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Effect of Aminoethylcysteine Ketimine Decarboxylated Dimer, a Natural Sulfur Compound Present in Human Plasma, on *Tert*-Butyl Hydroperoxide-induced Oxidative Stress in Human Monocytic U937 Cells

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Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) is a natural sulphur compound present in human plasma and urine and in mammalian brain. Recently, it has been detected in many common dietary vegetables. The aim of the present study was to evaluate the ability of AECK-DD to affect cellular response of U937 human monocytic cells to *tert*-butyl hydroperoxide-induced oxidative stress. AECK-DD was incorporated into cells, as confirmed by GC-MS analyses, without any cytotoxic effect. A 24 h treatment with 50 and 250 μM AECK-DD resulted in the incorporation of 0.10 ± 0.01 and 0.47 ± 0.08 ng AECK-DD $\times 10^6$ cells, respectively. U937 cells pretreated with AECK-DD (in the range 4–100 μM) showed an increased resistance to *tert*-butyl hydroperoxide-induced necrotic death, as revealed by a higher percent of survival measured at all incubation times with respect to control cells. Moreover, the protective effect exhibited by AECK-DD is significantly stronger with respect to that obtained with other common antioxidants (*N*-acetyl cysteine and trolox) and comparable, although somewhat higher, to that of vitamin E. This effect seems to be due to the ability of AECK-DD to reduce glutathione depletion and to inhibit lipid peroxidation during *tert*-butyl hydroperoxide treatment. It can be concluded that AECK-DD protects cultured human monocytic cells against *tert*-butyl hydroperoxide-induced oxidative stress and subsequent cell death, likely through an antioxidant action inside the cell. Due to its presence in both human plasma and urine, AECK-DD may play a role in the modulation of oxidative processes *in vivo*.

Keywords: Aminoethylcysteine ketimine decarboxylated dimer; U937; *tert*-Butyl hydroperoxide; Oxidative stress; Natural antioxidants

Abbreviations: AECK-DD, aminoethylcysteine ketimine decarboxylated dimer; *t*-BOOH, *tert*-butyl hydroperoxide; PBS, Dulbecco's phosphate-buffered saline; NAC, *N*-acetyl cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; CAT, catalase; CumOOH, cumene hydroperoxide; HPLC-ECD, high performance liquid chromatography-electrochemical detector; GC-MS, gas chromatography-mass spectrometry; LDH, lactate dehydrogenase; NADH, β-nicotinamide adenine dinucleotide, reduced form; TBA-RS, thiobarbituric acid-reactive substances; DETAPAC, diethylenetriaminepentaacetic acid; DCF, 2′7′-dichlorofluorescein

INTRODUCTION

An impairment of the antioxidant defence system is considered to be critically involved in a number of pathological conditions including cardiovascular diseases, atherosclerosis, cancer, inflammation and cataract.[1–2] Diet can contribute to the overall redox balance being a source of natural antioxidants, considered to be major health-protecting factors.

Aminoethylcysteine ketimine decarboxylated dimer (AECK-DD) is a natural sulphur-containing

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tri-cyclic compound, member of a family of sulphur-containing amino acids.[3] It has been detected in human plasma at micromolar concentration,[4] in human urine[5] and in mammalian cerebellum.[6] Moreover, AECK-DD has been described to be associated to lipoproteins isolated from human plasma.[4] In spite of its widespread presence in biological samples, a biochemical route leading to the *in vivo* synthesis of this molecule has not been demonstrated. Recently, the presence of AECK-DD in some common vegetables present in human diet has been reported[7] and this might account for a dietary supply of this compound. AECK-DD possesses a strong antioxidant activity: it has been reported to interact *in vitro* with reactive oxygen and nitrogen species (hydrogen peroxide, superoxide anion, hydroxyl radical, peroxynitrite and its derivatives).[8–11] Its scavenging activity has been described to be comparable to that of alpha-tocopherol and more potent than ascorbic acid and glutathione. Moreover, AECK-DD has been reported to possess a protective effect on copper-induced oxidation of low density lipoprotein at concentrations comparable to those found in human plasma.[12]

In this work, we evaluate the effect of AECK-DD supplementation on the response of cultured human monocyte (U937) cells to oxidative injury induced by *tert*-butyl hydroperoxide (*t*-BOOH), an organic hydroperoxide widely used as model compound to induce oxidative stress.[13,14] U937 is a well characterized cell line and the response of this cell line to various inflammatory agents has been well documented.[15,16] Monocyte cells, as circulating cells, are easily affected by dietary-derived peroxides and antioxidants present in plasma. It has been recognized that monocyte-endothelium adhesion is a crucial early event in atherogenesis and that plasma antioxidants can prevent or reduce it.[17–21] Moreover, monocyte cells, due to their involvement in inflammatory processes, are likely to find themselves *in vivo* in an oxidative environment.

