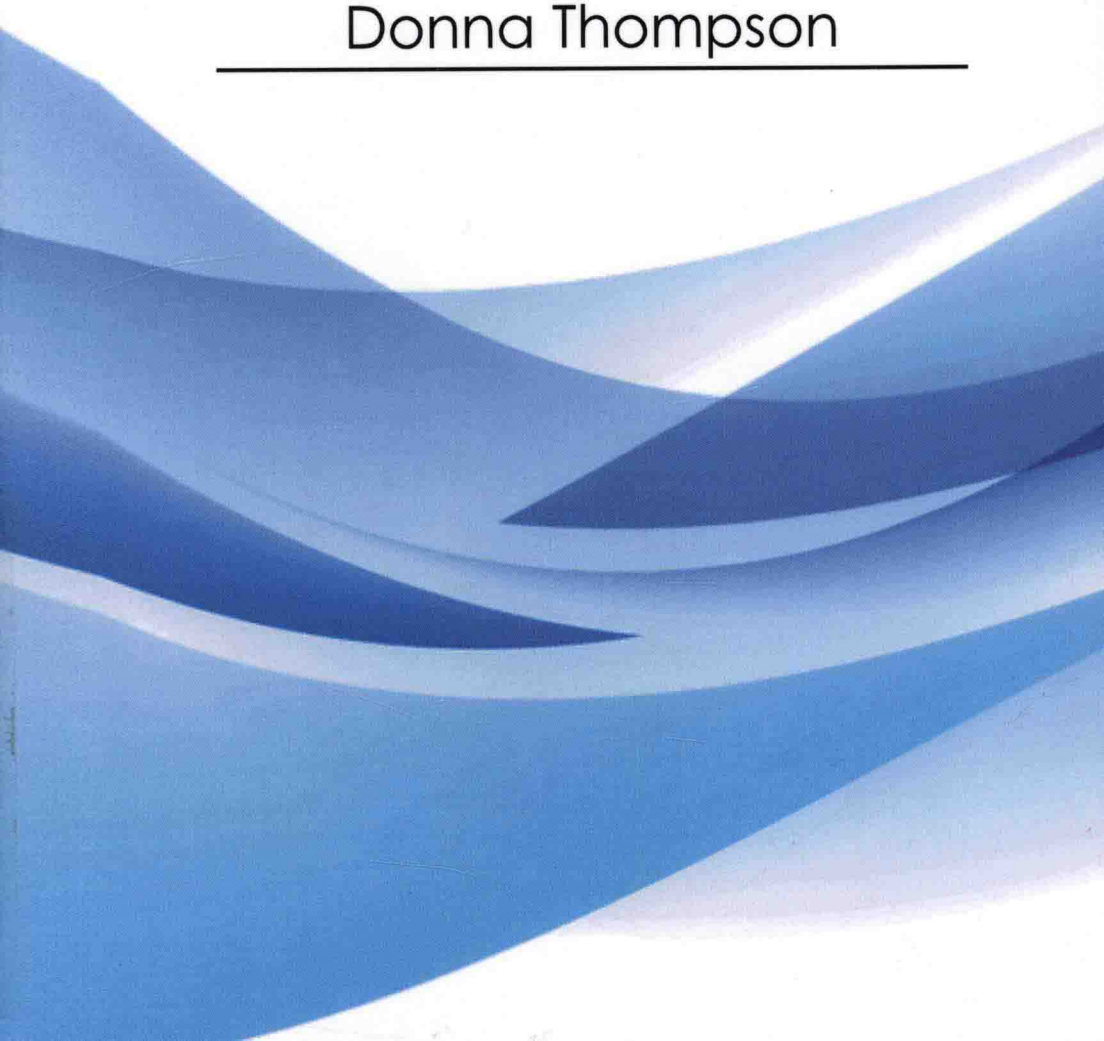
Lipid Peroxidation
Current Topics

Donna Thompson



Lipid Peroxidation: Current Topics

Edited by **Donna Thompson**

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Preface

This book was inspired by the evolution of our times; to answer the curiosity of inquisitive minds. Many developments have occurred across the globe in the recent past which has transformed the progress in the field.

