Increased oxidative stress in kwashiorkor

Mark J. Manary, MD, Christiaan Leeuwenburgh, PhD, and Jay W. Heinecke, MD

To test the hypothesis that kwashiorkor is associated with increased oxidative stress, urinary concentrations of 2 oxidized amino acids, *o,o'*-dityrosine and *ortho*-tyrosine, were measured by gas chromatography–mass spectrometry. Children with kwashiorkor, with or without infection, had a 3- to 7-fold increase in urinary *o,o'*-dityrosine and a 1.5- to 2-fold increase in *ortho*-tyrosine when compared with well-nourished children. This observation raises the possibility that oxidative damage to proteins and other biologic targets plays a role in the clinical manifestations of kwashiorkor. (J Pediatr 2000;137:421-4)

Kwashiorkor, a severe form of protein-energy malnutrition, is characterized clinically by edema. Potassium deficiency, dietary protein deficiency, hypoalbuminemia, and renal electrolyte wasting have been implicated in the pathogenesis of the edema.[1] Oxidative damage to cell membranes may also play a role in causing edema.[2] Indeed, concentrations of the antioxidants vitamin E, β-carotene, and glutathione are lower in children with kwashiorkor than in well-nourished children or children with marasmus.[3-6] Also, erythrocyte membranes isolated from children with kwashiorkor show increased susceptibility to oxidative stress and ion-pump dysfunction, possibly as a result of oxidative stress in situ.[7] Moreover, invading pathogens trigger oxidant production by white blood cells,[8] and infection often precipitates the clinical symptoms of kwashiorkor in malnourished children.

Analysis of healthy and diseased tissues for specific markers offers a powerful approach to studying oxidative damage in vivo. Such markers have been identified as stable products of protein oxidation through in vitro studies. *o,o'*-Dityrosine appears when a tyrosyl radical or hydroxyl radical cross-links tyrosine residues, and *ortho*-tyrosine forms when a hydroxyl radical oxidizes phenylalanine residues.[9] Quantification of these oxidized amino acids in urine may serve as a noninvasive measure of oxidative stress in vivo because levels vary in parallel with those of protein-bound oxidized amino acids in animal models.[10,11]

To explore the potential role of oxidative stress in kwashiorkor, a highly sensitive and specific method, gas chromatography–mass spectrometry, was used to quantify amounts of *o,o'*-dityrosine and *ortho*-tyrosine in urine of children with kwashiorkor.

METHODS

Study participants, children attending the Queen Elizabeth Central Hospital in Blantyre, Malawi, were assigned to 3 groups, 2 of which were control groups: (1) children with kwashiorkor, (2) children with cerebral malaria, and (3) well-nourished children from the outpatient immunization clinic (Table). Children with cerebral malaria were studied because serious systemic infection may cause increased oxidative stress.[8] The well-nourished children had no clinical signs of illness and no hematuria, pyuria, or parasites in the urine.

Children with kwashiorkor were considered to have infection if they had bac-

Table. Anthropometric characteristics of the study groups

	Well-nourished (M = 4, F = 6)	Cerebral malaria (M = 3, F = 3)	Kwashiorkor (M = 5, F = 3)	Kwashiorkor with infection* (M = 6, F = 11)
Age (mo)	36 ± 10	32 ± 16	35 ± 12	38 ± 11
Weight/age z score[†]	−1.9 ± 0.9	−2.1 ± 0.8	−4.3 ± 0.6[‡]	−3.9 ± 0.6[‡]
Weight/height z score[†]	−1.0 ± 1.1	−1.2 ± 0.7	−2.8 ± 1.0[‡]	−2.4 ± 0.6[‡]

Results are expressed as mean ± SD.
M, Male; *F,* female.
*Infections: 8 pneumonia, 5 malaria, 3 dysentery, 1 sepsis.
[†]One unit is 1 SD from the US National Center for Health Statistics standards.
[‡]Significantly different (*P* < .001) from well-nourished children.

teremia, a thick blood film with malaria parasites, a positive serology for human immunodeficiency virus, pneumonia with an infiltrate on chest radiograph, or clinical dysentery. Patients with bacteruria or pyuria were excluded because urinary tract infection might confound the results of the urine analyses. Severity of edema and rash, duration of edema, serum albumin concentration, and outcome were recorded for each child with kwashiorkor. The Health Sciences Research Committee of Malawi and the Human Studies Committee of Washington University approved this study.