Since AECK-DD is present in human plasma, human monocytic cells culture may represent a good model for investigating the ability of this molecule to protect these circulating cells from oxidative injury. Understanding the effect of growing these cells in a AECK-DD-supplemented medium on their redox balance and antioxidant defence potential may help to clarify the role of this compound in the modulation of oxidative processes *in vivo*.

MATERIALS AND METHODS

Chemicals

AECK-DD was prepared according to Antonucci *et al.*,[10]. 1,1,3,3-tetramethoxypropane, *t*-BOOH, *N*-acetyl cysteine and *o*-phthaldialdehyde were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Vitamin E-acetate was purchased by Fluka (Buchs, Switzerland). Trolox was purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). All other reagents were of the highest grade obtainable.

Cell Titer 96 aqueous one solution cell proliferation assay was purchased from Promega Corporation (Madison, WI, USA).

Cell Culture and Antioxidants Supplementation

Human monocytic U937 cells were routinely cultured at 37°C under 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 40 μg/ml gentalin with or without addition of AECK-DD or other antioxidants as specified. AECK-DD, vitamin E-acetate and trolox were dissolved in ethanol, filtered and added to cells 24 h before experiments. A 24 h preincubation time for both AECK-DD and vitamin E-acetate was selected on the basis of uptake experiments (data not shown). AECK-DD-treatment (50 μM) resulted in the incorporation of 0.10 ± 0.01 ng AECK-DD $\times 10^6$ cells. Vitamin E-acetate-treatment (50 μM) of U937 cells resulted in about 5.5-fold increase of basal vitamin E content (1.31 ± 0.22 ng vitamin E $\times 10^6$ cells before treatment, 7.20 ± 0.14 ng $\times 10^6$ cells after treatment) as measured by HPLC.[22] The presence of ethanol (up to 0.08% final concentration) had no effect on all variables studied herein. NAC was dissolved in Dulbecco's phosphate buffered saline (PBS) and neutralized with sodium hydroxide, then added to the culture medium 6 h before experiments.

U937 cells were maintained in log phase (cell number kept between 0.2 and 1×10^6 cells/ml) throughout the study.

Viability Assays

Cell viability was assessed by Trypan blue exclusion and cell lysis by counting the total cell number. For cytotoxicity assay, cells (0.3×10^6 ml) were incubated in complete medium with or without AECK-DD (from 25 μM to 0.8 mM) for 24 h. Cell viability was measured by lactate dehydrogenase (LDH) leakage from damaged cells into culture medium and expressed as a percentage of total cellular activity. LDH activity was determined at 30°C as the change in absorbance at 340 nm, using 0.18 mM NADH and 0.72 mM pyruvate as substrates in 50 mM K-phosphate buffer, pH 7.4.

Proliferation Assay

To test cell proliferation rate, a commercial reagent (cell Titer 96 aqueous one solution cell proliferation assay)

based on a colorimetric method was used. The method consists in an enzyme-based color change in which a tetrazolium compound (MTS) is converted to a brown derivative by cellular mitochondrial dehydrogenases. Control cells or cells supplemented with AECK-DD (50 μM) were plated out in individual wells of 24-well Falcon plates at a density of 50×10^3 cells/well, in a final volume of 0.5 ml. After 24 or 48 h incubation in the presence or absence of AECK-DD, 40 μl of cell titer 96 aqueous one solution cell proliferation reagent was added to each well. After 45 min incubation at 37°C, optical density at 490 nm was recorded. Blanks of culture medium without cells were also run. The quantity of coloured product measured is directly proportional to the number of living cells in culture.

Cell Treatment with *t*-BOOH

Before adding *t*-BOOH, cells were rinsed twice with PBS to wash out AECK-DD (or any other antioxidant tested) from the medium and resuspended in the same buffer at concentration of 2×10^6 cells/ml, then incubated at 37°C in *t*-BOOH at the final concentration of 500 μM, unless otherwise specified. In all experiments, parallel controls were performed without *t*-BOOH.

Biochemical Analyses

After incubation, cells were rinsed twice with PBS, resuspended in 10 mM Tris–HCl buffer, pH 6.0 containing 0.5 mM diethylenetriaminepentaacetic acid (DETAPAC) at 6×10^6 cells/ml and aliquots were withdrawn for glutathione and thiobarbituric acid-reactive substance (TBA-RS) analyses. Reduced and oxidized glutathione (GSH and GSSG, respectively) were measured by high performance liquid chromatography (HPLC) with fluorimetric detection after derivatization with *o*-phthaldialdehyde.[23] Oxidative damage to lipids was assessed by measuring TBA-RS using HPLC with fluorimetric detection.[24] 1,1,3,3-Tetramethoxypropane was used as standard.

Proteins were determined according to Lowry *et al.*,[25] glutathione peroxidase (GPX) activity was assayed following the procedure described by Flohé and Gunzler[26] using both hydrogen peroxide and cumene hydroperoxide as substrate. Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik.[27] Catalase activity was measured according to Cohen *et al.*[28].

