TOCOL PROPHYLAXIS FOR TOTAL-BODY IRRADIATION: A PROTEOMIC ANALYSIS IN MURINE MODEL

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Abstract—The aim of this study was to analyze the changes in mouse jejunal protein expression in response to prophylactic administration of two promising tocols, γ-tocotrienol (GT3) and α-tocopherol succinate (TS), as radiation countermeasures before irradiation to elucidate the molecular mechanism(s) of their radioprotective efficacy. Mice were administered GT3 or TS (200 mg kg−1) subcutaneously 24 h prior to exposure to 11 Gy 60Co γ-radiation, a supralethal dose for mice. Jejunum was harvested 24 h post-irradiation. Results of the two-dimensional differential in-gel electrophoresis (2D-DIGE), coupled with mass spectrometry, and advanced bioinformatics tools suggest that the tocols have a corresponding impact on expression of 13 proteins as identified by mass spectrometry. Ingenuity Pathway Analysis (IPA) reveals a network of associated proteins involved in inflammatory response, organismal injury and abnormalities, and cellular development. Relevant signaling pathways including actin cytoskeleton signaling, RhoA signaling, and Rho family GTPase were identified. This study reveals the major proteins, pathways, and networks involved in preventing the radiation-induced injury in gut that may be contributing to enhanced survival.

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INTRODUCTION

NUCLEAR DISASTER, caused by either accidental catastrophe or deliberate release of radioactive material, would result in a serious public health challenge and put first responders at risk of potential exposure. Injury resulting from total- or partial-body exposure to ionizing radiation manifests as acute radiation syndrome (ARS) and is characterized by hematopoietic (2–6 Gy), gastrointestinal (6–8 Gy), or neurovascular (>8 Gy) sub-syndromes (Hall and Giaccia 2012). Both colony-stimulating factors, granulocyte colony-stimulating factor (G-CSF/Filgrastim and PEGylated G-CSF/PEGfilgrastim) and granulocyte-macrophage colony-stimulating factor (GM-CSF/sargramostim) have been approved by the US Food and Drug Administration (FDA) and have been procured for the Strategic National Stockpile (Vendor Managed Inventory) to combat hematopoietic ARS (H-ARS) in the event of radiological emergency (Farese et al. 2013; Farese and MacVittie 2015; Hankey et al. 2015; National Institute of Allergic and Infectious Diseases 2015; US FDA 2015, 2018; Singh and Seed 2018). These agents provide significant benefit to subjects exposed to radiation; however, they have also been shown to produce potentially adverse consequences that should be taken into consideration. For instance, G-CSF administration following treatment with drugs that damage bone marrow stem cells worsens long-term stem cell damage through disproportionate differentiation stimulation (van Os et al. 2000). Additional apprehensions arise from G-CSF’s role in exacerbating delayed lung damage in animal models of ARS (Adachi et al. 2003; Aeolus Pharmaceuticals 2012). Furthermore, these agents can only be used as radiomitiors (after radiation exposure) and not as radioprotectors (prior to exposure). Therefore, additional agents, particularly radioprotectors, need to be developed to improve survival for groups like first responders who are at high risk of radiation exposure. Finally, these agents must be effective, safe, inexpensive to produce and store, stable under ambient conditions, and easily administered.
For the development of such agents to combat radiological or nuclear threats, human efficacy studies are neither ethical nor feasible. Approval of such drugs will be based on the FDA Animal Rule (US FDA 2015, 2016). In addition, extrapolation of the human effective doses for such drugs needs to be based on animal models according to the Animal Rule (US FDA 2015, 2016).

Vitamin E analogs, collectively known as tocols (for structures see Singh et al. 2013), are antioxidant molecules currently under investigation for their radioprotective potential (Singh et al. 2013, 2016). Both in vitro and in vivo studies have demonstrated that tocols can reduce the acute effects of radiation on radiosensitive tissues within the hematopoietic and gastrointestinal systems, ultimately increasing overall survival in radiation-exposed victims (Berbee et al. 2009, Ghosh et al. 2009, Singh et al. 2013). These agents are efficacious as radioprotectors only when administered intraperitoneally (Singh et al. 2013). Mechanism of action studies indicate that most of the tocols’ conventional antioxidant functions reduce radiation-induced free radicals, which limits the extent of macromolecular damage to DNA, RNA, and proteins (Singh et al. 2013). Treatment with tocols has also been shown to increase circulating levels of radioprotective cytokines like G-CSF and interleukin-6 (Singh et al. 2010, 2014c; Kulkarni et al. 2013). These cytokines lead to the mobilization of hematopoietic progenitor cells into the peripheral circulation, which reduces or prevents radiation-induced neutropenia, thrombocytopenia, and monocytopenia (Singh et al. 2014b). Although most of the tocols have demonstrated radioprotective efficacy, γ-tocotrienol (GT3), α-tocopherol succinate (TS), and δ-tocotrienol are more promising radioprotectors compared to others (Ghosh et al. 2009; Li et al. 2013; Singh et al. 2014a). All three agents have dose reduction factors of approximately 1.3 (Hasegawa and Landahl 1970; Singh et al 2014a). In addition to their antioxidant activity, cellular mechanisms may play a role in their radioprotective efficacy.