Lipid peroxidation is essentially the oxidative degradation of lipids. This book focuses on current advances made in lipid peroxidation. The data compiled in this book has been contributed by researchers with extensive experience in various fields of study. We hope that the matter provided here is comprehensible to a wide audience, not only experts but also students interested in the above stated topics. This book presents modern professional views on the subject of lipid peroxidation. It discusses various topics related to lipid peroxidation in health and diseases.

This book was developed from a mere concept to drafts to chapters and finally compiled together as a complete text to benefit the readers across all nations. To ensure the quality of the content we instilled two significant steps in our procedure. The first was to appoint an editorial team that would verify the data and statistics provided in the book and also select the most appropriate and valuable contributions from the plentiful contributions we received from authors worldwide. The next step was to appoint an expert of the topic as the Editor-in-Chief, who would head the project and finally make the necessary amendments and modifications to make the text reader-friendly. I was then commissioned to examine all the material to present the topics in the most comprehensible and productive format.

I would like to take this opportunity to thank all the contributing authors who were supportive enough to contribute their time and knowledge to this project. I also wish to convey my regards to my family who have been extremely supportive during the entire project.

Editor

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Lipid Peroxidation in Health and Disease

Lipid Peroxidation After Ionizing Irradiation Leads to Apoptosis and Autophagy

Juliann G. Kiang, Risaku Fukumoto and Nikolai V. Gorbunov

Additional information is available at the end of the chapter

1. Introduction

A living cell is a dynamic biological system composed primarily of nucleic acids, carbohydrates, lipids, and proteins that structurally and functionally interact with many other molecules--organic and inorganic--to carry out normal cell metabolism. Exposure of a cell to radiation can both directly and indirectly alter molecules within the cell to affect cell viability. Radiation energy absorbed by tissues and fluids is dissipated by the radiolysis of water molecules and biomolecules [1-3]. These reactions result in redox-reactive products such as hydroxyl radical (HO*), hydrogen peroxide (H_2O_2), hydrated electron (e^-_{aq}), and an array of biomolecule-derived carbon-, oxygen-, sulfur-, and nitrogen-centered radicals (i.e., RC*, RO*, RS*, and RN*) that can in turn lead to the formation of organic peroxides and superoxide anion radicals (O_2^{*-}) in the presence of molecular oxygen [3, 4].

While the strongly electrophilic HO* has the capacity to damage molecules like polypeptides, amino acids, and polyunsaturated fatty acids (PUFAs) directly, the alterations caused by peroxide and superoxide radicals are usually produced indirectly via Fenton-type reactions [1-3, 5]. It is the interaction of these radiation-induced free radicals with important biomolecules within the cell that is the basis of the cellular sensitivity to radiation.

Free radical reactions generated after short-term radiation exposure are often quickly terminated by antiradical/antioxidant redox cycles. However, in certain cases and certain cellular environments, free radicals can initiate self-propagating chain reactions that can magnify the effects of the initial oxidations induced by radiation, leading to major disruptions that affect basic cell function [4, 6-9]. This especially true in cellular structures rich in poly-unsaturated fatty acids (PUFAs), such as cellular membranes, where radiation exposure can induce lipid peroxidation chain reactions that trigger reactions both within and beyond of the membrane [see 10-13]. Although the chemical mechanisms by which radiation induces the formation of redox-reactive products in cells are fairly well-

understood, many of the mechanisms by which they impact specific cellular processes to produce radiation injury are only beginning to be elucidated.

Lipid peroxidation is a process in which free radicals remove electrons from lipids, producing reactive intermediates that can undergo further reaction. Cellular membranes, because of their high lipid content, are especially susceptible to damage. Because lipid peroxidation reactions can alter the structure and function of critical membrane lipids, they can lead to cell injury and cell death.

Lipid peroxidation reactions take place in three steps. The first step is initiation, which produces a fatty acid radical. In polyunsaturated fatty acids, methylene groups next to carbon-carbon double bonds possess especially reactive hydrogen atoms. Lipid peroxidation is most commonly initiated when reactive oxygen species (ROS) such as OH· and HO_2 interact with a reactive methylene hydrogen atom to produce water and a fatty acid radical:

$$PUFA + OH\cdot \rightarrow PUFA^* + H_2O \quad (1)$$

The second step is propagation. Molecular oxygen reacts with the unstable lipid radical to produce a lipid peroxyl radical. This radical is also unstable; it reacts readily with an unsaturated fatty acid to regenerate a new fatty acid radical as well as a lipid peroxide. The generation of the new fatty acid radical in this step reinitiates the cycle. For this reason, this series of reactions is referred to as a lipid peroxidation chain reaction.

$$PUFA^* + O_2 \rightarrow PUFAOO^* \quad (2)$$

$$PUFAOO^* \rightarrow \text{Fenton} \rightarrow HO\text{-}PUFA^* + OH^- \quad (3)$$

The third step is termination. As the chain reaction continues, an increasing concentration of lipid radicals are produced, thereby increasing the probability two lipid radicals will react with each other, which can produce a non-radical species. This constitutes a chain-breaking step, which in combination with the activity of natural radical scavenging molecules in cellular systems ultimately quells the chain reaction.