Intracellular pro-oxidants content was determined spectrofluorometrically following the formation of fluorescent derivative 2′,7′-dichlorofluorescein (DCF).[29]

Characterization of Intracellular AECK-DD by GC-MS Analyses

After incubation in the presence of AECK-DD, U937 cells ($20-40 \times 10^6$ cells) were pelleted by centrifugation, washed twice with 50 ml PBS and resuspended in 0.5 ml 0.5% Triton X-100 in PBS. After sonication (15 s) to facilitate lysis, cell suspensions were extracted with chloroform (2×2 ml) by 5 min vortexing and centrifuged at 2000*g* for 5 min. The organic layers were combined and evaporated under nitrogen flow. The dried residue was dissolved in 0.1 ml absolute methanol and purified by HPLC as described elsewhere.[3] HPLC eluate was dried, dissolved in a 20 μl acetone–hexane mixture (1:1 v/v) and analyzed by GC-MS without derivatization (1 μl injection volume) in the Total Ion mode. GC-MS analyses were performed on a Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA).

Chromatographic separations were carried out on a Agilent HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d.) coated with cross-linked 5% phenyl–methyl siloxane (film thickness 0.25 μm) as stationary phase. Injection mode: splitless at a temperature of 260°C. Column temperature program: 70 (1 min) then to 280°C at a rate of 10°C/min and held for 15 min. The carrier gas was helium at a constant flow of 1.0 ml/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy and a mass range scan from m/z 50 to 350; ion source 280°C, ion source vacuum 10^{-5} Torr. When the selected ion monitoring acquisition mode was used, the dwell time was 100 ms.

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis was performed using a one-factor analysis of variance (ANOVA) and Scheffe's method for multiple comparison. Probability of $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of Intracellular AECK-DD by GC-MS Analyses

Recently, we demonstrated the incorporation of AECK-DD in human monocytic U937 cells following *in vitro* supplementation, by means of HPLC-ECD.[30] The cellular content of AECK-DD was found to be 0.10 ± 0.01 and 0.47 ± 0.08 ng × 10^6 cells for cells grown in the presence of 50 and

