Impaired Deformability of Copper-Deficient Neutrophils

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We have previously shown that dietary copper deficiency augments neutrophil accumulation in the lung microvasculature. The current study was designed to determine whether a diet deficient in copper promotes neutrophil chemoattraction within the lung vasculature or if it alters the mechanical properties of the neutrophil, thus restricting passage through the microvessels. Sprague-Dawley rats were fed purified diets that were either copper adequate (6.3 μg Cu/g diet) or copper deficient (0.3 μg Cu/g diet) for 4 weeks. To assess neutrophil chemoattraction, bronchoalveolar lavage fluid was assayed for the neutrophil chemokine macrophage inflammatory protein-2 (MIP-2) by enzyme-linked immunosorbent assay. Neutrophil deformability was determined by measuring the pressure required to pass isolated neutrophils through a 5-μm polycarbonate filter. The MIP-2 concentration was not significantly different between the dietary groups (Cu adequate, 435.4 ± 11.9 pg/ml; Cu deficient, 425.6 ± 14.8 pg/ml). However, compared with controls, more pressure was needed to push Cu-deficient neutrophils through the filter (Cu adequate, 0.150 ± 0.032 mm Hg/sec; Cu deficient, 0.284 ± 0.037 mm Hg/sec). Staining of the filamentous actin (F-actin) with FITC-Phalloidin showed greater F-actin polymerization and shape change in the Cu-deficient group. These results suggest that dietary copper deficiency reduces the deformability of neutrophils by promoting F-actin polymerization. Because most neutrophils must deform during passage from arterioles to venules in the lungs, we propose that copper-deficient neutrophils accumulate in the lung because they are less deformable.
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Introduction

The lung microcirculation is known to contain a significantly larger concentration of neutrophils compared with the systemic circulation (1). In the noninflamed lung, this "marginated" pool of neutrophils is thought to be dependent on the geometry of the pulmonary capillary bed, as well as the ability of the neutrophils to deform while moving through the capillary segments (1). In the inflamed lung, chemokines are produced and neutrophils are "sequestered" by an adhesive interaction between the circulating neutrophils and the vascular endothelium followed by migration into the lung parenchyma (1).

Copper is an essential micronutrient that is an important regulator of neutrophil-endothelial cell interactions. Inadequate copper concentrations have proinflammatory effects on neutrophils and endothelial cells both *in vitro* and *in vivo*. *In vitro* studies have shown that neutrophils from copper-deficient rats pass more readily through endothelial cell monolayers (2, 3), migrate farther in response to IL-8 (3), adhere more to P-selectin (3), and express more CD11b (3) than copper-adequate controls. Copper-chelated lung microvascular endothelial cells have a greater concentration of F-actin compared with controls, both with and without histamine stimulation (3). Using the *in vivo* rat model, we have shown that dietary copper deficiency causes an accumulation of neutrophils (indexed by myeloperoxidase) in the lung microvasculature that is 3–4 times greater than that seen in control rats (2, 4). However, the mechanism of this enhanced neutrophil accumulation is not known.

The current study was designed to determine whether a diet deficient in copper promotes neutrophil chemoattraction within the lung vasculature or if inadequate copper alters the mechanical properties of the neutrophil, thus restricting passage through the capillary bed. We have previously suggested that stimulation of a CD11b/CD18-independent pathway may be the mechanism by which neutrophils preferentially accumulate in the lungs of copper-deficient rats (2). Supporting this hypothesis is data showing that migration of copper-deficient neutrophils is greater than controls to CD11b/CD18-independent IL-8 (3). Therefore,

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the first aim of this study was to determine whether the increased accumulation of neutrophils in the lungs of copper-deficient rats was due to augmented pulmonary production of the MIP-2 chemokine because it is the functional IL-8 correlate in the rat.

