BIOLOGICAL TRACE ELEMENT RESEARCH 4, 191–197 (1982)

The Effect of Copper on Glutathione Metabolism in Human Leukocytes

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Accepted March 26, 1982

Abstract

Leukocytes incubated with Cu(II) showed a decrease in both glutathione reductase activity and reduced glutathione content. The glucose 6-phosphate dehydrogenase activity under the same conditions was not affected. Serum albumin added to mixtures prevented the loss of enzyme activity, while D-penicillamine and L-histidine had little effect. Prior oxidation of the cell-reduced glutathione did not diminish the enzyme inhibitory action of Cu(II). The amount of regeneration of reduced glutathione in leukocytes previously treated with diamide to oxidize their reduced glutathione was a function of Cu(II) concentration in the media. No evidence was obtained that elevated serum ceruloplasmin levels in rabbits, nor incubation of leukocytes in vitro with ceruloplasmin, affect leukocyte glutathione reductase activity. It was proposed that the major mechanism by which copper affects glutathione metabolism in leukocytes is by inhibition of glutathione reductase.

Index Entries Glutathione metabolism, effect of Cu on; glutathione reductase, effect on Cu on; leukocyte, effect of Cu on: ceruloplasmin, effect on leukocyte glutathione reductase; D-penicillamine, effect on Cu toxicity.

Introduction

Like several other metals, copper plays an ambivalent role in cell function. In animals it can be either inflammatory or anti-inflammatory (*1*). Although it is an essential nutrient because it is a part of certain enzymes, it can be toxic to cells such

0163-4984/82/6900-0191$02.00

as erythrocytes when its serum concentration is elevated, as in Wilson's disease (*2*). Its toxicity for erythrocytes is usually explained by its direct oxidant action on GSH* or by its inhibition of enzymes such as glutathione reductase and glucose 6-phosphate dehydrogenase necessary for regeneration of GSH from GSSG. In keeping with an essential role of GSH in cell function is the finding of Johnson et al. (*3*) that incubation of dog erythrocytes with a chemical oxidant for GSH decreases their survival time in vivo. Another possibility for its toxicity is that oxygen radicals, which can damage cell structures, are generated upon reoxidation of Cu(I) by oxygen (*4*). In any case it appears that when intracellular copper exceeds a certain concentration, it can cause toxicity.

Because copper toxicity in animals is usually seen as hemolytic anemia, the action of copper on erythroctyes has been much studied. Comparable studies with leukocytes are lacking. Leukocytes (neutrophils) contain millimolar amounts of glutathione, almost all of which is GSH (*5*), which makes them good candidates for the action of copper. In the present study the effect of copper on the glutathione reductase activity and GSH content of human buffy coat cells has been investigated.

Materials and Methods

Leukocytes were prepared from washings obtained during preparation of packed human erythrocytes at the University Hospital blood bank. The washings, to which were added 1000 units of heparin per 100 mL, were centrifuged at 1000*g* for 15 min and the buffy coat removed by aspiration. Further separation of leukocytes from contaminating erythrocytes was obtained by sedimenting the erythrocytes at 1*g* in 6% dextran-0.15*M* NaCl and by hypotonic lysis. Cell number was measured using a hemocytometer slide and cells stained with gentian violet in dilute acetic acid. Protein content of leukocytic extracts was measured by the Lowry et al. method (*5*). *E. coli* (0.55:B5) lipopolysaccharide W was purchased from Difco Corp. Detroit, MI. Human ceruloplasmin, diamide [azodicarboxylic acid bis (dimethylamide)] and *Clostridium perfringens* neuraminidase were obtained from Sigma Chem. Co., St. Louis, MO.

Reaction mixtures contained 0.5–2 $\times$ 10^7 leukocytes in Tris-buffered saline, pH 7.5. For enzyme assays, cells were collected by centrifugation, washed once with Tris-buffered saline, resuspended in 0.1*M* Tris-HCl buffer, pH 7.5, and frozen and thawed three times. An aliquot of the supernatant after centrifugation of the lysate was used for enzyme assay. For both glutathione reductase and glucose 6-phosphate dehydrogenase the change in light absorption at 340 nm measured between 1 and 5 min was used to determine enzyme activity. The glutathione reductase assay mixture contained 1 m*M* GSSG, 0.1 m*M* NADPH, in either 0.1*M* Tris-HC1, 5 m*M* EDTA buffer, pH 7.5, or 0.1*M* Tris-HC1 buffer, pH 7.5. The difference in activity obtained with and without EDTA at 30°C was taken as a measure of copper inactivation of the enzyme. With no copper added to mixtures,

*The following abbreviations are used: GSH, reduced glutathione; GSSG, oxidized glutathione; EDTA, ethylenediamine tetracetic acid.

this difference in experiments reported varied from 0 to 15% of the total activity (EDTA buffer). All enzyme assays were done twice, once with EDTA in buffer solutions, and once without EDTA. In some assays, which were repeated 2 or 3 times, results were reproduced within ±5% error. All experiments were done at least twice using different leukocyte preparations. The results of a typical experiment are presented. The glucose 6-phosphate dehydrogenase assay mixture contained 0.1m*M* $NADP^+$ and 0.4 m*M* glucose 6-phosphate in the buffers just described. Cell GSH was measured as described by Oliver et. al. (*6*) and total glutathione as described by Tietze (*7*).