To understand the biological pathways and networks regulating the cellular radiation response and the complete molecular mechanism(s) underlying GT3 and TS radioprotective properties, this study analyzes the differential protein expression in jejunum from mice treated with GT3 or TS and exposed to γ-radiation using two-dimensional differential in-gel electrophoresis (2D-DIGE) coupled with mass spectrometry and advanced bioinformatics tools.

MATERIALS AND METHODS

Animals and animal care

Harlan Laboratories, Inc. (Indianapolis, IN) supplied the male, 6- to 8-wk-old CD2F1 mice. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-International accredited facility, eight per cage, in rooms maintained at 21±2 °C, relative humidity of 50±10%, with a 12 h light/dark cycle and 10–15 cycles of fresh air per hour. Certified rodent rations (Teklad Rodent Diet, Harlan Laboratories, Inc.) and acidified water (HCl, pH = 2.5–2.8) were provided to the mice ad libitum. The study was performed according to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, US National Academy of Sciences (National Research Council of the National Academy of Sciences 2011). All animal procedures were completed according to a protocol approved by the Institutional Animal Care and Use Committee.

Experimental groups

Two different mouse experiments were conducted: one with GT3 and the other with TS. In each experiment, there were three groups; untreated and sham irradiated, vehicle-treated and irradiated, and tocol-treated (GT3 or TS) and irradiated. There were eight mice in each treatment group.

Irradiation of mice

Irradiation boxes consisted of compartmentalized Plexiglas boxes, designed to hold eight mice. Mice were exposed to a bilateral, midline dose of 11 Gy 60Co γ-radiation at a dose rate of 0.6 Gy min\(^{-1}\). Radiation dosimetry was established primarily on the alanine/EPR (electron paramagnetic resonance) system, currently accepted as one of the most accurate methods for relatively high radiation doses and used for intercomparison between national metrology institutions. The calibration curves (spectrometer e-Scan, Bruker Biospin, Inc., Madison, WI) used in dose measurements are based on standard alanine calibration sets procured from the US National Institute of Standards and Technology (NIST, Gaithersburg, MD) as described earlier (Nagy 2000; Singh et al. 2013). The alanine dosimeters obtained from NIST had been calibrated in terms of absorbed dose to water using the US national standard radiation sources. Identical alanine dosimeters were placed midline within mouse phantoms (Plexiglas 2.5 cm in diameter, 7.5 cm length) and irradiated for predefined periods of time. Measurement of their EPR signals using the calibration curve were constructed with alanine dosimeters from NIST-provided dose rates to water in the core bodies of mice. A small correction was subsequently applied for the difference in mass energy absorption coefficients between water and soft tissue.

Drug preparation and administration

Yasoo Health, Inc. (Johnson City, TN) supplied GT3 formulation in 5% Tween-80 in saline. TS was purchased from Sigma-Aldrich (St. Louis, MO) and suspended in polyethylene glycol (PEG)-400 and Tween-80. GT3 and TS formulations were adjusted to a final concentration of 200 mg kg\(^{-1}\) in 0.1 mL total volume. Equal volumes of olive...
oil were administered as the vehicle (olive oil in 5% Tween-80 in saline for GT3 or PEG-400 and Tween-80 for TS). A Luer-Lock syringe with a 23 G needle was used for all drug administrations. Subcutaneous injections were administered at the nape of the neck at 24 h before irradiation.

**Tissue collection and sample preparation**

At 24 h post-radiation exposure, mice were anesthetized using isoflurane and humanely euthanized by exsanguination. The jejunum sections (about 5 cm long) were collected and flushed with phosphate-buffered saline (PBS), flash frozen on dry ice (−78.5 °C), and stored at −80 °C. Tissue samples were homogenized in 2D lysis buffer consisting of 30 mM Tris-HCl (pH 8.8), 7 M urea, 2 M thiourea, and 4% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate) detergent. Final concentrations were adjusted to 4–8 mg mL−1 of protein.

