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Asp-Ala-His-Lys (DAHK) Inhibits Copper-Induced Oxidative DNA Double Strand Breaks and Telomere Shortening

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Both DNA and the telomeric sequence are susceptible to copper-mediated reactive oxygen species (ROS) damage, particularly damage attributed to hydroxyl radicals. In this study, ROS-induced DNA double strand breaks and telomere shortening were produced by exposure to copper and ascorbic acid. Asp-Ala-His-Lys (DAHK), a specific copper chelating tetrapeptide d-analog of the N-terminus of human albumin, attenuated DNA strand breaks in a dose dependent manner. d-DAHK, at a ratio of 4:1 (d-DAHK:Cu), provided complete protection of isolated DNA from double strand breaks and, at a ratio of 2:1 (d-DAHK:Cu), completely protected DNA in Raji cells exposed to copper/ascorbate. Southern blots of DNA treated with copper/ascorbate showed severe depletion and shortening of telomeres and Raji cell treated samples showed some conservation of telomere sequences. d-DAHK provided complete telomere length protection at a ratio of 2:1 (d-DAHK:Cu). The human albumin N-terminus analog, d-DAHK, protects DNA and telomeres against copper-mediated ROS damage and may be a useful therapeutic adjunct in ROS disease processes.

Key Words: **Asp-Ala-His-Lys; DAHK; albumin; copper; DNA damage; reactive oxygen species; hydroxyl radical; hydrogen peroxide; telomere; transition metal.**

Reactive oxygen species (ROS) have been implicated in the pathogenesis of disease processes such as chronic inflammation, cancer, cardiovascular disease, Alzheimer's Disease, and aging (1–6). ROS damage to DNA can lead to strand breaks, base modifications, point mutations, altered methylation patterns, and DNA-protein cross linking (5, 6). Copper, iron, and other transition metals, in the presence of reducing agents, catalyze the production of ROS such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) through both the Haber-Weiss and Fenton reactions (7). $OH^{\cdot}$ is considered the most reactive and damaging ROS and is capable of producing all the above DNA lesions (5). Previous investigations have reported that $OH^{\cdot}$ induced, single- and double-strand DNA breaks occur during site-specific copper ion reactions *in vitro* and during excessive copper exposure *in vivo* (8–10).

Telomeres, which are repeats of the hexanucleotide TTAGGG, exist at the ends of chromosomal DNA to form a "protective cap" against degradation, chromosomal rearrangement, and allow the replication of DNA without the loss of genetic information (11). The classical theory of cellular aging, or senescence, involves the DNA polymerase end replication problem (12). DNA polymerase is unable to replicate the terminal end of the lagging strand during DNA replication resulting in the loss of 30–500 base pairs (13, 14). Somatic cells are unable to replace these lost telomeric repeats leading to progressive telomere shortening during a cell's replicative life. Senescence is manifested when telomere length reaches a critical threshold (11). Premature senescence has been documented in human fibroblasts exposed to oxidative stress (15). Examination of the length of fibroblast telomeres after several population doublings under conditions of higher oxidative stress, reveals telomere lengths similar to senescence under normal conditions (14). These data suggest that ROS-induced DNA damage in the telomere sequence may play an important role in telomere shortening and senescence.

Abbreviations used: DAHK, Asp-Ala-His-Lys; ROS, reactive oxygen species; $OH^{\cdot}$, hydroxyl radical; H_2O_2, hydrogen peroxide; $O_2^{\cdot -}$, superoxide; Cu, copper; Fe, iron; PBS, phosphate buffered saline; IMDM, Iscove's modified Dulbecco's media; FCS, fetal calf serum; TAE, Tris acetic acid EDTA; DIG, digoxigenin; 8-oxo-dG, 8-oxo-deoxyguanosine.

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Asp-Ala-His-Lys (DAHK) is the N-terminal four amino acids of human albumin and is a strong binding site for the transition metals copper, nickel and cobalt (16, 17). An alteration in the transition metal binding capacity of albumin appears to signal ischemic disease states associated with ROS damage (16, 18, 19). We have previously observed that d-DAHK prevents the *in vitro* formation of copper-induced free radicals, including $OH^{\bullet}$ (unpublished data). In this study, we examined the ability of d-DAHK to protect DNA and telomeres from ROS damage induced by copper and ascorbic acid.