The disparity between the size of the neutrophils (6–8 μm) and the pulmonary capillary (5.5 μm) requires neutrophils to deform during transit through the pulmonary capillaries (5). Deformability of the neutrophil is mediated by the rapid assembly of filamentous actin (F-actin) from soluble actin (G-actin) at the cell periphery, which increases the rigidity of the neutrophils and transit time through the lung (6). Because augmented F-actin polymerization has been seen in both copper-deficient endothelial cells (3) and platelets (7), we hypothesized that F-actin would also be greater in neutrophils from copper-deficient rats. Based on this hypothesis, the second aim of the study was to examine the mechanical deformation of copper-deficient neutrophils.

Materials and Methods

Animals and Diet. The use of animals was approved by the University of Louisville Animal Care and Use Committee. Male weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12:12-hr light:dark cycle. The rats were given free access to distilled water and to one of two purified diets for 4 weeks. The basal diet was a casein-sucrose-cornstarch–based diet (TD84469, Teklad Test Diets; Madison, WI) containing all known essential vitamins and minerals except for copper and iron. The copper-adequate diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of $CuSO_4 \cdot H_2O$/kg of diet. The copper-deficient diet was the same except for replacement of copper with cornstarch in the mineral mix. Diet analysis by inductively coupled argon plasma emission spectrometry (Liberty II model; Varian Associates, Sugarland, TX) indicated that the copper-adequate diet contained 6.18 mg copper/kg and the copper-deficient diet contained 0.29 mg copper/kg. Parallel assays of the National Institute of Standards and Technology (Gaithersburg, MD) reference samples (citrus leaves, no. 1572) yielded values within the specified range, which validated our copper assays.

Bronchoalveolar Lavage (BAL) Fluid Content of MIP-2. Animals ($n = 4$, Cu adequate; $n = 4$, Cu deficient) were anesthetized ip with sodium pentobarbital (50 mg/kg of body weight). The BAL fluids were collected by instilling and withdrawing 5 ml of sterile phosphate-buffered saline (PBS) three times from the lungs *via* an intratracheal cannula. The BAL content of MIP-2 was measured using an enzyme-linked immunosorbent assay (ELISA) purchased from R&D Systems (Minneapolis, MN).

Neutrophil Isolation. Animals ($n = 10$, Cu adequate; $n = 11$, Cu deficient) were anesthetized as previously explained. The isolation method has been previously described (3). The blood was drawn by venipuncture of the vena cava using syringes that contained sodium citrate anticoagulant at a ratio of one part anticoagulant to nine parts blood. A total of 6.5 ml of blood and anticoagulant mixture was carefully layered on top of the same volume of Polymorphprep (Axis-Shield PoC AS; Oslo, Norway) in a 15-ml conical tube and centrifuged at 600 *g* for 45 mins at room temperature. After the centrifugation, neutrophils were carefully collected from the lower band at the sample/medium interface using a Pasteur pipette. Red blood cells were lysed by the addition of 0.3 ml of deionized water for 30 secs, and osmolarity was restored by adding 0.3 ml of 2× concentrated saline. The cells were washed twice in PBS containing 0.5% bovine serum albumin. The neutrophils were then counted using a hemocytometer, and the sample was normalized to a concentration of 10^6 neutrophils/ml. We have previously used this method and reported a high cell purity in both dietary groups (3).

Deformability Assay. Leukocyte deformability was analyzed by recording the pressure generated when isolated neutrophils were pumped at a constant rate through polycarbonate filters containing 5-μm pores (Nucleopore Polycarbonate; Whatman, Inc., Clifton, NJ). The filtration device consisted of a 13-mm microanalysis filter holder (Nucleopore Corp., Pleasanton, CA) connected to a disposable plastic syringe positioned in a constant-rate infusion pump (Model 2400; Harvard Apparatus, Southnatick, MA). A stopcock was positioned on the pump side of the filter and connected to a pressure transducer (Deltran, Midvale, UT). Pressure values were recorded on a polygraph recorder (Model 500; Linear Scientific, Reno, NV), and the rate of pressure increase was calculated from the pressure curve. The apparatus was calibrated using a mercury manometer. All samples were analyzed at a flow rate of 0.2 ml/min.