**Fig. 1.** Differential protein expression in mouse jejunum following prophylactic vitamin E treatment before radiation exposure. (A) Two-dimensional difference in gel electrophoresis (2D-DIGE) of jejunum tissue lysate from nontreated (red) and TS-treated (green) animals receiving TBI. Samples are labeled with Cy5 (red) or Cy3 (green) fluorescent dye; internal control (pooled sample) is labeled with Cy2 (yellow). (B) Three-dimensional densitometric visualization of gel spot IDs 87 and 74, corresponding to peroxiredoxin-1 and phosphoglycerate mutase 1. Left: tocol succinate-treated, Right: nontreated.

**Table 2.** Table of correspondingly up/down-regulated proteins following treatment with isoforms of vitamin E before radiation exposure. (A) Heat map displaying relative upregulation (red) or downregulation (green) of jejunum proteins expressed as ratio of irradiated to irradiated and drug treated mice. (B) Average protein expression fold change ratio of irradiated to unirradiated and irradiated and drug-treated mice. GT3 (n = 6) expression ratio is followed by TS (n = 3) in each column. Selection criteria for irradiated to irradiated and drug-treated mice ratio included average fold change of 1.5 or greater and p<0.05. NT = no treatment, ND = not detected.
for analysis. The protein lysate was quantified using the bicinchoninic acid assay. Samples were frozen at −80 °C until analysis.

**Protein identification**

Jejunal lysates were labeled with two different Cy dyes (Cy3 or Cy5). As an internal control, lysates of eight mice from vehicle control and tocol-treated groups were pooled and labeled with Cy2. Samples were separated by 2D-DIGE, and all 2D Gels were run in triplicate as described earlier (Kongara et al. 2010). The 2D gels were then analyzed using an Amersham Biosciences 2D-gel system (Amersham Biosciences, Piscataway, NJ). Images were scanned using Typhoon TRIO and analyzed by ImageQuantTL software (GE Healthcare, Chicago, IL). In-gel and cross-gel analysis employed Decyder software version 6.5 (GE Healthcare). Between 150 and 200 protein spots per gel were selected for identification using Ettan Spot picker (Amersham Biosciences).

The selected spots were then digested in buffer containing sequencing grade modified trypsin protease at 37 °C. The digested peptides were desalted and incorporated into α-cyano-4-hydroxy-cinnamic acid matrix and spotted on a MALDI (matrix assisted laser desorption/ioniziation) plate. MALDI time of flight tandem mass spectrometry (MALDI-TOF/TOF) was performed using an Applied Biosystems Proteomic Analyzer Spectrometer. Protein identification was based on peptide fingerprinting mass mapping and peptide fragmentation mapping. Peptide mass and associated fragmentation spectra were submitted to a GPS Explorer work station equipped with MASCOT search engine (Matrix Science, Boston, MA) to identify proteins from a primary sequence database.

Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA) was then used to integrate the proteins into pathways and networks based on biological and/or functional relevance to the other proteins as well as the significance value of uploaded proteins. The groups of proteins were first considered for each individual drug treatment before common proteins of interest were identified. Networks and signaling pathways were informed by searching for relationships based on the specific tissue type being considered, mouse small intestine.

**Statistical analysis**

Statistical significance was assessed by Student’s t-test analysis. Significant change of protein abundance was defined as...
as at least 1.5-fold difference with a \( p \) value < 0.05 (\( n = 3 \)). For MALDI-TOF/TOF analysis, only those proteins with a protein score confidence interval percentage or ion confidence interval percentage of >95% were considered significant.

**RESULTS**

The aim of this study was to analyze the changes in the protein expression profiles for two promising radioprotectants under development, GT3 and TS, in irradiated mice to better understand the molecular mechanism of their radioprotective efficacy. Mice were treated with GT3, TS or respective vehicle 24 h prior to supralethal irradiation (11 Gy, which causes gastrointestinal injury in addition to hematopoietic sub-syndrome). The jejunum was collected 24 h post-irradiation, and the fold-change protein expression profile was determined by 2D-DIGE. The spot detection and investigation of protein abundance in each gel provided ~2,500 proteins of interest. From each gel, about 100–200 spots with isoelectric points ranging between pI 4 and 9 and molecular weights ranging from 10 to 150 kDa were digested, separated, and identified using MALDI-TOF/TOF and the MASCOT search engine (Fig. 1). From the studies performed independently with GT3 and TS, the names of common proteins showing a statistically significant (\( p<0.05 \)) fold difference of 1.5 or greater with ratio of total-body irradiation (TBI) to unirradiated mice are presented (please see Supplemental Digital Content 2, Table 1, http://links.lww.com/HP/A178). Of this group of 26 total proteins reflecting a change with irradiation, tocol prophylaxis was found to maintain the levels of 13 proteins with respect to unirradiated mice (Fig. 2). Basal expression of cytochrome b5 was not detected in one of the two studies, denoted as not detected (ND) in the figure. The group of proteins showing maintained expression levels with tocol treatment include proteins involved in the structure and remodeling of cytoskeleton, protein synthesis, catalysis of reduction of hydrogen peroxide and organic hydroperoxides, regulation of apoptosis, modulation of cell-cell and cell-matrix interactions, and endosomal sorting.