There are four types of radiation capable of ionizing, and thus damaging, target molecules via mechanisms such as lipid peroxidation: alpha particles, beta particles, gamma rays, and neutrons. Gamma radiation has been shown to increase lipid peroxidation in a variety of biological systems. After gamma radiation exposure, levels of the lipid peroxidation indicator MDA have been shown to increase in brain [14], liver [15-19], lens [20], serum [21], and skeletal muscle [22] of rats as well as in bacteria [23].

The possibility of exposure to radiation doses significant enough to cause lipid peroxidation leading to tissue injury is more than a hypothetical hazard. It is estimated that more than 50% of cancer patients receive radiotherapy at some point during the course of their disease, and these exposures can injure normal tissues [24, 25]. Potentially harmful radiation exposures after a nuclear power plant accident are also possible, either as a plant worker or a citizen who lives in or moves through fallout areas. Such exposures are unlikely; however,

as the Fukushima, Japan, reactor incident showed, the threat is still very real. The threat of general exposure to radiation via a nuclear or radionuclide-based terrorist device is unfortunately also a real-world scenario. In order to provide public health protection in such cases, it is important to understand more about how radiation affects cells and tissues and learn how to ameliorate radiation injury.

Living organisms have evolved a variety of free radical-scavenging molecules to help protect the cell membrane from damage. Endogenous antioxidants include the enzymes superoxide dismutase (SOD), catalase, and peroxidase. Other antioxidants such as exogenously derived vitamin E can also play a role.

Free radical-mediated lipid peroxidation is harmful not only because damaged lipids disrupt membrane structure and function, but also because the process produces potentially mutagenic and carcinogenic byproducts [26]. One such product is the highly reactive carbonyl compound, malondialdehyde (MDA), which can react with deoxyadenosine and deoxyguanosine in DNA to form DNA adducts, primarily pyrimido[1,2-a]purin-10(3H)-one (M_1G) [23, 26]. M_1G toxicity has been demonstrated in experiments with glutathione peroxidase 4 knockout mice, which have a diminished capacity to protect themselves from lipid peroxidation and thus M_1G toxicity; mice with this lethal phenotype do not survive past embryonic day 8 [27].

Lipid peroxidation reactions can occur at the both the cell membrane and mitochondria membranes, and either can subsequently trigger cell death through apoptosis and/or autophagy [28]. Apoptosis is typically executed by caspases, which are cysteine aspartic acid-specific proteases that cleave an amino acid sequence-motif located N-terminal to a specific aspartic acid residue. Caspases can be broadly divided into two functional subgroups: (1) those activated during apoptosis (caspases -2, -3, -6, -7, -8, -9, and -10) ; and (2) those implicated in the processing of proinflammatory cytokines during responses (caspases -1, -4, and -5) [29-31].

The apoptosis process can follow two pathways, extrinsic and intrinsic. The extrinsic pathway involves the activation of pro-caspase-8 by external, typically molecular signals such as FAS ligand binding to FAS or TNF binding to TNF receptors on the cell membrane. Through a series of steps pro-caspase-8 becomes activated caspase-8, which then acts on the mitochondrial membrane either directly or via the activation of caspase-3. Subsequent steps then follow those described for the intrinsic pathway. The intrinsic pathway involves mitochondria directly. When mitochondria are under stress, cytochrome c is released from the mitochondria. The released cytochrome c conjugates with Apf-1and caspase-9 tin the cytoplasm to form apoptosomes, which in turn activate caspase-3 and -7. Activated caspase-3 then activates caspase-2, -6, -8, and -10. It should be noted that caspase-independent apoptosis pathways also exist, such as the intrinsic, apoptosis inducible factor (AIF) pathway and the intrinsic, mitochondria-derived endonuclease G-related pathway [29-31].

