Cancer Letters 282 (2009) 25–29

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

In vivo amelioration of adriamycin induced oxidative stress in plasma by gamma-glutamylcysteine ethyl ester (GCEE)

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 19 February 2009
Accepted 25 February 2009

Keywords:
Adriamycin
Doxorubicin
Oxidative stress
Plasma
GCEE
Chemobrain

ABSTRACT

Adriamycin (ADR) is a common chemotherapeutic known to generate significant amounts of reactive oxygen species (ROS). Although ROS generation is one of several means by which ADR attacks cancerous tissues, oxidative stress-related toxicity has been documented in several non-targeted organs as a result of anthracycline chemotherapy. Oxidative damage to tissues has been shown in the past to be minimized with co-administration of various antioxidants. Gamma-glutamylcysteine ethyl ester (GCEE) is an antioxidant and precursor to glutathione that has been shown to successfully defend brain against ADR-induced oxidative stress. The current study shows ADR *in vivo* also causes oxidative stress in plasma in the form of protein oxidation [indexed by protein carbonyls and protein bound 3-nitrotyrosine] and lipid peroxidation [indexed by protein-bound-4-hydroxynonenal]. All three markers of oxidative stress are significantly suppressed with *in vivo* co-administration of GCEE. This work further supports the concept that administration of GCEE can protect patients undergoing anthracycline chemotherapy from non-targeted oxidative damage.

1. Introduction

Adriamycin (ADR) is an antineoplastic anthracycline used to treat solid tumors and various forms of cancer, including leukemia, lymphoma, breast, lung, ovarian, and liver cancers, among others. Although ADR is an effective chemotherapeutic, dosage in patients is limited clinically due to severe cardiac toxicity [1,2]. ADR is well known to produce large amounts of reactive oxygen species (ROS), which may be lethal to tumors and cancerous cells. However, unchecked ROS generation typically leads to oxidative stress. Oxidative stress is manifested in protein oxidation [3,39], lipid peroxidation [4,5], DNA and RNA oxidation [39], all of which generally lead to the dysfunction of biomolecules. ADR has been documented to cause oxidative damage in several organs, such as heart [6] and kidney [7], testes [8,9], liver [10], and brain [11].

Because non-targeted oxidative stress is a consequence of ADR administration [12], antioxidant intervention may be appropriate. The effects of ADR on brain are somewhat unconventional due to the fact that the drug itself is incapable of crossing an intact blood brain barrier (BBB) [13]. The mechanism of ADR-induced cognitive dysfunction appears to involve an increase in peripheral levels of tumor necrosis factor alpha (TNFα), which migrates across the BBB to cause deletrious effects, including oxidative stress [11,14–16].

The oxidative stress caused by ADR in brain is halted by peripheral co-administration of anti-TNF antibody [14] or gamma-glutamylcysteine ethyl ester (GCEE) [15]. GCEE is an antioxidant and glutathione (GSH) precursor capable of easily crossing cell membranes and the BBB. N-acetylcysteine (NAC), also a GSH precursor, has also been shown to ameliorate ADR-induced behavioral dysfunction in rats, most likely by augmenting brain GSH levels and quenching oxidative stress [17].

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doi:10.1016/j.canlet.2009.02.047

Plasma is the liquid component of blood in which blood cells, platelets, lymphocytes as well as a vast array of proteins and lipids are found. Plasma contains many carrier proteins whose function is to chaperone needed materials, for instance cholesterol, to cells and tissues including heart and brain. The ROS generated by ADR may cause oxidative modifications to plasma proteins, which would most likely affect their functionality; *in vivo* protection in plasma with GCEE may preserve the health of cancer patients both during and post cessation of chemotherapy. The current study was designed to test the hypothesis that ADR induces oxidative stress in plasma *in vivo* assessed by protein carbonyls, protein-bound-4-hydroxynonenal (HNE), and 3-nitrotyrosine (3NT), and that these indices of oxidative stress are ameliorated by co-administration of GCEE.