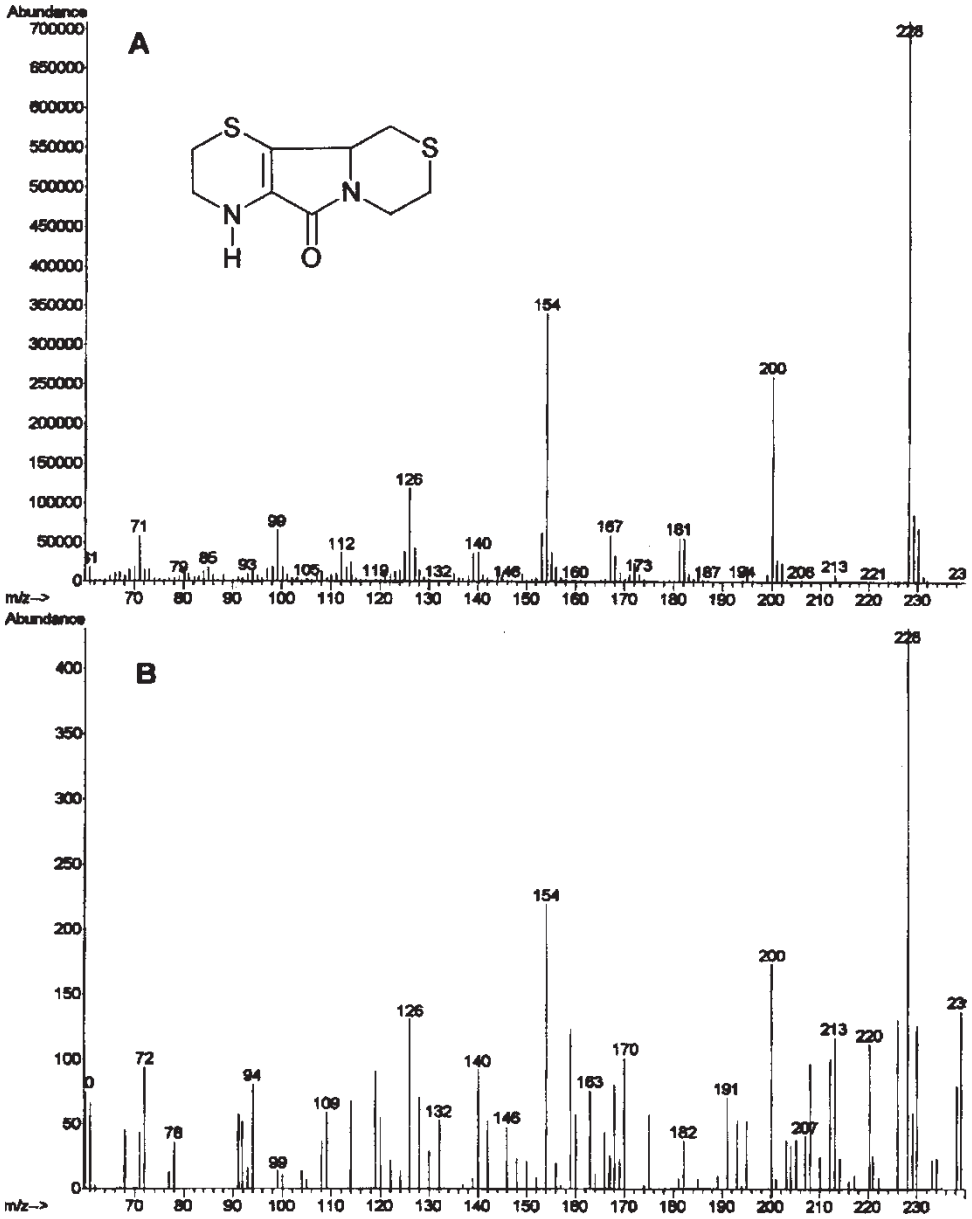

FIGURE 1 Characterization of intracellular AECK-DD by mass spectra analyses. Mass spectra of (A) standard AECK-DD and (B) a cellular extract prepared from AECK-DD-supplemented cells, as obtained by electron impact at 70 eV. The formula structure of AECK-DD is shown. Experimental conditions as reported in "Materials and Methods" section.

250 μM AECK-DD, respectively. In this study, we present further evidences for identification of AECK-DD inside the cells by GC-MS analyses, following a 24 h incubation time. Figure 1A shows the mass spectrum of pure standard AECK-DD showing a fragmentation pattern where the molecular ion m/z 228 and the other typical ions, m/z 200, 154 and 126, respectively, are the most abundant (42.2% for m/z 200, 48.8% for m/z 154 and 16.6% for m/z 126). The mass spectrum of the cellular extract sample (Fig. 1B) shows the same fragmentation pattern of standard AECK-DD and similar relative intensities of the more abundant ions (44.4% for m/z 200, 52.2% for m/z 154 and 22.2% for m/z 126).

Cytotoxicity of AECK-DD

To evaluate possible cytotoxic effects of AECK-DD, U937 cells were incubated in the presence or absence (control) of AECK-DD, ranging from 25 to 800 μM and the release of LDH was measured after 24- and 48-h incubation periods. An increased release of LDH into the culture medium is indicative of a cellular and membrane damage. No cytotoxic effect of AECK-DD was observed up to 800 μM AECK-DD

in the culture medium, at both 24- and 48-h incubation, in comparison with control cells (9.1 ± 0.4% and 9.2 ± 0.4% LDH leakage at 24 and 48 h, respectively, for control cells; 9.6 ± 1.7% and 5.6 ± 1.1% LDH leakage at 24 and 48 h, respectively, for cells treated with 800 μM AECK-DD).

Effect of *t*-BOOH on Cell Survival and Modulation by AECK-DD. Comparison with other Antioxidants

The effect of increasing *t*-BOOH concentrations ranging from 100 to 500 μM on survival of U937 cells is shown in Fig. 2A. A dose-dependent effect was clearly evident. Moreover, the survival curves obtained were very similar to those previously reported from our lab.[31] To test the efficacy of AECK-DD in affecting cellular response to oxidative challenge, we chose the concentration of 500 μM *t*-BOOH to induce a substantial oxidative stress. From now on, these conditions will be reported as *t*-BOOH treatment. In this experimental conditions, cell lysis, estimated by counting the total cell number, was undetectable in the first 3 h of *t*-BOOH treatment and did not exceed 20% at 5 h incubation.

The effect of 24-h supplementation with 4, 10, 50 or 100 μM AECK-DD on U937 cells viability during *t*-BOOH treatment was measured by Trypan blue exclusion over a 5 h incubation time (Fig. 2B). AECK-DD-treated cells showed a higher, dose-dependent resistance to *t*-BOOH treatment with respect to