Autophagy is a catabolic process involving the bulk degradation of cellular constituents in lysosomes [32]. Autophagy under normal conditions is a cytoprotective process involved in

tissue remodeling, recovery, and rejuvenation. Autophagy dynamics in mammalian cells are well-described in several recent reviews [33-37]. The autophagic pathway is complex. To date there are over thirty genes identified in mammalian cells as regulators of various steps of autophagy. The misregulation of the autolysosomal pathway during autophagy can eventually cause cell death either by triggering apoptosis in apoptosis-sensitive cells or as a result of destructive self-digestion [38]. Light chain 3 (LC3) is a protein involved in the formation of autophagosomes in mammalian cells that serves as a biomarker for occurrence of autophagy [13].

Given the widespread potential for cellular damage from lipid peroxidation after exposure to ionizing radiation, we hypothesize that ionizing radiation-induced lipid peroxidation leads to caspase-mediated apoptotic cell death and LC-3-mediated autophagic cell death. The objective of this current chapter is to provide evidence of this hypothesis. We used human Jurkat T cultured cells and mouse ileum to investigate the relationship between lipid peroxidation and cell death both *in vitro* and *in vivo*.

2. Experimental procedures and technical approach

2.1. Cell culture

Human Jurkat T cells (American Type Cell Collection, Rockville, MD, USA) were grown in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) containing RPMI 1640 medium supplemented with 0.03% glutamine, 4.5 g/L glucose, 25 mM HEPES, 10% fetal bovine serum, penicillin (50 μg/mL), and streptomycin (50 U/mL) (Gibco/BRL, Gaithersburg, MD, USA). Cells were incubated in a 5% CO2 atmosphere at 37 °C and fed every 3-4 d.

2.2. Animal

CD2F1 male mice (25-30 g) were purchased from Harlan Laboratories (Indianapolis, IN). All mice were randomly assigned to experimental groups. Eight mice were housed per filter-topped polycarbonate cage (MicroIsolator) in conventional holding rooms. Rooms were provided 20 changes per hour of 100% fresh air, conditioned to 72 ± 2 °F and a relative humidity of 50 ± 20%. Mice were maintained on a 12-h light/dark, full-spectrum light cycle with no twilight. Research was conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-International (AAALAC-I). All procedures involving animals were reviewed and approved by the Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee.

2.3. Radiation exposure

2.3.1. Cell culture

Radiation exposures were conducted using AFRRI's ^{60}Co source. Cells suspended in growth medium were placed in 6-well plates (5 x 10^6 cells/ml; 2 ml per well) and exposed to ^{60}Co gamma-radiation at various total doses using a dose rate of 0.6 Gy/min. Cells were then returned to the incubator in a 5% CO_2 atmosphere at 37 °C for the specified time.

2.3.2. *Animals*

For survival experiments, mice (n=16 per group) received 9.25 Gy (equivalent to $LD_{90/30}$) of total-body ^{60}Co gamma-photon radiation administered at a dose rate of 0.6 Gy/min. Sham-treated mice were handled identically but received no radiation. After treatment, mice were returned to their original cages and survival was monitored for 30 d. Body weight and facial dropsy were assessed. Mean survival times (ST_{50}) were observed. The moribund mice found during the observation period were euthanized in accordance with recommendations [39, 40] and guidelines [41]. For mechanistic experiments, mice also received 9.25 Gy (n=6 per group). At specified time points after irradiation, mice were euthanized in accordance with recommendations [39, 40] and guidelines [41]. Interested tissues were harvested 1 and 7 d after irradiation and stored at -80°C until use for biochemical assays and western blots. Ileum was also prepared for immunofluorescence assessment.