Urine was collected from each of the children with kwashiorkor or malaria by sterile catheter and from well children by standard clean void technique. A 2-mL sample of urine was mixed with 1 mL of antioxidant buffer (1% phenol and 100 μmol/L diethylenetriaminepentaacetic acid), immediately frozen, and subsequently stored at −70°C until analysis was performed.

Urine samples were analyzed for *o,o'*-dityrosine, *ortho*-tyrosine, tyrosine, and phenylalanine as previously described.[11] Briefly, carbon 13–labeled internal standards of each analyte were added to freshly thawed urine. Amino acids were extracted from the sample by solid-phase chromatography on a C-18 column, eluted with methanol, dried, and converted to *n*-propyl, heptafluorobutyryl derivatives. Derivatized amino acids were quantified by isotope dilution negative-ion electron capture gas chromatography/mass spectrometry with selected ion monitoring. Urinary creatinine concentration was determined with a standard diagnostic kit (Sigma Chemical Co, St Louis, Mo).

The amounts of *o,o'*-dityrosine and *ortho*-tyrosine were normalized to the precursor amino acid and to the concentration of creatinine. The former measures the fraction of amino acid that has been oxidized, and the latter corrects for differences in glomerular filtration rate between subjects. Differences between group means were compared by using the Kruskal-Wallis test. Statistical analyses and anthropometric *z* scores were determined by using Epi Info 6 (Centers for Disease Control and Prevention, Atlanta, Ga).

RESULTS

Selected ion monitoring gas chromatography/mass spectrometric analysis of urine of the subjects demonstrated negative ions derived from *o,o'*-dityrosine and *ortho*-tyrosine that co-eluted with ions derived from authentic ^{13}C-labeled internal standard. When normalized to creatinine concentration, the urine of the children with kwashiorkor contained 3-fold more *o,o'*-dityrosine than that of the well-nourished children

(Fig 1, *A*). Infection did not further increase the oxidized amino acids in the urine of the malnourished children. The difference between the children with kwashiorkor and the healthy control subjects became greater, a 7-fold increase, after *o,o´*-dityrosine was normalized to its precursor amino acid, tyrosine (Fig 2, *A*).

Children with kwashiorkor and infection had similar levels of *o,o´*-dityrosine as those with kwashiorkor without infection. Seven of the 17 children with kwashiorkor were infected with HIV; their urine contained 5.6 ± 2.0 nmol *o,o'*-dityrosine/mole tyrosine compared with 6.7 ± 1.4 for the HIV-negative children. The normalized level of *o,o'*-dityrosine in the urine of children with malaria was 1.5- to 3-fold higher than that of healthy, well-nourished children (Fig 1, *A,* and Fig 2, *A*).

There was little elevation in the urine content of *ortho*-tyrosine in either the kwashiorkor or malaria groups when the results were normalized to creatinine concentration (Fig 1, *B*). When the *ortho*-tyrosine content was normalized to renal excretion of its precursor amino acid, phenylalanine, however, the level was ~1.5-fold higher in the children with kwashiorkor (with or without infection) than in the healthy children (Fig 2, *B*). The levels of *o,o´*-dityrosine and *ortho*-tyrosine did not correlate with the duration or severity of edema, muscle wasting, severity of rash, or serum albumin concentration.

DISCUSSION

Urinary levels of *o,o'*-dityrosine, a marker of damage by tyrosyl radicals, were higher in children with kwashiorkor than in well-nourished, healthy children. Dityrosine also was elevated in urine from children with cerebral malaria, though to a lesser extent. In contrast, there was little difference in levels of *ortho*-tyrosine, a marker for damage by hydroxyl radicals. Previ-

ously, the amount of protein-bound *o,o'*-dityrosine, but not *ortho*-tyrosine, was found to be elevated in atherosclerosis and aging.[9,10] These observations suggest that oxidative stress is prevalent in children with kwashiorkor.

Urinary compounds are normalized to creatinine concentration to correct for differences in the glomerular filtration rate of subjects. However, creatinine excretion diminishes in malnourished children because of muscle wasting. Therefore the oxidized amino acids were also normalized to their precursor amino acids, tyrosine and phenylalanine. Both approaches indicated that urinary levels of *o,o'*-dityrosine are markedly elevated in kwashiorkor, whereas levels of *ortho*-tyrosine are only slightly higher. The consistent failure of *ortho*-tyrosine to reach higher levels in the urine of children with kwashiorkor suggests that malnutrition has little effect on the renal excretion of oxidized amino acids. Collectively, these observations suggest that the increase in urinary *o,o'*-dityrosine in kwashiorkor reflects an increase in oxidative stress rather than an alteration in renal excretion of the amino acid.