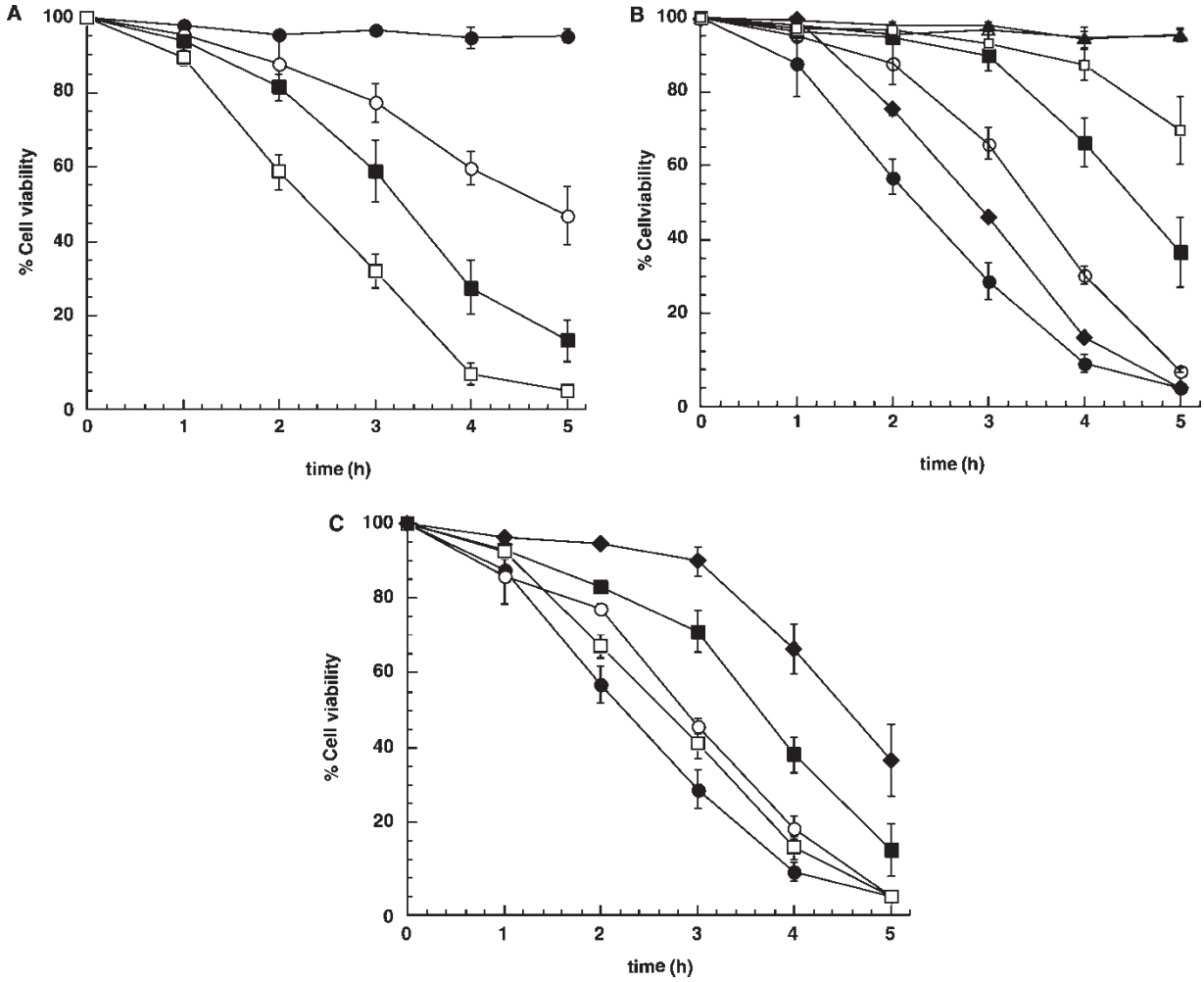

FIGURE 2 Survival curves of U937 cells. Cells (2×10^6 ml) were incubated in PBS at 37°C. At various times, viability was determined by Trypan blue exclusion. Data are mean ± SD of three independent experiments. (A) survival curves of U937 cells in response to increasing concentrations of *t*-BOOH. Closed circles: no *t*-BOOH, open circles: +100 μM *t*-BOOH, closed squares: +200 μM *t*-BOOH, open squares: +500 μM *t*-BOOH. (B) effect of AECK-DD on survival curves of U937 cells in response to 500 μM *t*-BOOH treatment. AECK-DD-treated and control cells were incubated with or without 500 μM *t*-BOOH. Open triangles: control cells, no *t*-BOOH, closed triangles: 50 μM AECK-DD-treated cells, no *t*-BOOH, closed circles: control cells + *t*-BOOH, closed rhombi: 4 μM AECK-DD-treated cells + *t*-BOOH; open circles: 10 μM AECK-DD-treated cells + *t*-BOOH; closed squares: 50 μM AECK-DD-treated cells + *t*-BOOH; open squares: 100 μM AECK-DD-treated cells + *t*-BOOH. (C) effect of antioxidants on survival curves of U937 cells in response to *t*-BOOH treatment. Control cells or antioxidant-treated cells were incubated with 500 μM *t*-BOOH. Closed circles: control cells, open squares: NAC-treated cells (1 mM), open circles: trolox-treated cells (50 μM), closed squares: vitamin E-treated cells (50 μM), closed rhombi: AECK-DD-treated cells (50 μM).

control cells. After 2 h incubation with *t*-BOOH, a 56.9 ± 4.8% viability was observed for control cells while, at the same time, values of 75.2 ± 1.4%, 87.5 ± 5.4%, 94.5 ± 1.1% and 98.8 ± 1.9% were observed for 4, 10, 50 and 100 μM AECK-DD, respectively. After 3-h incubation with *t*-BOOH, viability was 28.8 ± 5.1% for control cells, 46.0 ± 0.7%, 66.0 ± 4.4%, 89.9 ± 4.1 and 92.9 ± 4.7% for 4, 10, 50 and 100 μM AECK-DD, respectively. From these results, a protective, dose-dependent, effect of AECK-DD on cell survival was evident for all the concentration tested, in the range 4–100 μM.