Neutrophil F-Actin Content. Neutrophils were isolated using the method previously described. Once isolated, the cells were incubated with 5 ng/ml of fluorescein isothiocyanate (FITC)-Phalloidin (Sigma Chemical Co., St. Louis, MO) and 100 μg/ml of lysopalmitoylphosphatidylcholine dissolved in 3.7% para-formaldehyde for 30 mins at 4°C in the dark (3). After incubation, the cells were washed three times with PBS. The images of the formed intracellular F-actin were recorded with a confocal laser-scanning microscope (FV10-SW; Olympus, Melville, NY) with a 100× objective and equipped with a 495-nm excitation and 513 emission filter.

Image Analysis. All confocal images were analyzed using Matrox Inspector software (Version 4.1; Dorval, Quebec, Canada). The software was used to determine F-actin polymerization by measuring the mean pixel intensity

of each cell. The software was also used to measure the area (A) and perimeter (P) of each cell, which were used to calculate the parameter of compactness ($C = P^2/4\pi A$), or roundness, of the cell. The variation in compactness indicates the deviation of the shape in the image from that of a disc (C for disc = 1; Ref. 8).

Statistical Analysis. All data are expressed as means ± SE. Data were analyzed with a one-way ANOVA. Differences were considered significant when $P < 0.05$.

Results

Establishment of Copper Deficiency. Our model of copper deficiency is well established. The degree of copper deficiency is determined by measuring hepatic copper content. In the current study, rats fed the copper-deficient diet for 4 weeks had significantly lower hepatic copper concentrations than the copper-adequate group (2.10 ± 0.25 μg Cu/g vs. 12.70 ± 0.37 μg Cu/g dry weight, respectively).

BAL Fluid Content of MIP-2. To determine if dietary copper deficiency promotes neutrophil chemoattraction in the lung, BAL fluids were analyzed for the presence of the neutrophil-attracting chemokine, MIP-2, by ELISA. The neutrophil chemokine MIP-2 is critical in multiple models of inflammatory lung injury (9–11). The data show that there was no difference in BAL concentrations of MIP-2 between the dietary groups (Fig. 1). These results suggest that the increased lung accumulation of neutrophils in copper-deficient rats is not caused by alterations in pulmonary expression of the MIP-2 chemokine.

Deformability Assay. To determine if there were structural changes to neutrophils induced by copper deficiency, neutrophil deformability was assessed by measuring the pressure required to push neutrophils through a 5-mm pore polycarbonate filter. The results were determined by comparing the rate of pressure development between neutrophils from the two dietary groups. The rate of pressure increase was significantly greater ($P < 0.042$) in the copper-deficient group ($n = 8$) compared with the copper-adequate group ($n = 8$; Fig. 2). These results suggest that neutrophils in copper-deficient rats are less able to deform and pass through the lung microvasculature.

Neutrophil F-Actin Content. To investigate the mechanism of decreased neutrophil deformability seen in the copper-deficient group (Fig. 2), the content of F-actin was assayed in isolated neutrophils from the two dietary groups. Copper-adequate neutrophils stained with FITC-Phalloidin showed a modest amount of F-actin in the shape of a ring around the edge of the cell (Fig. 3), whereas the copper-deficient cells showed a more diffuse and intensely stained area of F-actin throughout the cell (Fig. 3). The degree of F-actin polymerization was quantitated by measuring cell mean fluorescent intensity. Neutrophils from copper-deficient rats had significantly greater ($P < 0.006$) mean fluorescent intensity compared with neutrophils from copper-adequate rats (Fig. 4).

The shape and size of the neutrophils from copper-deficient rats were also very different from neutrophils from copper-adequate rats. The copper-adequate neutrophils showed little deviation from the expected round shape, described as compactness with a circle having a value equal to 1. Neutrophils from copper-deficient rats showed obvious areas of elongation and protrusions, and the compactness was significantly less ($P < 0.00008$) than the controls (Fig. 5). The results suggest that the decreased deformability of the copper-deficient neutrophils is caused by the diet-induced increase in F-actin polymerization and shape change.