Evaluating these proteins as a group with IPA revealed a network of common proteins involved in cell cycle, gene expression, and associated with cardiovascular disease (Fig. 3). The proteomic analysis suggests a network of related proteins simultaneously up- or down-regulated in response to prophylactic vitamin E isoform treatment given prior to 11 Gy irradiation, maintaining the levels associated with unirradiated animals. Further investigation of this group of proteins showing a change in expression in response to...
irradiation reveals relationships including actin cytoskeleton signaling (Fig. 4), RhoA signaling (Fig. 5), and Rho family GTPase signaling (Fig. 6).

We also considered the vitamin E isoform treatments individually. When jejunal lysate from healthy mice was compared to that of mice exposed to 11 Gy and prophylactically administered the vehicle, 48 proteins including isomers of the same proteins were differentially expressed. When jejunal lysate from healthy mice was compared to that of mice exposed to 11 Gy and prophylactically administered TS, 18 proteins were differentially expressed. Finally, when jejunal lysate from irradiated mice were compared to that of TS-treated/irradiated mice, 77 proteins were differentially expressed.

Radiation exposure caused the differential expression of 48 of the 108 jejunal proteins (irradiated vs. unirradiated). Of these 48 proteins, all could be identified, and 34 had ratios with the same directionality between the two group comparisons (irradiated vs. unirradiated and irradiated vs. drug-treated/irradiated), indicating the drug’s ability to prevent protein expression alteration by radiation exposure (please see Supplemental Digital Content 2, Table 2, http://links.lww.com/HP/A178). The top five diseases and disorders for which these proteins are involved are inflammatory response, cancer, organismal injury and abnormalities, gastrointestinal disease, and hepatic system disease. The molecular and cellular functions to which these proteins pertain are cell death and survival, cell morphology, cellular assembly and organization, as well as cellular compromise and development (please see Supplemental Digital Content 2, Table 2, http://links.lww.com/HP/A178). The top five pathway canonical pathways: signaling by Rho family GTPases, glycolysis I, xenobiotic metabolism signaling, 14-3-3-mediated signaling, and retinol biosynthesis. The top five network-associated functions are cellular compromise (cellular stress and immune response), cell morphology, and cellular assembly and organization (please see Supplemental Digital Content 2, Table 2, http://links.lww.com/HP/A178).

Considering the GT3 treatment independently, when jejunal lysate from healthy mice was compared to that of mice exposed to 11 Gy and prophylactically administered the vehicle, 142 proteins, including isomers of the same proteins, were differentially expressed. When jejunal lysate from healthy mice was compared to that of mice exposed to 11 Gy and prophylactically administered GT3, 21
proteins were differentially expressed. When jejunal lysate from irradiated mice was compared to that of GT3-treated/irradiated mice, 65 proteins were differentially expressed. Radiation exposure caused the differential expression of 142 of the 163 jejunal proteins (irradiated vs. unirradiated). Of these 142 proteins, 112 could be identified and 52 showed ratios with the same directionality between the two group comparisons (irradiated vs. unirradiated and irradiated vs. drug-treated/irradiated), indicating a drug-effect, preventing protein expression alteration by radiation exposure (please see Supplemental Digital Content 2, Table 4, http://links.lww.com/HP/A178). IPA analysis of jejuna proteins yielded the five top canonical pathways including calcium signaling, actin cytoskeleton signaling, epithelial adherens junction signaling, RhoGDI signaling, and agranulocyte adhesion and diapedesis (please see Supplemental Digital Content 1, Figure 3, http://links.lww.com/HP/A177). The second top network-associated functions are inflammatory response, nutritional disease, and free radical scavenging (please see Supplemental Digital Content 1, Figure 4, http://links.lww.com/HP/A177).