MATERIALS AND METHODS

Reagents. The synthetic d-analog of human albumin N-terminus, d-DAHK, was obtained from Bowman Research Ltd. (Newport, Wales, UK). TeloTAGG Telomere Length Assay and X-ray film were purchased from Roche Molecular Biochemicals (Mannheim, Germany). DNeasy tissue kits were purchased from Qiagen (Valencia, CA). Hybond-N+ nylon membrane was ordered from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

DNA treatments. DNA strand breaks were measured using a modified method of Asaumi (20). Raji cells, a Burkitt lymphoma derived cell line, were grown in IMDM with 10% FCS at 10% CO_2 and 37°C. Genomic DNA was isolated using DNeasy tissue kits (Qiagen) following the manufacturer's protocol. One microgram genomic DNA was incubated per reaction with $CuCl_2$, ascorbic acid, and/or d-DAHK in 10 mM sodium phosphate pH 7.4. Final concentrations were as follows: $CuCl_2$ = 10 μM, 25 μM, and 50 μM; ascorbic acid = 25 μM, 50 μM, and 100 μM; d-DAHK = 50 μM, 100 μM, and 200 μM. Total reaction volumes of 20 μl in 0.2 ml PCR tubes were incubated at 37°C for 2 h. Following the incubation, strand breaks were visualized by immediately adding 5 μl of loading dye [0.25% (w/v) bromophenol blue and 40% (w/v) sucrose] and loading on a 0.5% TAE agarose gel. Gels were then run at 70V for 90 min and stained using 2 μg/ml ethidium bromide for 30 min. Prior to photographing, gels were rinsed in TAE for 10 min.

Cell treatments. Raji cells were washed with PBS (10 mM phosphate buffered saline; 138 mM NaCl; 2.7 mM KCl, pH 7.4). 1.5×10^6 cells were put into 5 ml PBS containing $CuCl_2$, ascorbic acid, and/or d-DAHK. Final concentrations were as follows: $CuCl_2$ = 10 μM, 25 μM, and 50 μM; ascorbic acid = 100 μM, 250 μM, and 500 μM; d-DAHK = 50 μM, 100 μM, and 200 μM. The cells were then incubated at 37°C for 2 h. Following the incubation, genomic DNA was isolated using DNeasy columns. DNA damage was visualized by 0.5% TAE agarose gel electrophoresis.

Telomere length assay. To examine telomere damage, the TeloTAGG Telomere Length Assay (Roche) was used according to manufacturer's recommendations: digesting 1 μg of genomic DNA per reaction using *Hin*fI and *RSA*I. Samples were then run on a 0.8% TAE agarose gel at 70V for 2 h. Southern blots were performed and probed using a DIG labeled telomere specific oligonucleotide. For cell treated samples, genomic DNA was used as described above. For DNA treated samples, reactions were setup as above, brought to 200 μl with PBS, and isolated using DNeasy spin columns prior to restriction digestions.

RESULTS AND DISCUSSION

Copper ions, an essential part of chromatin (21), are present within DNA (22) and may participate in oxidative DNA damage (8, 10, 23). In the presence of ascorbate or other reducing agents, copper can lead to the production of ROS by catalyzing the following reactions (24):

$$2\,Cu^{2+} + \text{ascorbate} \rightarrow 2\,Cu^{+} + \text{dehydroascorbate} + 2\,H^{+} \quad [1]$$