It is difficult to exclude the possibility of infection in malnourished children, but the comparison of infected and uninfected children revealed little difference in the amounts of *o,o'*-dityrosine or *ortho*-tyrosine. There was a smaller but significant elevation in excreted oxidized urinary amino acids in children with cerebral malaria, a serious systemic infection. These observations suggest that infection alone is unlikely to account for the differences in *o,o'*-dityrosine or *ortho*-tyrosine observed in this study.

What is the source of *o,o'*-dityrosine and *ortho*-tyrosine in urine? One possible source could be oxidatively damaged cytosolic or membrane-bound proteins, which are targeted for proteolytic degradation.[12] In this case, oxidative damage to membrane proteins, such as ion channels, might account for the abnormal ion gradients seen in the red and white
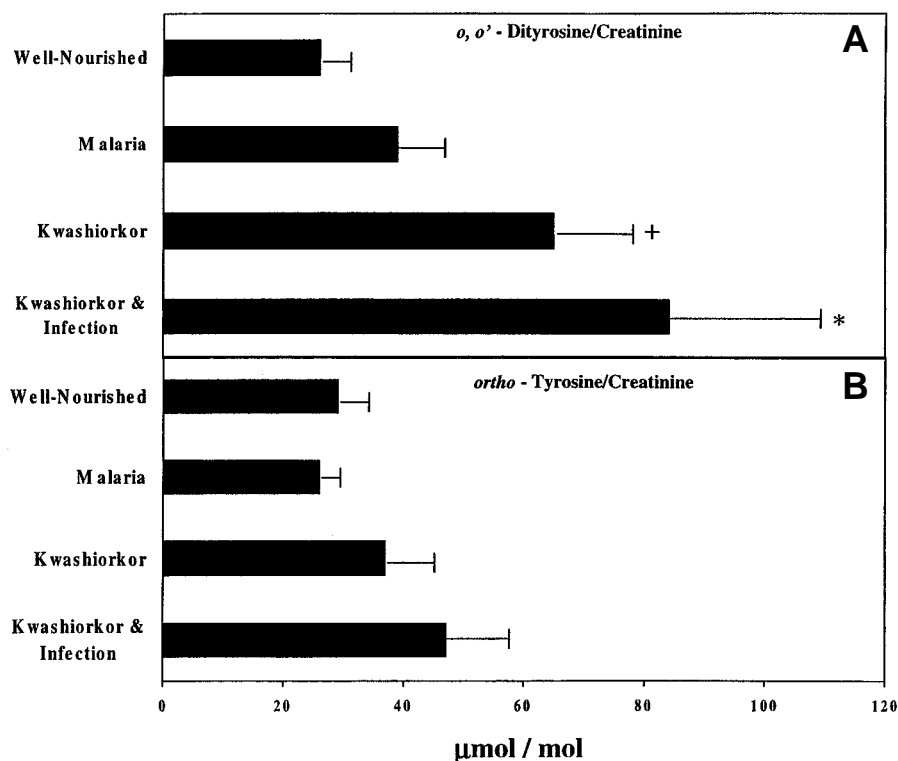
Fig 1. Relative amounts of *o,o'*-dityrosine (**A**) and *ortho*-tyrosine (**B**) in urine normalized to creatinine content. [+]*P* < .05 and [*]*P* < .01 compared with well-nourished subjects.
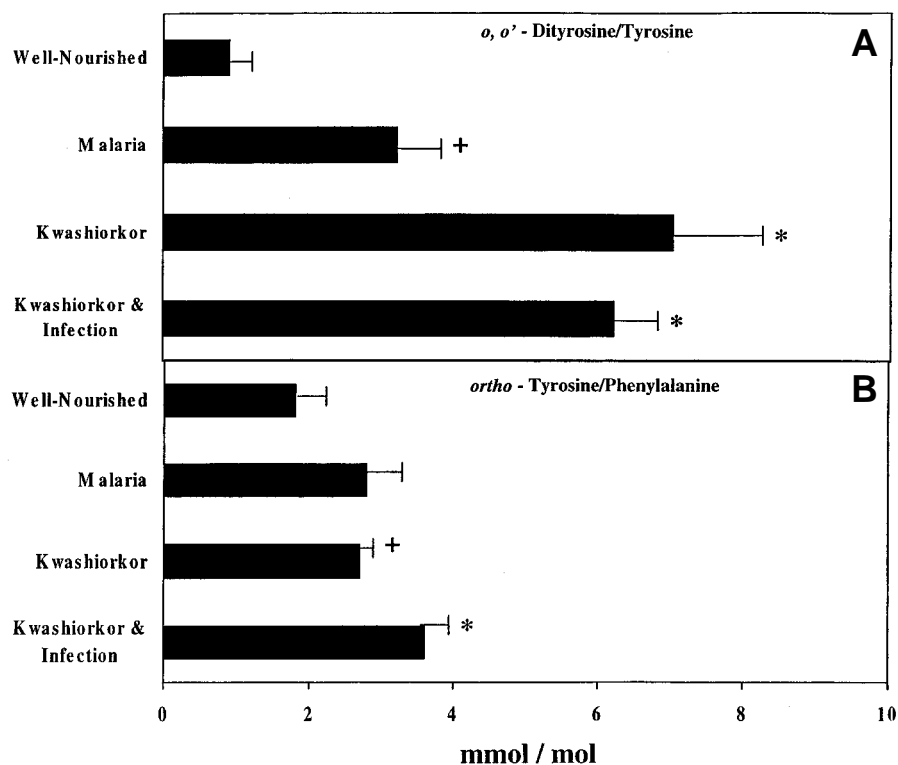
Fig 2. Relative amounts of *o,o'*-dityrosine (**A**) and *ortho*-tyrosine (**B**) in urine normalized to the precursor amino acid, tyrosine or phenylalanine, respectively. [+]*P* < .05 and [*]*P* < .01 compared with well-nourished subjects.