**DISCUSSION**

Proteome-wide analysis by 2D-DIGE has broadened our understanding of cellular responses to damage-causing agents like ionizing radiation revealing vital cellular proteins, pathways, and networks that operate during the
damage response (Stickel et al. 2014). Recent studies suggest a non-conventional mechanism of action through the release of radioprotective cytokines and growth factors (G-CSF) that facilitates the immune response and hematopoietic progenitor cell mobilization into peripheral circulation. The various intracellular and extracellular molecular factors, signaling pathways, and potential direct and indirect targets of tocols that contribute to enhanced survival in mice are still not known. Therefore, the present study examines the GT3 and TS mediated maintenance of protein expression levels to examine the cellular responses to radiation using a high throughput 2D-DIGE approach. The identified list of proteins was further analyzed using the web-based software application IPA.

By combining the control groups of the two tocol studies, we identified 26 common proteins showing a change in expression due to radiation exposure. Interestingly, 13 of these changes in protein expression were found to have been corrected by the tocol prophylaxis, suggesting that the mechanism(s) of action for both tocols might be the same. Several proteins identified can be classified as cytoskeletal proteins or proteins involved with the remodeling and development of the cytoskeleton: dihydrosymidase-related protein 2, ezrin, elongation factor 2, and plastin-1. Alternatively, cytoplasmic actin 2 was upregulated with TBI, and tocol treatment reversed this effect. An impact on cytoskeletal dynamics appears to be consistent with previous findings (Gabrys et al. 2007; Cheema et al. 2018). Of note, antioxidant enzyme peroxiredoxin-1 levels were decreased with irradiation, and this change was prevented with tocol treatment. While peroxiredoxin-1 has been found to be upregulated in human HT29 colon cancer and rat C6 glioma cells in response to irradiation, perhaps the oncologic status explains the difference in response in healthy tissue (Chen et al. 2002). A major regulator of apoptotic pathways, cell cycle progression, signal transduction, and checkpoint activation, 14-3-3 protein zeta/delta, was found to be downregulated with irradiation, and the change in expression was prevented with tocol treatment (Brunet et al. 2002). Finally, vacuolar protein-sorting-associated protein 25 was found to be downregulated following irradiation, indicating a change in endosomal sorting activity, which in turn would impact normal lysosomal degradation (Im et al. 2009). Tocol treatment appeared to prevent this change in activity.

When we turn to consider the group of proteins that were modified following irradiation and these changes were prevented by tocol treatment, IPA reveals a network of common proteins involving inflammatory response, organismal injury and abnormalities, and cellular development. Further proteomic analysis identifies three major signaling pathways impacted by irradiation and attenuated with tocol treatment: actin cytoskeleton, RhoA, and Rho family GTPases signaling. When the tocol studies are considered individually, we see similar networks at play. In the TS study, we found multiple proteins representing a network related to functions including cellular compromise, cell morphology, and cellular assembly and organization. Furthermore, we found a network of associated functions including cancer, neurological disease, and organismal injury and abnormalities as might be expected with radiation exposure. Considering the GT3 study, the top network revealed associated functions of cell signaling, post-translational modification, and protein synthesis that appears to be consistent with the TS study. Interestingly, another top network of associated functions includes inflammatory response and free radical scavenging in response to TBI.

Recent publications from Byrum et al. (2016, 2017) note proteomic changes in the plasma and urine proteomes in response to TBI in a nonhuman primate model. These studies suggest that the intestinal damage may be detected rapidly by sampling the plasma and/or urine (Byrum et al. 2016, 2017). Histopathology of jejunum from irradiated and tocol-treated animals (specifically GT3 and TS) has been published earlier, and there is definite improvement in the gut of tocol-treated and irradiated animals compared with irradiated animals receiving no treatment (Berbee et al. 2009; Singh et al. 2012). Such improvement may be related to change in the protein expression. Tocols in combination with radiation exposure can be pro-oxidative (lipid peroxidation), and such lipid peroxidation may influence proteomes. Considered as a whole, the present study demonstrates the inflammation-associated and antioxidant proteomic changes in response to prophylactic use of GT3 and TS, warranting further investigation of such prophylactic treatment toward radioprotection and their mechanism(s) of action.

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**Author contributions**—VKS conceived and planned the experiments. OOF and SYW carried out the experiments. ER performed the IPA analysis. ER, VAR, and VKS prepared the manuscript. All authors discussed the results and contributed to the final manuscript.

**References**


Berbee M, Fu Q, Boerma M, Wang J, Kumar KS, Hauer-Jensen M. Gamma-tocotrienol ameliorates intestinal radiation injury and