$$Cu^{+} + O_2 \rightarrow O_2^{\bullet -} + Cu^{2+} \quad [2]$$

$$Cu^{+} + O_2^{\bullet -} + 2\,H^{+} \rightarrow Cu^{2+} + H_2O_2 \quad [3]$$

$$Cu^{+} + H_2O_2 \rightarrow OH^{-} + OH^{\bullet} + Cu^{2+} \quad [4]$$

While iron (Fe) is found at higher concentrations physiologically, oxidation by copper and H_2O_2 is 50 times faster than iron (7, 25). Due to the negative charge of the sugar phosphate backbone, cations can loosely bind DNA. Site-specific binding of copper ions within base pairs may be important to the regulation of DNA biosynthesis (26). Unlike Fe catalyzed reactions, $OH^{\bullet}$ scavengers do not prevent copper-mediated oxidative DNA damage suggesting that ROS generation occurs in close proximity to the copper ions and DNA (27). The reactivity of $OH^{\bullet}$ is so great that, presumably, $OH^{\bullet}$ interactions only occur at or near the site of $OH^{\bullet}$ production (5, 28). Oikawa *et al.* (27) have shown that the following copper-mediated ROS reaction also occurs and that the resulting DNA-copper-peroxide complex may be even more damaging to DNA than $OH^{\bullet}$,

$$Cu^{+} + H_2O_2 \rightarrow Cu^{+}OOH + H^{+}.$$

As expected, copper and ascorbic acid alone showed no ability to cause strand breaks in our experiments. When $CuCl_2$ and ascorbic acid were combined, a dose-dependent accumulation of lower molecular weight DNA fragments was seen, presumably the result of double strand breaks. These double strand breaks were attenuated by d-DAHK in a dose dependent manner (Fig. 1). At molar ratios of 1:1 (50 μM d-DAHK to 50 μM copper) and 2:1, some strand breaks were apparent. By elevating the d-DAHK:copper ratio to 4:1, no strand breaks were detected. We observed similar results in Raji cells treated with copper and ascorbic acid (Fig. 2). A lower ratio of 2:1 (d-DAHK to copper) provided complete protection to DNA in cell treated samples. It is reasonable to expect that DNA treated samples would require higher d-DAHK levels due to competition for copper with DNA and proximal $OH^{\bullet}$ attack. The separation of DNA and copper would be critical in these samples necessitating the need for elevated d-DAHK. In cell treated samples, damage would be attributable to H_2O_2. H_2O_2 is freely diffusible, can penetrate to the nucleus, and has been shown to

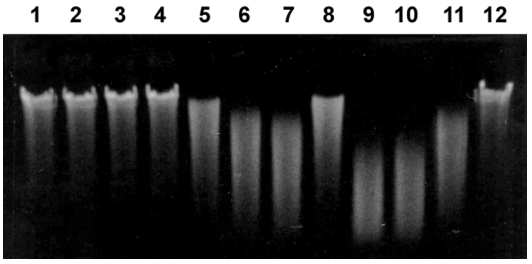

FIG. 1. ROS induced DNA double strand breaks in genomic DNA and d-DAHK attenuation. Isolated genomic DNA from Raji Burkitt cells was treated with $CuCl_2$ and ascorbic acid for 2 h at 37°C in 10 mM phosphate buffer pH 7.4. After incubation, 1 μg/well DNA was resolved on 0.5% TAE agarose gel and visualized with Ethidium Bromide. Lane 1, no treatment; lane 2, $CuCl_2$ 50 μM; lane 3, ascorbic acid 100 μM; lane 4, d-DAHK 200 μM; lane 5, $CuCl_2$ 10 μM + ascorbic acid 50 μM; lane 6, $CuCl_2$ 25 μM + ascorbic acid 50 μM; lane 7, $CuCl_2$ 50 μM + ascorbic acid 50 μM; lane 8, $CuCl_2$ 50 μM + ascorbic acid 25 μM; lane 9, $CuCl_2$ 50 μM + ascorbic acid 100 μM; lane 10, $CuCl_2$ 50 μM + ascorbic acid 100 μM + d-DAHK 50 μM; lane 11, $CuCl_2$ 50 μM + ascorbic acid 100 μM + d-DAHK 100 μM; lane 12, $CuCl_2$ 50 μM + ascorbic acid 100 μM + d-DAHK 200 μM.

damage DNA in fibroblasts (15, 29). Entrance of H_2O_2 into the cell may lead either to the formation of DNA peroxide complexes with native metals or to the release of sequestered metal stores that, combined with endogenous reducing agents (GSH, NADH, and ascorbic acid), would drive the production of $OH^{\cdot}$. One possible mechanism of d-DAHK would be the chelation of copper ions, thereby preventing production of $OH^{\cdot}$ and H_2O_2. Another mode of protection may be the formation of d-DAHK-copper-peroxide complexes, which would absorb the $OH^{\cdot}$ damage, "mop-up" peroxides, and perhaps, in cell treated samples, keep H_2O_2 outside the cell.