To obtain elevated ceruloplasmin content in blood rabbits were injected intravenously with 50 μg of *E coli* endotoxin. Animals were bled and ceruloplasmin measured in their serum according to the method of Ravin (*8*). Each determination was done in duplicate and the results averaged. Maximum serum ceruloplasmin levels were obtained 2–3 days after endotoxin injection. Leukocytes were separated from the blood by mixing it with an equal volume of 6% dextran–0.15*M* NaCl and allowing the erythrocytes to sediment at 1*g*. Leukocyte extracts were prepared and glutathione reductase assays carried out in 0.1*M* Tris-HCl buffer, pH 7.5 as previously described. Copper inhibition of the enzyme was judged by doing the assay with and without 300 μ*M* D-penicillamine. This concentration of D-penicillamine reverses copper inhibition of the enzyme (*9*).

Results

Leukocytes incubated with Cu(II) showed a decrease in glutathione reductase activity that was related to the Cu(II) concentration in the media (Table 1). The glucose 6-phosphate dehydrogenase activity measured under the same conditions was

TABLE 1
Inhibition of Glutathione Reductase in Leukocytes Incubated with Different Amounts of Copper[a]

Cu(II)	Inhibition
μ*M*	%
—	[b]
6.4	0
12.8	0
22	20
48	40
64	55

[a]Mixtures were incubated 30 min at 30°C. Glutathione reductase activity was measured in cell extracts prepared from the leukocytes with and without EDTA in buffer solutions. The value obtained in EDTA buffer was taken as 100% activity to calculate percent inhibition.

[b]The specific activity of the extract prepared from leukocytes incubated with no copper was equal to ΔE_{340} of 0.015/min/10^6 cells.

not affected. Serum albumin and EDTA added to incubation mixtures prevented the loss of enzyme activity, while D-penicillamine and L-histidine had little effect (Table 2). The addition of D-penicillamine together with serum albumin reversed, in part, the blocking effect of serum albumin on the inhibitory action of Cu(II).

Because the amount of Cu(II) available for reaction with glutathione reductase can be diminished through its reaction with cell GSH, the effect of first oxidizing cell GSH with diamide on Cu(II) inhibition of the enzyme was examined. As can be seen in Table 3 there was a small increase in the percent inhibition of glutathione reductase after diamide treatment of leukocytes.

Incubation of leukocytes with Cu(II) resulted in a loss of GSH, which was related to the amount of Cu(II) added (Table 4). The total glutathione content of the cells did not change during the incubation. To examine the effect of copper on the ability of leukocytes to regenerate their GSH, cells were first incubated with diamide to oxidize their GSH. They were then incubated with different amounts of Cu(II) and their GSH content measured. As can be seen in Table 5, impairment of GSH regeneration was first obtained with 36 μM Cu(II). The effect of added glucose in these experiments depended on the leukocyte preparation; with some preparations it increased the rate and with others it had no effect. This presumably results from the different amounts of glycogen in cells. In another experiment, using leukocytes with a low GSH content, which was increased several-fold upon incubating the cells with glucose, it was found that 60 μM Cu(II) completely prevented the increase, indicating that it is the copper and not the diamide that prevents GSH regeneration.

Ceruloplasmin, a copper-binding glycoprotein, contains greater than 90% of the copper found in serum. Two kinds of experiments were done to test its possible effect on leukocyte glutathione reductase. In the first experiment the ceruloplasmin content of rabbit blood was elevated by injecting the animals with bacterial lipopolysaccharide. The glutathione reductase activity was then measured in ex-

TABLE 2
Effect of Different Materials on the Copper Inhibition of Glutathione Reductase in Leukocytes[a]

Additions	Inhibition, %
EDTA, 4 mM	5(55)
D-Penicillamine, 1 mM	45(55)
L-Histidine, 3 mM	30(50)
Bovine serum albumin, 0.8 mg/mL + D-penicillamine, 0.5 mM	0(70), 25(70)
Rabbit serum, 1.0 mL	45(45)

[a]Mixtures were incubated 30 min at 30°C and all contained 53 μM Cu(II). The number in parenthesis is the percent inhibition in that particular experiment with 53 μM Cu(II) and the number outside the parenthesis is the percent inhibition with copper plus the indicated addition. Determination of percent inhibition was described in Table 1. The specific activity of extracts prepared from leukocytes with no copper or other additions was comparable to that given in Table 1.