2.4. Western blots

To investigate amounts of caspase-3 and LC3 proteins, ileum was minced, mixed in 100 μL Na^+ Hanks' solution containing protease inhibitors, sonicated, and centrifuged at 8000 x g for 10 min. The supernatant was collected and total protein was determined with Bio-Rad reagent (Bio-Rad, Richmond, CA, USA). Aliquots containing 20 μg of protein in tris buffer (pH=6.8) containing 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol were resolved on SDS-polyacrylamide slab gels (Novex precast 4-20 % gel; Invitrogen, Grand Island, NY, USA). After electrophoresis, proteins were blotted onto a PDVF nitrocellulose membrane (type NC, 0.45 μm; Invitrogen), using a Novex blotting apparatus and the manufacturer's protocol. After blocking the nitrocellulose membrane by incubation in tris-buffered saline-0.5% tween20 (TBST) containing 3% nonfat dried milk for 90 min at room temperature, the blot was incubated for 60 min at room temperature with monoclonal antibodies directed against caspase-3 and LC3 at a concentration of 1 μg/ml in TBST - 3% dry milk. The blot was then washed 3 times (10 min each) with TBST before incubating the blot for 60 min at room temperature with a 1000X dilution of species-specific IgG peroxidase conjugate (Santa Cruz Biotechnology) in TBST. The blot was washed 6 times (5 min each) in TBST before detection of peroxidase activity using the Enhanced Chemiluminescence Plus kit (Amersham Life Science Inc., Arlington Heights, IL, USA). IgG levels were not altered by radiation; we therefore used IgG as a control for protein loading. Protein bands of interest were quantitated densitometrically and normalized to IgG.

2.5. Nitric oxide measurements

Nitric oxide (NO) production was measured under acidic conditions as nitrite, using a commercial kit (Biomedical Research Service, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, NY, USA; www.bmrservice.com).

2.6. Lipid peroxidation measurements

Malondialdehyde (MDA), a lipid peroxidation end product, was measured colorimetrically using a commercial lipid peroxidation assay kit (CalBiochem, San Diego, CA).

2.7. Detection and analysis of caspase-3/7 activity by confocal microscopy

The Magic Red® Caspase Detection kit (MP Biomedicals; Solon, OH, USA) was used for the detection of caspase-3/7 activity, following the manufacturer's protocol. Briefly, about 2 x 10^5 cells were stained in the presence of up to 300 µl of OPTI-MEM I medium (Invitrogen). Cells were seeded onto #1 borosilicate glass slides with 4-well chambers (Fisher Science Education, Hanover Park, IL). An LSM 5 PASCAL Zeiss laser scanning confocal microscope (Carl Zeiss MicroImaging; Thornwood, NY, USA) with a 100×/1.3 NA Plan Apochromat oil objective was used to scan the signals. Each resulting image was provided with a simultaneous scan of differential interference contrast (DIC).

2.8. Immunofluorescence staining and image analysis

Small intestine specimens (5 per each of animal groups) collected at necropsy were processed for the immunofluorescence analysis and analyzed using fluorescence confocal microscopy [42]. Donkey normal serum and antibody were diluted in phosphate buffered saline (PBS) containing 0.5% BSA and 0.15% glycine. Any nonspecific binding was blocked by incubating the samples with purified donkey normal serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:20. The primary antibodies were raised against CD15 (mouse monoclonal biotin conjugated IgM from eBioscience), MAP LC3and AD4 (vendors indicated above). This was followed by incubation with secondary fluorochrome-conjugated antibody and/or streptavidin-AlexaFluor 610 conjugate (Molecular Probes, Inc., Eugene, OR, USA), and with Heochst 33342 (Molecular Probes) diluted 1:3000. The secondary antibodies used were AlexaFluor 488 and AlexaFluor 594 conjugated donkey IgG (Molecular Probes Inc.) Negative controls for nonspecific binding included normal goat serum without primary antibody or with secondary antibody alone. Five confocal fluorescence and DIC images of crypts (per specimen) were captured with Zeiss LSM 7100 microscope. Immunofluorescence image analysis was conducted as described previously [43]. The index of spatial correlation (r) of proteins was determined by multiple pixel analysis for pairwise signal interaction of green and red channels. Paneth cell identification was conducted by i) their spatial localization in crypts; ii) presence of immunoreactivity to CD15 and AD4, which are specific to this epithelial phenotype; iii) spatial appearance of the immunoreactivity to CD15, AD4, and the FISH reactivity to AD4 mRNA (see above) in morphologically identified Paneth cells.

2.9. Solutions

Na^+ Hanks' solution contained in mM: 145 NaCl, 4.5 KCl, 1 .3 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES (pH 7.40 at 24 °C). Na^+ Hanks' stop buffer contained in mM: 50 tris-HCl, 1% NP-40, 0 .25% Na^+-deoxycholate, 150 NaCl, 1 EDTA, 1 phenylmethanesulfonyl fluoride, 1 Na_3VO_4, 1 NaF, along with aprotinin, leupeptin, and pepstatin (10 µg/mL each). Na^+ Hanks' wash buffer contained in mM: 1 EDTA, 1 phenylmethanesulfonyl fluoride, 1 DTT, 1 Na_3VO_4, 1 NaF, along with aprotinin, leupeptin, and pepstatin (10 µg/mL each).