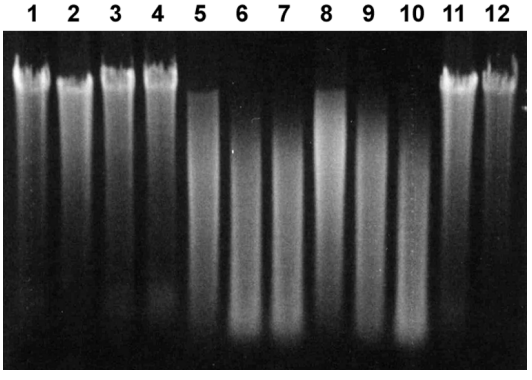

FIG. 2. d-DAHK attenuation of ROS induced DNA double strand breaks in Raji cells treated with $CuCl_2$ and ascorbic acid. Raji Burkitt cells were treated with $CuCl_2$ and ascorbic acid for 2 h at 37°C in 10 mM PBS pH 7.4. After incubation, genomic DNA was isolated and 1 μg/well DNA was resolved on 0.5% TAE agarose gel. Lane 1, no treatment; lane 2, $CuCl_2$ 50 μM; lane 3, ascorbic acid 500 μM; lane 4, d-DAHK 200 μM; lane 5, $CuCl_2$ 10 μM + ascorbic acid 500 μM; lane 6, $CuCl_2$ 25 μM + ascorbic acid 500 μM; lane 7, $CuCl_2$ 50 μM + ascorbic acid 500 μM; lane 8, $CuCl_2$ 50 μM + ascorbic acid 100 μM; lane 9, $CuCl_2$ 50 μM + ascorbic acid 250 μM; lane 10, $CuCl_2$ 50 μM + ascorbic acid 500 μM + d-DAHK 50 μM; lane 11, $CuCl_2$ 50 μM + ascorbic acid 500 μM + d-DAHK 100 μM; lane 12, $CuCl_2$ 50 μM + ascorbic acid 500 μM + d-DAHK 200 μM.

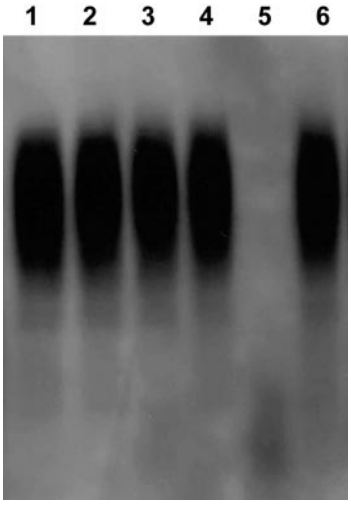

FIG. 3. Telomere Southern Blot of DNA treated with $CuCl_2$ and ascorbic acid. Genomic DNA was treated with $CuCl_2$ and ascorbic acid followed by *Hin*fI and *Rsa*I restriction digestion to remove all but telomere sequence. DNA was resolved on 0.8% TAE agarose gel and transferred to nylon membrane then probed with DIG labeled telomere specific oligo. Lane 1, no treatment; lane 2, $CuCl_2$ 50 μM; lane 3, ascorbic acid 100 μM, lane 4, d-DAHK 200 μM; lane 5, $CuCl_2$ 50 μM + ascorbic acid 100 μM; lane 6, $CuCl_2$ 50 μM + ascorbic acid 100 μM + d-DAHK 200 μM.

Prior reports suggest that oxidative DNA damage may be directed at G-C rich areas, including telomeres. Rodriguez *et al.* reported that copper induced ROS damage primarily targets in guanine bases (30). Strong, preferential binding of Cu (II) to the G-C pair has been reported at the N-7 and O-6 of guanine plus the N-3 of cytosine (23). DNA peroxide complexes formed at these positions are believed to direct $OH^{\cdot}$ attack to adjacent bases (27). In addition, GGG in telomeric DNA has been shown to be sensitive to copper mediated ROS damage (31).