TABLE 3
Effect of Diamide on Copper Inhibition of Glutathione Reductase in Leukocytes[a]

Additions	Inhibition, %
—	12[b]
Diamide, 300 nmol	0
Cu(II), 32 nmol	18
Diamide + Cu(II)	32

[a]Two-mL mixtures, which contained diamide, were incubated 5 min at 37°C before adding copper. All mixtures were then incubated 30 min at 37°C. Determination of percent inhibition was described in Table 1. The specific activity of extracts prepared from leukocytes incubated with no additions was comparable to that given in Table 1.

[b]This inhibition with no copper added is presumably a result of nonspecific metal ion attachment to the enzyme during its preparation.

tracts of their leukocytes with and without D-penicillamine. The D-penicillamine was used to reverse any copper inhibition of the enzyme. As can be seen in Table 6, there is no evidence for copper inhibition of the enzyme even in rabbits with three times the normal serum ceruloplasmin level. The enzyme specific activity of leukocytic extracts likewise showed no relationship to the ceruloplasmin content of the blood from which the leukocytes were obtained. It varied from ΔE_{340} of 0.017–0.020/min/mg of protein. In the second type of experiment, human ceruloplasmin (25 μM with respect to copper) was incubated 1 h with leukocytes in the manner described in Table 1. Again, no inhibition was observed. Prior treatment of the ceruloplasmin with bacterial neuraminidase at pH 5.0 did not alter its inhibitory capacity.

TABLE 4
Reduced Glutathione Content of Leukocytes Incubated with Different Amounts of Copper[a]

Cu(II)	GSH
μM	nmol
0	18.9
16	17.9
48	11.3[b]
96	6.8

[a]Mixtures were incubated 30 min at 30°C.

[b]This value is considered significantly lower than the control at $< 0.1\%$ probability level.

TABLE 5
Effect of Different Amounts of Copper on Regeneration of Reduced Glutathione in Leukocytes[a]

Incubation, h at 30°C	Cu(II), μ*M*	GSH, nmol
0	0	0
2	0	19.2
2	9	19.0
2	22	20.5
2	36	15.5[b]
2	60	2.6

[a]All mixtures contained 5 m*M* glucose. The cells were pretreated with 100 nmol diamide/reaction mixture for 15 min at 30°C before adding copper.

[b]This value is considered significantly lower than the control at 0.1% probability level.

Discussion

The experiments reported here show that if Cu(II) concentration is high enough in the media, it can enter leukocytes and affect the activity of glutathione reductase and GSH content. Even at the highest Cu(II) concentration used, only about 50% inhibition of glutathione reductase was observed. That this concentration of Cu(II) completely prevented GSH regeneration suggests that the inhibition is actually greater in the intact cell. After breakage of cells in copper-free media there was probably some reversal of inhibition. That prior oxidation of cell GSH with diamide did not diminish the inhibitory action of copper on glutathione reductase indicates that Cu(II), and not Cu(I) or some product formed by its reaction with oxygen, is the reactive species. Also, that the inhibition found in this experiment was comparable to the inhibition obtained with the cell GSH intact, indicates that little intracellular copper reacts with cell GSH. For this reason, it is proposed that a major mechanism for the depletion of leukocyte GSH by Cu(II) is the inhibition of glutathione reductase, which prevents GSH regeneration. It is unlikely that the concentrations of Cu(II) used in these studies to see effects on glutathione reductase and GSH content in leukocytes are ever attained in any in vivo situation. In humans the normal serum copper level is about 18 μ*M* of which 17 μ*M* is ceruloplasmin. In patients with an inflammatory disease such as rheumatoid arthritis, it is elevated to about 27 μ*M* (*9*); however, again most of the copper is ceruloplasmin, which judged from this study is not available to the leukocyte. If copper plays a role in the inflammatory process, ceruloplasmin is probably the copper donor. For ceruloplasmin to be recognized and ingested by leukocytes, some modification of its carbohydrate side chains will be necessary. Previously, it has been found that glycoproteins with a terminal mannose residue are bound and presumably ingested by alveolar macrophages (*10*). If any type of leukocyte is perturbed by altered copper composition in tissue fluids, the macrophage seems the

TABLE 6
Effect of D-Penicillamine on Glutathione Reductase Isolated from Leukocytes of Rabbits with Different Serum Levels of Ceruloplasmin

Ceruloplasmin, mg%	Percent of control[a]
17	130
26	100
27	105
29	95
37	90
61	115
63	110

[a][Enzyme activity measured with 300 μ*M* D-penicillamine/enzyme activity measured without D-penicillamine] × 100.

most likely because of its pinocytoic activity, which would allow it to internalize a modified ceruloplasmin. This possibility is now being investigated.

Acknowledgments

Supported by a research grant, AM 21447, from the National Institutes of Health, US Public Health Service. Thanks are due to Diane Auger and Mei Hing Siu for their technical assistance.

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