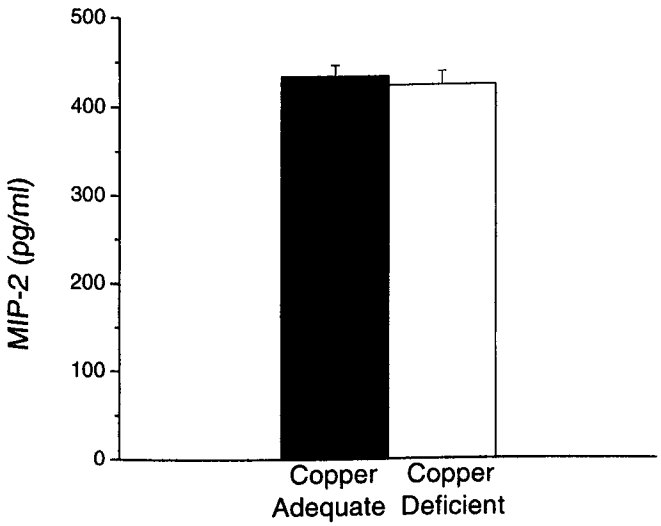

Figure 1. Effects of copper deficiency on bronchoalveolar lavage fluid content of macrophage inflammatory protein-2 (MIP-2). Values are means ± SE. $n = 4$ rats per group.

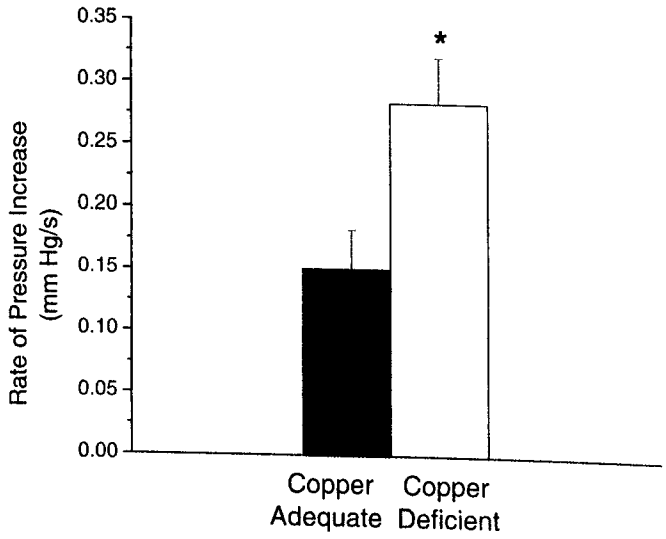

Figure 2. Effects of copper deficiency on neutrophil deformability as indexed by the rate of pressure increase required to push neutrophils through a 5-μm polycarbonate filter. Values are means ± SE. $n = 8$ rats per group. *$P < 0.05$ compared with the copper-adequate control group.

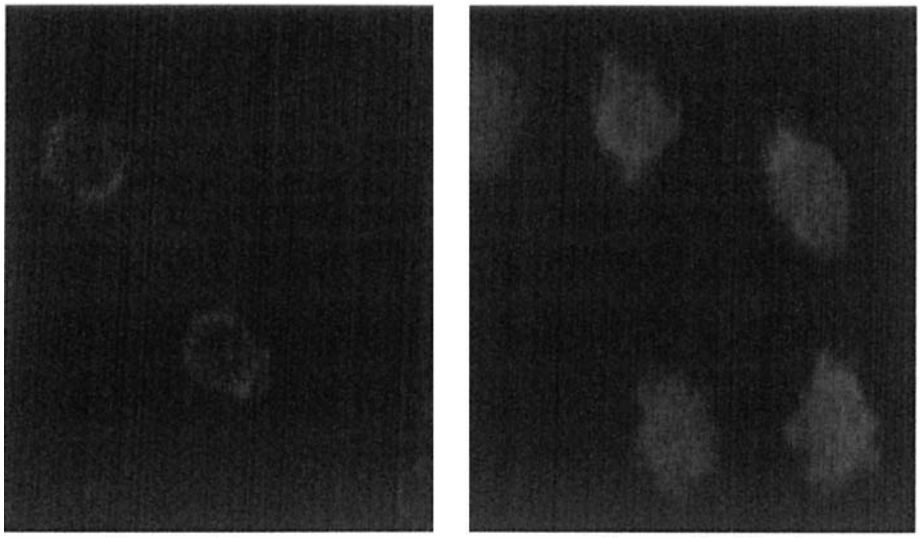

Figure 3. Representative confocal microscopy images of copper-adequate and -deficient neutrophils stained with FITC-Phalloidin to demonstrate F-actin polymerization and localization.

