focuses on the mechanism of cardiac hypertrophy and cardiomyopathy, as well as substrate utilization with reference to energy, other factors provide fertile ground for consideration in the context of this model.

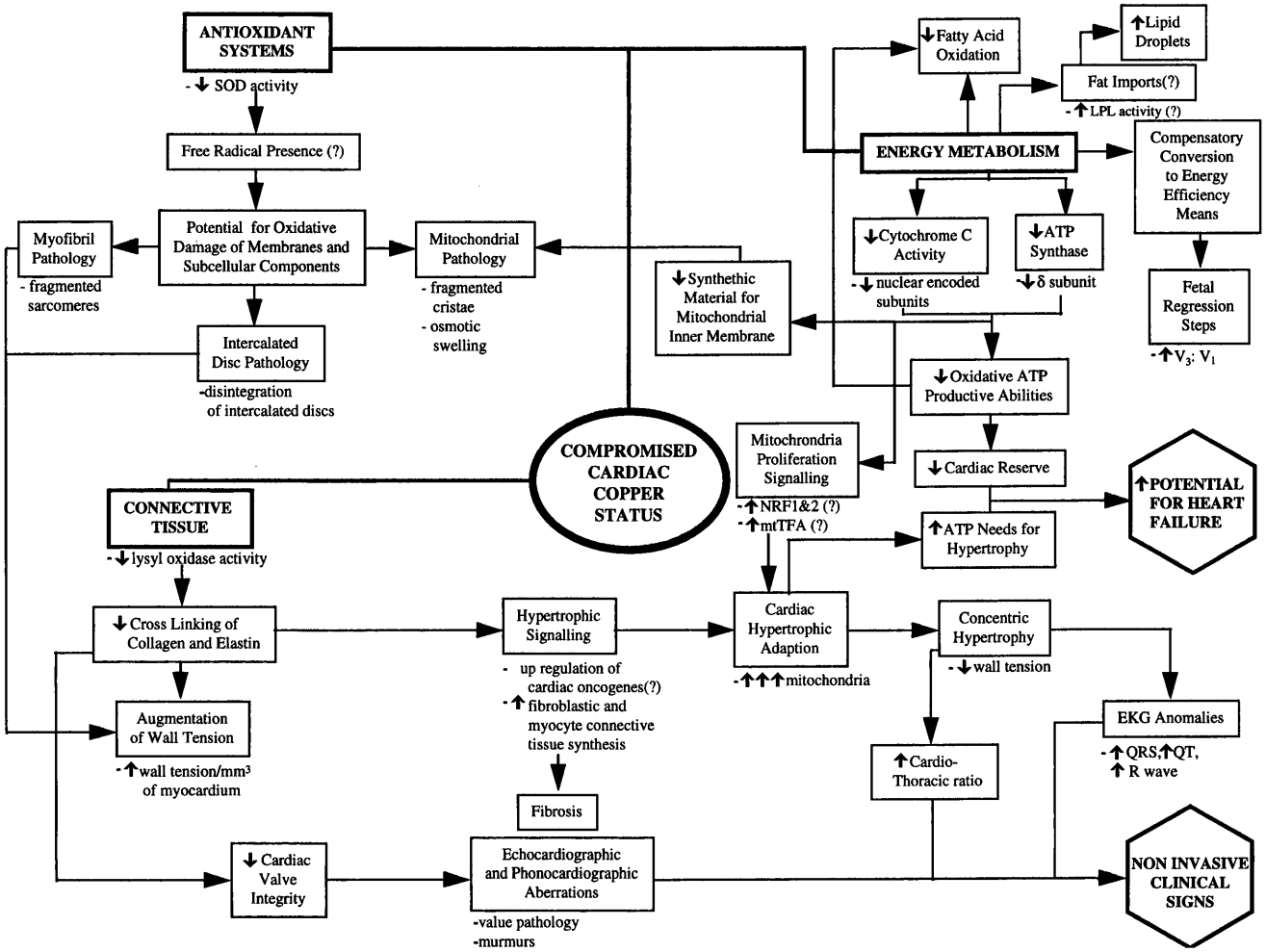

Figure 2. Model of critical events leading to cardiac hypertrophy and cardiomyopathy under conditions of copper deficiency. The primary focus of the model is the influence upon copper-containing metalloenzymes. Three components appear affected under conditions of compromised cardiac copper status: (i) antioxidant systems *via* decreased Cu,Zn-SOD activity, (ii) connective tissue pathology, including both fibrillar collagens and basement membrane proteins, *via* decreased activity of lysyl oxidase, and (iii) impaired energy metabolism and compromised CCO activity and peptide structure and possibly that of ATP synthase. Altered activity of these critical enzymes leads to an array of consequences and secondary changes culminating in cardiac failure and clinical signs of failure.

The role dietary carbohydrate type is known to have a role in that fructose or sucrose exacerbate the signs of heart disease with copper deficiency in comparison with glucose or cornstarch as the predominant dietary carbohydrate (147–153). The mechanism by which this may occur remains obscure. The recent finding that higher levels of fat can have a similar deleterious effect could be due to enhanced copper excretion *via* biliary routes (91). The large body of knowledge with respect to lipoprotein metabolism and copper deficiency was not reviewed here, but the total sequelae of heart disease progression suggest that blood lipid levels and vessel pathology must be considered in the total framework of any disease condition (154, 155). That marginal copper intake in animals has been shown to result in cardiac defects leads to the question of whether humans may be at risk for heart disease if they consume marginal copper. Such studies support the recent consensus that a Recommended Dietary Allowance (RDA) for copper be established (156). More comprehensive studies that examine copper status and specific types of heart disease may provide insights into relevance of results obtained from animal studies. Specific electrocardiogram patterns, echocardiograms revealing valvular regurgitation and heart murmurs, are conditions, in addition to concentric hypertrophy in the absence of hypertension, that could be candidate symptoms indicating the need to evaluate further copper status in such afflicted individuals.

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