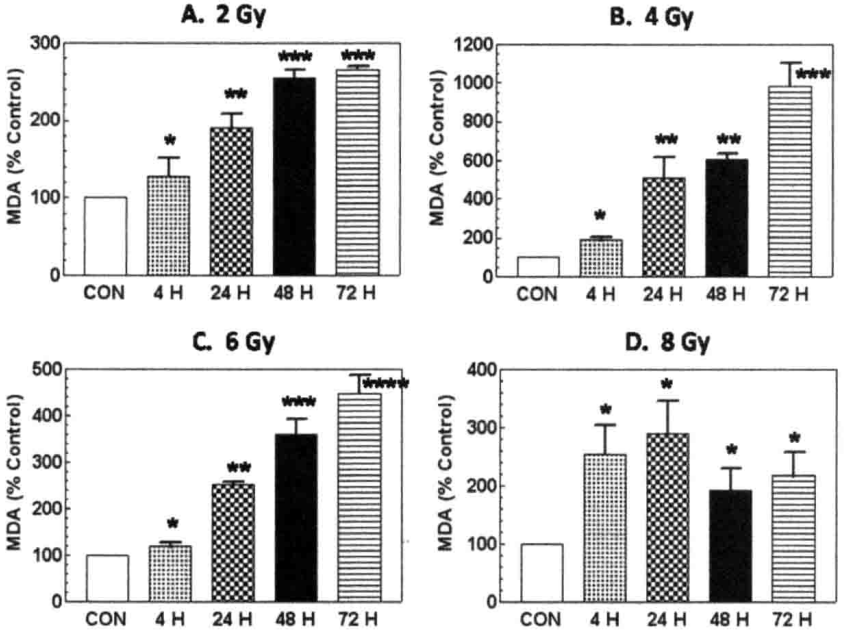
2.10. Statistical analysis

Results represent the mean ± s.e.m. One-way ANOVA, Studentized-range test, Bonferroni's inequality, and Student's t-test were used for comparison of groups with 5% as a significant level.

3. Response of human T cells to irradiation

3.1. Gamma radiation increased lipid peroxidation production

Cells were irradiated with 2, 4, 6, or 8 Gy; the lipid peroxidation marker MDA was measured in these cells at 4, 24, 48, and 72 h postirradiation. Figure 1 shows MDA levels increased in a radiation dose- and postirradiation time-dependent manner in cells receiving 2, 4, and 6 Gy (Fig. 1A-C). Cells receiving 8 Gy (Fig. 1D) had lipid peroxidation levels above the baseline at all times tested. But in cells receiving 6 Gy and 8 Gy, by 24 h postirradiation the levels were significantly lower than those observed in cells receiving 4 Gy (Fig. 1C vs. 1B and Fig. 1D vs. 1B). This observation may be a reflection of the drop in cell viability previously observed in cells after doses greater than 4 Gy.