2. Materials and methods

2.1. Animals

For this study, male B6C3 mice (2–3 months of age), approximately 30 g in size, housed in the University of Kentucky Central Animal Facility under 12-h light/dark conditions and fed standard Purina rodent laboratory chow *ad libitum*, were used. The animal protocols were approved by the University of Kentucky Animal Care and Use Committee.

2.2. Chemicals

Doxorubicin HCl (ADR) was purchased from Bedford Laboratories™. GCEE was purchased from Bachem (Torrance, CA). The OxyBlot Kit used for protein carbonyl determination was purchased from Chemicon International (Temecula, CA). Primary antibody for protein bound-HNE was purchased from Alpha Diagnostics (San Antonio, TX). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

2.3. Treatments

Mice were divided into four groups of five mice each. Two groups received saline by intraperitoneal (i.p.) injection, and the other two groups received GCEE in saline (150 mg/kg body weight) i.p. One group of saline-treated mice and one group of GCEE-treated mice received 20 mg/kg body weight ADR i.p. 4 h after saline or GCEE injection. Dosage and time for GCEE and ADR were based on prior studies [11,15,18]. After 72 h post i.p. injection of ADR, animals were sacrificed and whole blood was obtained via cardiac puncture of the left ventricle with a 1 cc syringe. Blood was transferred to 500 μl EDTA tubes and immediately centrifuged at 2500 rpm for 5 min to obtain plasma. Plasma was distributed into aliquots and frozen in liquid nitrogen until subsequent analysis.

2.4. Protein carbonyl assay

Plasma samples were diluted 10-fold with media I buffer containing 0.32 M sucrose, 0.6 mM $MgCl_2$, and 0.125 M Tris pH 8.0 with protease inhibitors, 4 μg/ml leupeptin, 4 μg/ml pepstatin A, and 5 μg/ml aprotinin. Diluted plasma samples (5 μl), 12% sodium dodecyl sulfate (SDS) (5 μl), and 10 μl of 10 times-diluted 2,4-dinitrophenylhydrazine (DNPH) from a 200 mM stock solution were incubated at room temperature for 20 min, followed by neutralization with 7.5 μl neutralization solution (2 M Tris in 30% glycerol). This neutralized solution (250 ng protein) was loaded in each well on a nitrocellulose membrane under vacuum using a slot–blot apparatus. The bicinchoninic acid (BCA, Pierce) assay was used for protein estimation. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 3 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 2 h. The membrane was washed three times in PBS following primary antibody incubation at intervals of 5 min each. The membrane was incubated following washing with an anti-rabbit IgG alkaline phosphatase-linked secondary antibody diluted in PBS in a 1:8000 ratio for 1 h. The membrane was washed for three times in PBS for 5 min and developed in Sigmafast tablets, [5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium substrate (BCIP/NBT substrate)]. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).

2.5. Protein-bound HNE assay

Samples (5 μl) of diluted plasma, 12% SDS (5 μl), and 10 μl of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were probed for protein-bound HNE in the same manner as discussed above, except that 500 ng of protein was loaded into each well of the slot blot apparatus.

2.6. 3-Nitrotyrosine (3NT) assay

Similarly, samples (5 μl) of diluted plasma, 12% SDS (5 μl), and 10 μl of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were probed for 3-NT in the same manner as described above, except that 500 ng of protein was loaded into each well of the slot blot apparatus.

2.7. Statistics

Data were analyzed using SPSS 8.0 software with one-way analysis of variance (ANOVA) used to assess statistical significance. *p*-values less than 0.05 were considered significant.

3. Results

3.1. ADR causes increased protein carbonyls in vivo which are abolished with administration of GCEE

Protein carbonyls are a marker for protein oxidation [5]. ADR produces a significant *in vivo* increase in the levels of protein carbonyls in plasma compared to mice treated with saline alone. This effect is not observed when mice are also treated with GCEE (Fig. 1).