In Fig. 2C, the protective effect of AECK-DD on U937 survival in response to *t*-BOOH treatment is compared to the one exerted by other well known antioxidants, such as vitamin E, trolox (a water-soluble analogue of vitamin E) and *N*-acetyl cysteine (NAC). U937 cells treated with 50 μM AECK-DD showed a higher resistance to *t*-BOOH-induced damage than cells treated with trolox at the same concentration. The protective effect of AECK-DD was comparable, although somewhat higher, to that exhibited by vitamin E at the same concentration. At 3 h incubation with *t*-BOOH, the survival was 89.9 ± 4.1%, 45.6 ± 2.0% and 71.0 ± 5.6% for AECK-DD-, trolox-, and vitamin E-treated cells, respectively, in comparison with a 28.8 ± 5.1% survival of control cells. For NAC, the lowest effective concentration to achieve a detectable protective effect was found to be 1 mM. However, also at this relative high concentration, the protective effect was quite low compared to that observed for cells treated with 50 μM AECK-DD (41.0 ± 4.1% survival at 3-h incubation for NAC-treated cells compared to 89.9 ± 4.1% obtained for AECK-DD-treated cells). Longer pre-treatment of cells with NAC (24 h) did not result in stronger protective effect with respect to that obtained with 6 h pre-treatment (data not shown). At 5-h incubation with *t*-BOOH, the survival was 36.6 ± 9.6% for AECK-DD-treated cells and 12.5 ± 7.1% for vitamin E-treated cells. At the same time, viability for NAC- and trolox-treated cells was undetectable. For the sake of clarity, blanks without *t*-BOOH were not shown in Fig. 2C.

Effect of AECK-DD on Proliferation Rate and Basal Levels of Intracellular Pro-oxidants, Glutathione, TBA-RS, GPX, GR and Catalase

The effect of AECK-DD on proliferation rates was studied by means of a colorimetric method, based on the conversion by cellular mitochondrial dehydrogenases of MTS, a tetrazolium compound, to a brown derivative, measured at 490 nm. AECK-DD at 50 μM in the culture medium did not significantly affect proliferation rates in U937 cells, as measured at both 24- and 48-h incubation times. The absorbance at 490 nm was 0.56 ± 0.02 and 0.58 ± 0.03 for control and AECK-DD-treated cells, respectively, at 24-h incubation; 0.85 ± 0.08 and 0.91 ± 0.05 for control and AECK-DD-treated cells, respectively, at 48-h incubation. Treatment of U937 cells with AECK-DD (50 μM) did not affect the basal levels of total and oxidized glutathione and TBA-RS (Table I). Moreover, no significant differences in the GPX activity, measured using both hydrogen peroxide and cumene hydroperoxide as substrate, were observed between control and AECK-DD-treated cells (Table I). GR and catalase activity were also similar in both groups of cells (Table I). Intracellular pro-oxidants, measured with the fluorescent probe DCF, were found significantly lower in AECK-DD-treated cells in respect to control cells ($p = 0.0043$)

Effect of *t*-BOOH Treatment on Glutathione and TBA-RS Content

Glutathione is one of the most abundant intracellular antioxidants and determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. Particularly, oxidative stress induced by *t*-BOOH treatment has been reported to lead to depletion and oxidation of intracellular glutathione.[13,14,32,33] Figure 3A shows the time-course of GSH depletion in U937 cells exposed to *t*-BOOH treatment. A rapid decrease of total intracellular glutathione was observed in control cells whereas in AECK-DD-treated cells

TABLE I Basal intracellular levels of total glutathione, GSSG, pro-oxidants, TBA-RS, GPX, GR and CAT in U937 cells

	Control cells	AECK-DD-treated cells
Total glutathione (nmol/mg)	26.7 ± 1.5	27.2 ± 3.3
GSSG (nmol/mg)	nd	nd
TBA-RS (pmol/mg)	177.7 ± 33.2	157.2 ± 43.5
GPX (CumOOH) (nmol/min/mg)	60.6 ± 3.1	55.9 ± 10.0
GPX (H_2O_2) (nmol/min/mg)	59.1 ± 2.1	59.8 ± 5.7
GR (nmol/min/mg)	33.9 ± 0.54	35.7 ± 2.2
CAT (μmol/min/mg)	43.3 ± 3.9	42.1 ± 1.8
DCF fluorescence (relative fluorescence/mg)	736.5 ± 37.0	605.0 ± 21.0*

Data, expressed per mg protein, are mean ± SD of five measurements. nd, not detectable. *Difference between control and AECK-DD cells are statistically significant (1-factor ANOVA, Scheffe's test; $p \leq 0.05$).