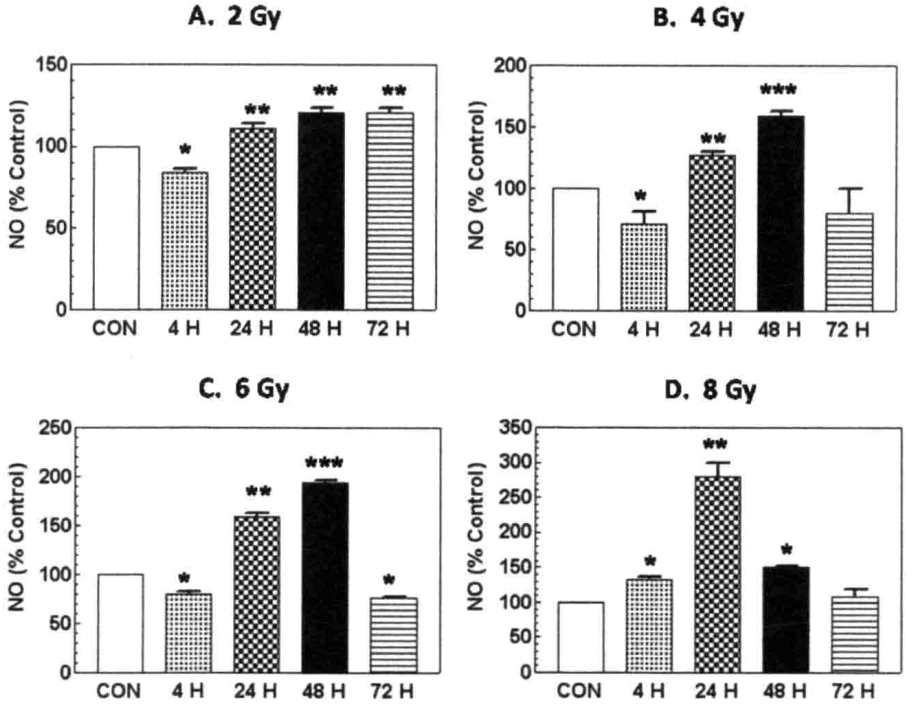


Jurkat T cells were exposed to gamma radiation at 2 (A), 4 (B), 6 (C), or 8 Gy (D) and allowed to respond for 4, 24, 48, or 72 h (n=3) before preparation of cell lysates. Lipid peroxidation as indicated by MDA was measured. Data are expressed relative to that of unirradiated controls. For panels A: *P<0.05 vs. control, 24 H, 48 H, and 72 H; **P<0.05 vs. control, 4 H, 48 H, and 72 H; ***P<0.05 vs. control, 4 H, and 24 H. For Panel B: *P<0.05 vs. control, 24 H, 48 H, and 72 H; **P<0.05 vs. control, 4 H, and 72 H; ***P<0.05 vs. control, 4 H, 24 H, and 48 H. For panels C: *P<0.05 vs. control, 24 H, 48 H, and 72 H; **P<0.05 vs. control, 4 H, 48 H, and 72 H; ***P<0.05 vs. control, 4 H, 24 H, and 72 H; ****P<0.05 vs. control, 4 H, 24 H, and 48 H. For panel D: *P<0.05 vs. control, determined by one way ANOVA and studentized-range test. CON: unirradiated controls

Figure 1. Gamma radiation increased lipid peroxidation in T cells.

3.2. Gamma radiation increased NO production

Because NO is known to react with O_2- to form peroxynitrite (ONOO-) that can oncrease lipid peroxidation [23], we measured NO production levels in irradiated Jurkat cells. Figure 2 shows NO production in irradiated cells. By 4 h postirradiation cells receiving 2, 4, or 6 Gy showed NO levels statistically lower than non-irradiated controls, but cells receiving 8 Gy showed an increase. By 24 and 48 h, all irradiated cells exhibited increased NO production, generally in a radiation dose-dependent manner. By 72 h postirradiation NO level returned to baseline in cells receiving 4, or 8 Gy, while NO levels in cells receiving 2 or 6 Gy remained above the baseline and had dropped below baseline, respectively.



Jurkat T cells were exposed to gamma radiation at 2 (A), 4 (B), 6 (C), or 8 Gy (D) and allowed to respond for 4, 24, 48, or 72 h (n=3) before preparation of cell lysates. NO production was measured. Data are expressed relative to that of unirradiated controls. For panel A: *P<0.05 vs. control, 24 H, 48 H, and 72 H; **P<0.05 vs. control and 4 H. For Panel B: *P<0.05 vs. control, 24 H, and 48 H; **P<0.05 vs. control, 4 H, 48 H, and 72 H; ***P<0.05 vs. control, 4 H, 24 H, and 72 H. For panel C: *P<0.05 vs. control, 24 H, and 48 H; **P<0.05 vs. control, 4 H, 48 H, and 72 H. ***P<0.05 vs. control, 4 H, 24 H, and 72 H. For panel D: *P<0.05 vs. control, 24 H, and 72 H; **P<0.05 vs. control, 4 H, 48 H, and 72 H, determined by one way ANOVA and studentized-range test. CON: unirradiated controls

Figure 2. Gamma radiation increased NO production in T cells.

3.3. Gamma radiation increased apoptosis

Lipid peroxidation occurring within the cell and mitochondrial membranes can trigger apoptosis [28]. Because increased caspase-3 and -7 are indicators of cells undergoing