3.2. Increases in protein-bound HNE in plasma with administration of ADR are lowered with GCEE

HNE is an indicator of lipid peroxidation [5], and HNE covalent binding to proteins generally results in lowered function. ADR produces a significant *in vivo* increase in the levels of protein-bound HNE in plasma compared to mice treated with saline alone. This effect is abrogated when mice are also treated with GCEE in addition to ADR (Fig. 2).

3.3. Increased 3NT levels in plasma following administration of ADR are lowered by GCEE

ADR produces a significant increase in the *in vivo* levels of 3NT, a marker of nitrosative stress [5], in plasma compared to mice treated with saline alone. This effect is abrogated when mice also were treated with GCEE in addition to ADR (Fig. 3).

4. Discussion

In addition to our prior studies in brain [11,14–16], other groups have shown that various oxidative stress parameters are enhanced in the periphery following administration of ADR [19–22]. In the current study, our findings support these prior studies showing increased oxidative damage in plasma due to ADR therapy in the form of protein oxidation and lipid peroxidation. To our knowledge, the current study is the first to show ADR causes nitrosative stress in plasma. ADR contains a quinone moiety that undergoes one electron redox cycling with molecular oxygen to generate large amounts of superoxide [23–25], which can react further to form highly reactive ROS such as hydroxyl or peroxynitrite. ROS in plasma have the potential to damage proteins, such as albumin, apolipoproteins, and transferrin, which function as transporters of important metabolites, or sequestration proteins in cells and tissues. Oxidative stress in plasma could contribute to unfavorable consequences for other organs, as all cells require nutrients, fatty acids, iron, hormones, among other substances, that may be insufficiently supplied by plasma if the delivery system in plasma is oxidatively disrupted. Oxidative damage to TNFα suppressors in blood, such as apolipoprotein A1 [26], may possibly explain the increase in peripheral TNFα, which is thought to mediate ADR-induced cognitive dysfunction [14,16]. Along the same lines, oxidized albumin has also been shown to trigger a respiratory burst in plasma monocytes that leads to a large increase in TNFα [27].

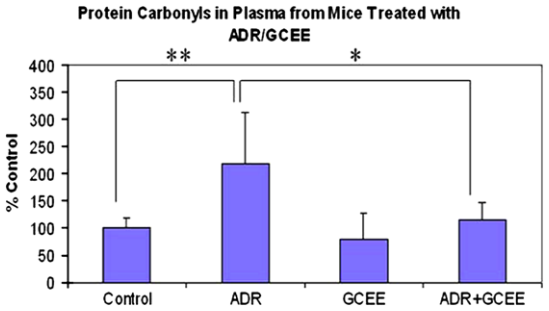

Fig. 1. Protein carbonyl analysis in plasma of mice treated with saline (control), ADR, GCEE, or ADR + GCEE. Treatment with ADR alone caused a large increase in the levels of protein carbonyls in plasma. GCEE co-administration with ADR quenched this effect. $^{*}p < 0.02$, $^{**}p < 0.01$. Mean ± SD are shown. $n = 5$.

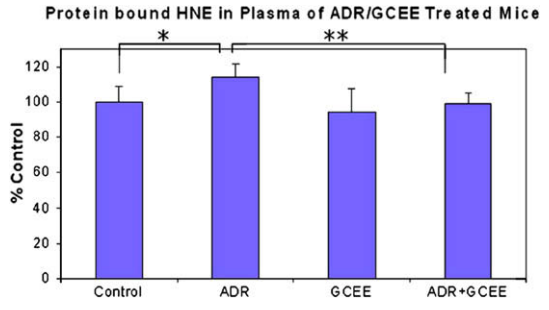

Fig. 2. Protein-bound HNE analysis of mice treated with saline (control), ADR, GCEE, or ADR + GCEE. Treatment with ADR alone caused an increase in the levels of protein-bound HNE in plasma. GCEE co-administration with ADR diminished this effect. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.03$. Mean ± SD are shown.