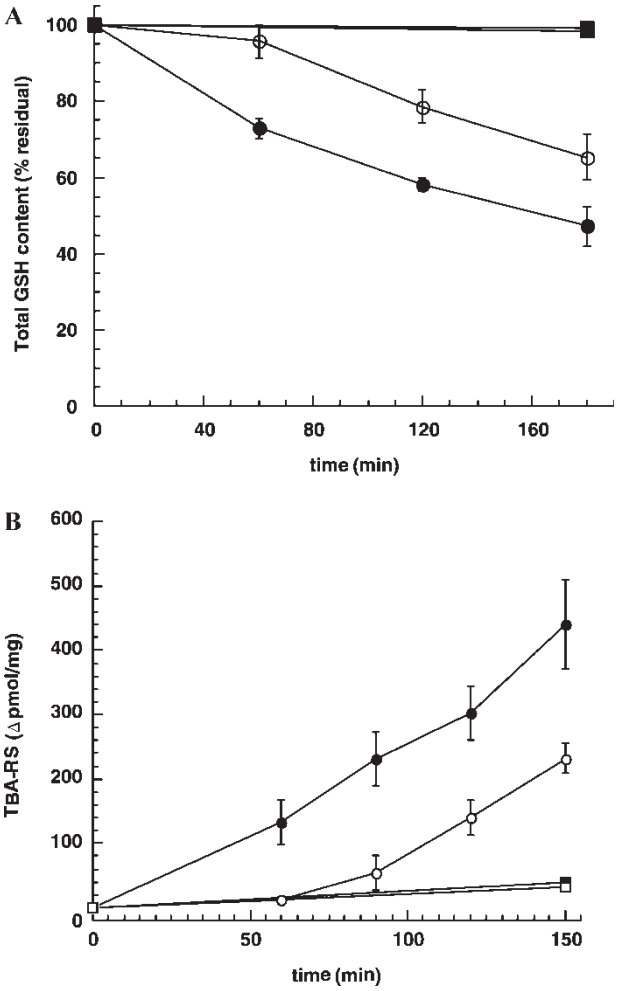

FIGURE 3 Effect of AECK-DD on glutathione content and TBA-RS production in U937 cells exposed to *t*-BOOH treatment. AECK-DD-treated (50 μM) (open symbols) or control cells (closed symbols) were incubated at 2×10^6 cells/ml with (circles) or without (squares) 500 μM *t*-BOOH in PBS at 37°C. Data presented are mean ± SD of three independent experiments. (A) Effect of AECK-DD on total intracellular glutathione content. Total glutathione was measured by HPLC as described in Materials and Methods section. (B) Effect of AECK-DD on intracellular TBA-RS production. TBA-RS formation was measured by HPLC as described in Materials and Methods section. Values represent the difference from time 0.

the loss of GSH was slowed down and preceded by a lag phase. At 1-h incubation, total glutathione content decreased to 72.9 ± 2.7% of initial value in control cells, while in AECK-DD-treated cells 95.8 ± 4.4% of initial total glutathione content was measured. Total glutathione levels remained significantly higher in AECK-DD-treated cells in respect to control cells also at longer incubation times. The loss of intracellular GSH was accompanied by a corresponding increase in intracellular GSSG, again more pronounced in control cells than in AECK-DD treated cells (data not shown). At 1-h incubation, GSSG was 3.9 ± 0.7 nmol/mg protein for control cells and 2.2 ± 0.2 nmol/mg protein for AECK-DD treated cells ($p = 0.015$). However, GSSG measurements, especially at long incubation times, are an insensitive indicator of its rate of formation, because GSSG is known to be rapidly transported outside in various cell types.[34] Moreover, in a previous study, we observed a specific extrusion of GSSG during *t*-BOOH treatment of U937 cells.[31]

To evaluate the effect of AECK-DD on oxidative damage to lipids induced by *t*-BOOH treatment, TBA-RS formation was measured. As shown in Fig. 3B, a fast increase in TBA-RS content was observed in control cells, whereas the rate of TBA-RS formation was significantly slowed down in AECK-DD-treated cells with respect to control cells. After 3-h incubation with *t*-BOOH, TBA-RS level in AECK-DD-treated cells was about 50% lower than control cells.

DISCUSSION

This study was undertaken to evaluate the effect of AECK-DD, a sulfur-containing compound with antioxidant activity present in human plasma and urine, in protecting human monocytic U937 cells from oxidative injury. AECK-DD was previously found to be incorporated into U937 cells by HPLC-ECD.[30] Here, the incorporation of AECK-DD inside the cells was confirmed by gas chromatography-mass spectrometry analyses. Moreover, AECK-DD did not display any cytotoxic effect up to 0.8 mM concentration in the culture medium. Supplementation of normal serum-containing growth medium with antioxidants has been described to affect cellular proliferation rate.[35,36] In our experiments, supplementation of growth medium with AECK-DD (50 μM) did not significantly affect the proliferation rate of U937 cells. Further, at this concentration, no pro-apoptotic effect has been observed by DNA fragmentation measurements (data not shown).