Discussion

Copper is an essential cofactor in both the structure and function of the cardiovascular system. A diet deficient in the trace mineral causes significant defects in the heart, blood vessels, and circulating blood cells (12). Neutrophils, in particular, are known to be very sensitive to suboptimal copper concentrations, even when traditional indices of copper deficiency are unaffected (13). The current study provides new information on the status of circulating neutrophils and their accumulation in the lungs of copper-deficient rats.

In the current study, the possibility of a dietary alteration in chemokine signaling within the lung was tested by assaying for the potent neutrophil chemoattractant MIP-2. The proinflammatory cytokine MIP-2 is released from type II alveolar epithelial cells and macrophages in response to inflammatory stimuli (14). Although the current study was performed in the absence of an inflammatory stimulus, we have demonstrated that a copper-deficient diet has several proinflammatory effects on both neutrophils and lung microvascular endothelial cells (3). Further, the lungs of copper-deficient rats are predisposed to exaggerated inflammatory responses (2, 4). Therefore, we hypothesized that proinflammatory signaling may be exaggerated in the copper-deficient lung due to a dietary effect alone. The current data, which show that there is no difference in MIP-2 release in the copper-deficient lung (Fig. 1), suggest that

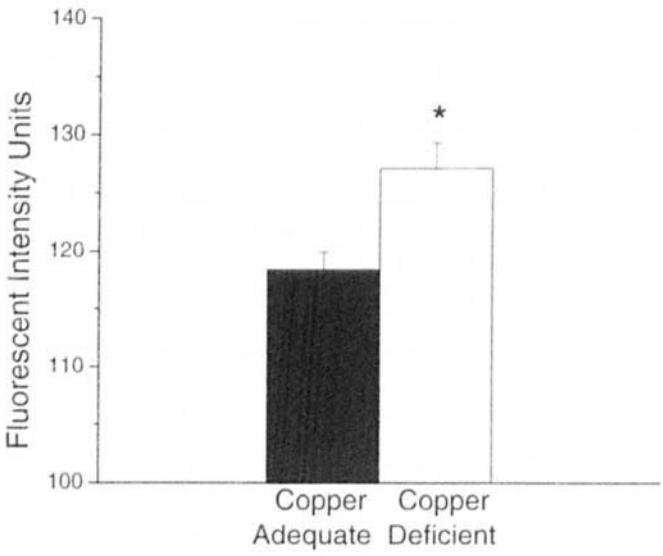

Figure 4. Effects of copper deficiency on F-actin polymerization in neutrophils. Fluorescent intensity of FITC-Phalloidin was used as an index to quantitate F-actin polymerization. Values are means ± SE. $n = 5$ rats per group. $^*P < 0.05$ compared with the copper-adequate control group.

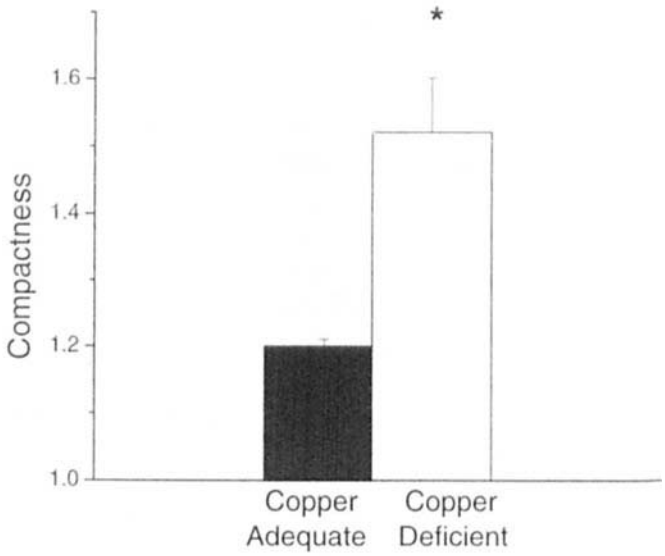

Figure 5. Effects of copper deficiency on shape change associated with cytoskeletal reorganization. Compactness is an index of roundness (see the Materials and Methods section), with a disc having a value of 1. Values are means ± SE. $n = 5$ rats per group. $^*P < 0.05$ compared with the copper-adequate control group.