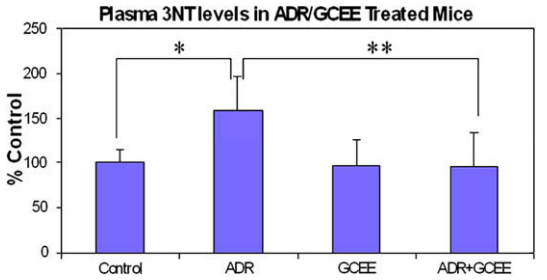

Fig. 3. 3NT analysis of plasma from mice treated with saline (control), ADR, GCEE, or ADR + GCEE. Treatment with ADR alone caused an increase in the levels of 3NT in plasma. GCEE co-administration with ADR quenched this effect. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$. Mean ± SD are shown.

ROS generated by ADR would presumably cause large amounts of protein oxidation due to the extremely high protein content in plasma (approximately 70 mg/ml). Free radical damage to iron carrier proteins such as transferrin could result in loosely bound or free iron ions that catalyze many ROS-generating reactions, thus creating a type of feed-forward cycle. ADR also complexes directly with iron to produce ROS [28,29]. HNE is a peroxidative product of arachadonic acid (AA), a component of the lipid bilayer in cell membranes and triglycerides. AA in plasma of mice treated with ADR could be attacked by ROS before its uptake into cells; however the oxidation of AA in erythrocyte or white blood cell membranes with subsequent diffusion or transport of HNE into plasma is also conceivable.

Increased protein nitration was also observed in the current study. Conceivably, the large amounts of superoxide produced by ADR redox cycling may react with NO present in the blood to form peroxynitrite, which further reacts to form free radicals that bind to tyrosine to form 3NT. All of these forms of oxidation and nitration were quenched with prior *in vivo* treatment with GCEE in our study.

GCEE is a precursor to GSH, one of the body's foremost defenses against oxidative insults. GCEE provides gamma-glutamyl-cysteine dipeptide for GSH synthesis attached to an ethyl ester moiety to increase lipophilicty and therefore, mobility across lipid bilayers and the BBB [15]. Administration of GCEE significantly increases GSH levels in brain

[15,18], hepatocytes [30], eyes [31], reperfused heart [32,33] and liver [34,35] as well as selenium-deficient heart [36]. Because products of intracellular oxidation or nitration are capable of diffusing or being transported into plasma, intracellular antioxidant protection conceivably could reduce the amount of oxidative stress products observed in plasma. However, GCEE also acts an antioxidant itself, in addition to augmenting GSH levels [18,37]. GCEE has been shown to directly scavenge peroxynitrite, therefore preventing nitration of tyrosine residues [18,38]. GCEE also intercepts the highly reactive hydroxyl radical, which is capable of perpetrating all three forms of oxidative or nitrosative stress measured in this study [18]. Therefore, increases in antioxidants both intracellularly and peripherally would serve to diminish levels of protein oxidation and nitration, as well as lipid oxidation in plasma.

As mentioned previously, ADR causes oxidative stress in many cells and tissues, including plasma, as seen in this study. To protect cancer patients from the physical rigors of oxidative stress, an ideal solution would be one in which a single antioxidant molecule could protect all cells and tissues from oxidative damage. GCEE is capable of affording that protection in several organs under various circumstances as stated above, either by augmenting GSH levels or scavenging ROS directly. While other molecules have been shown to abolish ADR induced oxidative stress in some organs, GCEE is advantageous in that is has been shown to easily cross the BBB to protect brain against oxidative stress, a requirement that renders some antioxidant molecules ineffective. As shown in this study, GCEE is also capable *in vivo* of protecting plasma proteins from oxidative modifications, thereby further warranting investigation into its use in patients receiving anthracycline chemotherapy.

Pending demonstration that GCEE administration does not compromise effective chemotherapy, the results presented here potentially support the notion that GCEE could be used as a protective co-administrant along with ADR in cancer patients as a preventative measure against peripheral non-targeted oxidative damage and chemotherapy-induced cognitive dysfunction. Additional studies to test this notion are required and are underway in our laboratory.

Conflict of interest

There were no conflicts of interest in this work.

Acknowledgement

This research was supported, in part, by funds from the University of Kentucky Comprehensive Cancer Center.

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