AECK-DD had no significant effects on basal content of total and oxidized glutathione and TBA-RS levels. Moreover, the activity of glutathione peroxidase and catalase, both involved in hydroperoxide detoxification, and glutathione reductase were similar in AECK-DD-treated cells and in control cells. Nevertheless, our results indicate that AECK-DD effectively protects U937 cells from oxidative injury, as revealed by the higher viability maintained with respect to control cells during *t*-BOOH treatment, for all the concentrations of AECK-DD tested (4–100 μM). AECK-DD (50 μM) was found to be more potent than trolox at the same concentration, and even more effective than 1 mM concentration of NAC. The protective effect of AECK-DD was comparable, although somewhat higher, to that of vitamin E at the same concentration. Due to its very low solubility in water, AECK-DD can

be assumed to be bound to hydrophobic regions of macromolecular constituents. On this regard, AECK-DD has been already described to be associated to plasma lipoproteins in humans.[4] Indeed, a strict association of AECK-DD to cellular lipids seems to be suggested by the need of chloroform extraction step to release AECK-DD from cellular homogenates. A role for lipid peroxidation in *t*-BOOH-induced cell death has been already described. It is reported that low concentrations of *t*-BOOH (less than 1.0 mM) lethally injure cultured cells by a mechanism that depends on the peroxidation of cellular lipids.[37–39] Therefore, an association of AECK-DD to cellular membranes could explain the high efficiency of AECK-DD in protecting cells against *t*-BOOH-induced oxidative injury, comparable, although somewhat higher to that of vitamin E, with respect to the other hydrophilic antioxidant tested (trolox and NAC). Moreover, in our study, AECK-DD-supplementation of U937 cells was found to significantly slow down the onset of lipid peroxidation during *t*-BOOH treatment, as revealed by TBA-RS measurement, with respect to control cells.

Altogether our results indicated that the ability of AECK-DD to protect human monocytic U937 cells from *t*-BOOH-induced oxidative stress seems to be mediated by its ability to maintain both intracellular glutathione levels and a reducing environment inside the cell, and to slow down the onset of lipid peroxidation. Oxidative stress results in a shift of intracellular thiols to disulfides and in glutathione depletion.[13,14,32,33] Thiol homeostasis determines critical aspects of cell function and response.[40,41] Thiol redox state affects cellular processes such as signal transduction, gene expression, proliferation, apoptosis, proteolytic processing, ubiquitination and degradation of proteins.[42–45] In our study, AECK-DD positively and directly affect glutathione redox status during *t*-BOOH-induced oxidative stress, both delaying glutathione depletion and lowering the level of GSSG formation. Glutathione plays an important role in the protection against oxidative stress, both indirectly, as substrate in glutathione peroxidase-catalyzed detoxification of hydrogen peroxide and organic peroxides, and directly, by reacting with free radicals and repairing free radical-mediated damages through electron-transfer reactions.[46,47] On the other hand, alkoxyl radicals may be produced from *t*-BOOH inside the cell and initiate a lipid peroxidation process.[32] The effect of AECK-DD on GSH and GSSG content during *t*-BOOH treatment cannot be ascribed to differences in basal levels of the glutathione-related enzymes activity (glutathione peroxidase and glutathione reductase) between AECK-DD-treated and control cells. Therefore, the protective effect of AECK-DD might be due to a direct quenching of free radicals, produced during *t*-BOOH treatment, by AECK-DD itself, thus partially sparing intracellular glutathione. AECK-DD has been reported to scavenge reactive oxygen and nitrogen species *in vitro*[8–11] and cysteic acid and taurine have been identified among the oxidation products of AECK-DD.[10] Moreover, our data indicate that AECK-DD is able to significantly reduce the intracellular level of pro-oxidant species in U937 cells in basal condition. On the other hand, we failed to reveal a direct interaction of AECK-DD with *t*-BOOH. In fact, the absorption spectrum of AECK-DD (50 μM), characterized by an absorption maximum at 308 nm, remained unchanged following a 2-h incubation with 10-fold excess *t*-BOOH (500 μM) in PBS at 37°C. Moreover, GC-MS analyses performed after 2- and 24-h incubation in the presence of *t*-BOOH didn't reveal any oxidation product of AECK-DD, such as sulfoxide or sulfone. From these observations, a direct peroxide reducing mechanism of AECK-DD seems unlikely to be primarily involved in the biological effects observed in our study, although it cannot be completely ruled out.

Our results demonstrate for the first time an antioxidant action of AECK-DD inside the cell, and the ability of this compound to modulate cellular response to oxidative challenge. Finally, even the lowest AECK-DD concentration tested in this study (4 μM) resulted effective against a severe oxidative stress induced by *t*-BOOH (500 μM). AECK-DD, at this concentration, delayed by about 45 min the 50% decrement in cell viability in respect to control cells. This concentration of AECK-DD is in the range of those measured in human plasma from healthy subjects in fasting conditions.[4] Moreover, due to the presence of AECK-DD in human diet, the physiological concentrations of this molecule in non fasting conditions may be expected to be even higher than those measured in fasting humans. Therefore, from these results, it can be suggested that, at the concentrations present in human plasma, AECK-DD might really play a significant role in the modulation of oxidative processes *in vivo*.

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