blood cells of children with kwashiorkor. The edema of kwashiorkor therefore might result from increased cation permeability across cell membranes damaged by oxidant stress.

The case-fatality rate in kwashiorkor with infection is higher than in other forms of malnutrition.[6] Developing therapeutic strategies to improve the antioxidant status of populations at risk for kwashiorkor may lower the number of malnutrition-related childhood deaths. Monitoring oxidized amino acids in the urine may be useful as a noninvasive technique to assess oxidative damage to proteins under disease conditions.

REFERENCES

1. Waterlow JC. Kwashiorkor revisited: the pathogenesis of oedema in kwashiorkor and its significance. Trans R Soc Trop Med Hyg 1984;78:436-41.
2. Golden MH, Ramdath D. Free radicals in the pathogenesis of kwashiorkor. Proc Nutr Soc 1987;46:53-68.
3. Becker K, Botticher D, Leichsenring M. Antioxidant vitamins in malnourished Nigerian children. Int J Vitam Nutr Res 1994;64:306-10.
4. Sive AA, Subotzky EF, Malan H, Dempster WS, De V, Heese H. Red blood cell antioxidant concentrations in kwashiorkor and marasmus. Ann Trop Paediatr 1993;13:33-8.
5. Jackson AA. Blood glutathione in severe malnutrition in childhood. Trans R Soc Trop Med Hyg 1986;80:911-3.
6. McLaren DS, Shirajian E, Loshkajian H, Shadarevian S. Short-term prognosis in protein-calorie malnutrition. Am J Clin Nutr 1969;22:863-70.
7. Okunade WG, Olorunsogo OO. Effect of reactive oxygen species on the erythrocyte calcium-pump function in protein-energy malnutrition. Biosci Rep 1992;12:433-43.
8. Klebanoff SJ. Oxygen metabolism and the toxic properties of phagocytes. Ann Intern Med 1980;93:480-9.
9. Leeuwenburgh C, Rasmussen JE, Hsu FF, Mueller D, Pennathur S, Heinecke JW. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. J Biol Chem 1997;272:3520-6.
10. Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW. Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo. Am J Physiol 1999;276:R128-35.
11. Leeuwenburgh CP, Hansen PA, Holloszy JO, Heinecke JW. Hydroxyl radical generation during exercise increases mitochondrial protein oxidation and levels of urinary dityrosine. Free Radical Biol Med 1999;27:186-92.
12. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997;272:20313-16.