Examination of the telomere in the genomic DNA samples in the present study showed double strand breaks in response to oxidative stress. DNA treated samples examined by Southern blot showed severely depleted and shortened telomere sequences (Fig. 3). Cell treatments showed damage to the telomere with some conservation of the sequence, even at the highest levels of copper and ascorbic acid used (Fig. 4), which may be attributed to ROS production outside the cells with the DNA sheltered inside the nucleus. d-DAHK protected the telomere from copper-mediated damage in these samples.

In addition to the double strand breaks detected in our experiments, other DNA lesions may be involved in ROS disease processes. Some cations, including copper, bound loosely to the phosphate backbone have been implicated in strand breaks while those coordinated in

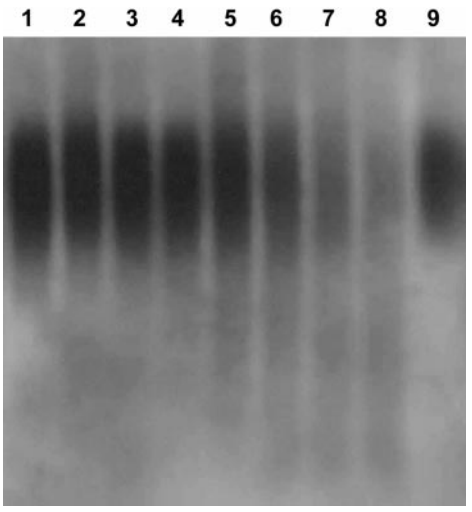

FIG. 4. Telomere Southern blot of Raji cells treated with $CuCl_2$ and ascorbic acid. Raji Burkitt cells were treated with $CuCl_2$ and ascorbic acid for 2 h at 37°C in PBS. Following incubation, genomic DNA was isolated and treated with *Hin*fI and *Rsa*I. Southern blot was then performed using telomere specific DIG labeled oligo. Lane 1, no treatment; lane 2, $CuCl_2$ 50 μM; lane 3, ascorbic acid 500 μM, lane 4, d-DAHK 200 μM; lane 5, $CuCl_2$ 50 μM + ascorbic acid 100 μM; lane 6, $CuCl_2$ 50 μM + ascorbic acid 250 μM; lane 7, $CuCl_2$ 50 μM + ascorbic acid 500 μM; lane 8, $CuCl_2$ 50 μM + ascorbic acid 500 μM + d-DAHK 50 μM; lane 9, $CuCl_2$ 50 μM + ascorbic acid 500 μM + d-DAHK 100 μM.

the helix cause base modifications (5, 30). 8-oxo-deoxyguanosine (8-oxo-dG) is a common DNA adduct produced by ROS, and may result in G → T point mutations, which are widely seen in mutated oncogenes (5). Episodes of increased copper and oxidative stress may direct DNA damage to G-C rich areas. In addition to telomeres, G-C rich areas exist at the 5′ end of many genes (32) hinting toward a site of oxidative damage in an area involved in gene regulation. Conditions such as acidosis occurring during myocardial ischemia or alterations of ceruloplasmin have been shown to mobilize free copper to catalyze local oxidative tissue and DNA damage (9, 33). Levels of 8-oxo-dG are reported to be three to four times higher in the DNA of ischemic rat hearts than in controls (34). In addition, chronic inflammation can produce areas of localized oxidative damage. Inflammatory cells, such as macrophages and neutrophils, release ROS that have been shown to damage the DNA of nearby cells (35). Nitric oxide and superoxide released from activated leukocytes can lead to the production of peroxynitrite, which is more reactive with 8-oxo-dG than unmodified bases and possibly exacerbates the damage (5).

While the exact mechanisms for ROS DNA damage have yet to be fully elucidated, d-DAHK appears to inhibit copper-induced DNA double-strand breaks by ROS in both genomic DNA and in the telomere sequence. d-DAHK, an analog of the N-terminus of human albumin, may prove to be a beneficial therapeutic compound to help prevent oxidative DNA damage in human disease.

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