the copper deficiency does not upregulate chemokine expression in the rat lung in the absence of any classical inflammatory stimuli.

In addition to chemoattraction, neutrophil deformability is a key factor in determining whether neutrophils pass through the lung microvasculature or become lodged in the smallest vessels (5). The biomechanic property of deformability is particularly important in the noninflamed lung where known adhesion molecules do not mediate neutrophil margination (1) and where extravasation of activated neutrophils occurs from capillaries instead of postcapillary venules (15). In our second study, the comparison of deformability between neutrophils from copper-adequate and -deficient rats demonstrates that the copper-deficient neutrophils are less deformable (Fig. 2) and, therefore, are less likely to readily pass through the lung microcirculation.

Neutrophil stiffening or decreased deformability is caused by the rapid assembly of filamentous F-actin from soluble G-actin, which increases the rigidity and viscosity of the neutrophil (16). Filamentous F-actin stained with FITC-Phalloidin was used in the current study to quantitate the cytoskeletal component in neutrophils from the two dietary groups. The results demonstrate both a significant increase in F-actin (Figs. 3 and 4) and a significant, spontaneous shape change in neutrophils from the copper-deficient group (Figs. 3 and 5). These results clearly correlate with the decreased deformability of the copper-deficient neutrophils seen in our study (Fig. 2) and suggest a proinflammatory activation of neutrophils in copper-deficient rats. The shape changes also suggest the occurrence of cytoskeletal remodeling, which is required for neutrophils to migrate to the borders of endothelial cells (17) and may account for the greater extravasation of copper-deficient neutrophils through endothelial cell monolayers (2, 3).

The mechanism of enhanced F-actin polymerization in the copper-deficient group has not been studied, but we have seen the same phenomenon in copper-deficient lung microvascular endothelial cells (3). Based on our experience with the copper-deficient rat model, a likely explanation for the change in intracellular F-actin relates to the regulatory role of nitric oxide (NO). The inhibition of NO synthesis is known to increase PMN sequestration in the rat lung (18). Further, exogenous NO has been shown to inhibit the sequestration of polymorphonuclear leukocytes in the lung by inhibiting F-actin assembly (19). We have previously shown that NO-mediated signaling is inhibited in the microcirculation of the copper-deficient rat (20–22). The mechanism of this inhibition appears to be both a reduction in the mobilization of intracellular endothelial calcium necessary for NO production (23) and the reduction of available NO by reaction with superoxide anion to produce peroxynitrite (23). Therefore, we propose that the lack of available NO in the copper-deficient circulation promotes the F-actin polymerization seen in this study (Fig. 4).

In summary, the current study addresses two possible mechanisms for the greater accumulation of neutrophils that occurs in the copper-deficient rat lung. The results demonstrate that although the inflammatory response in copper-deficient rat lung is exaggerated (4), there is no increase in the production of the chemokine MIP-2 in the absence of an inflammatory stimulus (Fig. 1). The results also demonstrate that although the MIP-2 chemokine signaling is not enhanced, the copper-deficient neutrophils are stiffer (Fig. 2) and, therefore, less likely to deform and pass through the small caliber lung capillaries. The data bolster our hypothesis that dietary copper deficiency causes proinflammatory changes in neutrophils (3). Further experimentation revealed that the reason for the loss of deformability is likely caused by greater polymerization of F-actin in the copper-deficient neutrophil (Fig. 4). Based on the results, we conclude that the excess accumulation of neutrophils in the copper-deficient rat lung is the result of changes in the biomechanic-deformability properties of the neutrophil.

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