DNA Damage in Mammalian Cells by Non-Thermal Atmospheric Pressure Microsecond Pulsed Dielectric Barrier Discharge Plasma is not mediated via Lipid Peroxidation

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Abstract: Lipid peroxidation in mammalian cells by non-thermal atmospheric pressure microsecond pulsed dielectric barrier discharge plasma was investigated. It has been shown earlier that plasma treatment of mammalian cells submerged in a shallow layer of culture medium can result in dose dependent DNA damage. We show that non-thermal plasma induces lipid peroxidation, measured by release of Malondialdehyde (MDA), in cells treated under medium and not under phosphate buffered saline (PBS). N-Diphenyl-Phenyl-enediamine (DPPD), a lipophilic antioxidant was used to block non-thermal plasma induced lipid peroxidation. We show that non-thermal plasma induces lipid peroxidation in mammalian cells, but non-thermal plasma induced lipid peroxidation does not lead to DNA damage in mammalian cells. Thus, one needs to consider other possible mechanisms for plasma induced DNA damage in mammalian cells such as ROS signaling or active transport of long-lived organic hydroperoxides.

Keywords: Dielectric Barrier Discharge, Lipid Peroxidation, Non-Thermal Plasma, MDA, Reactive Oxygen Species

1. Introduction

Non-thermal atmospheric pressure plasma is now being widely developed for various applications like surface sterilization, cell proliferation, modulation of cell attachment, blood coagulation, apoptosis, enhancement of cell transfection etc. Previously, we have shown using mammalian cells in culture that non-thermal dielectric barrier discharge (DBD) plasma has dose-dependent effects from increasing cell proliferation to inducing apoptosis and that these effects are primarily due to the formation of reactive oxygen species (ROS). It was also known that non-thermal plasma treatment of mammalian cells submerged in a shallow layer of culture medium could result in dose dependent DNA damage. We have specifically examined the induction of DNA damage by DBD plasma and shown that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium. In this sense plasma can create effects similar to ionizing radiation (IR). However, while IR penetrates through cell membranes creating ROS in the immediate vicinity of DNA, plasma treatment acts on cells through cell membrane. The question arises: By what mechanisms do non-thermal plasma effects reach across cell membrane? There are several possibilities. One involves peroxidation of lipid membrane with MDA as a by-product. The by-products of lipid peroxidation (MDA) have been known to create bulky adducts on DNA which is a form of damage requiring repair. Other mechanisms may involve ROS signaling and transport across membrane. The goal of this paper is to test the hypothesis that non-thermal plasma induced DNA damage in mammalian cells is created through lipid peroxidation.

2. Materials and Methods

Non-Thermal Plasma Treatment

Non-thermal DBD plasma was generated and applied to cells as described elsewhere. Plasma characteristics are described by Ayan et. al. MCF10A cells on glass cover slips were exposed to non-thermal plasma at various doses from 0.13 J/cm² to 7.8 J/cm². Two different approaches were used for non-thermal plasma-treatment of cells in vitro: direct and separated. Plasma treatment of cells, modes of treatment and the setup are described in detail elsewhere.

Cell Culture

Mammalian Breast Epithelial Cells (MCF10A) were maintained in high glucose Dulbecco’s Modified Eagle’s Medium-Ham’s F12 50:50 mixture (DMEM-Ham’s F12 50:50) supplemented with 5% donor horse serum (Cellgro, Mediatech, VA, USA), Epidermal Growth Factor (EGF, 100 µg/ml), Hydrocortisone (1 mg/ml), Cholera Toxin (1 mg/ml), Insulin (10 mg/ml) and Penicillin/Streptomycin (500 µl, 10000 U/ml penicillin and 10 mg/ml streptomycin). Media was changed every two days.

Lipid Peroxidation Assay

Bromotrichloromethane (BrCCl3, Sigma-Aldrich, St. Louis, MO, USA) was used as a known inducer of lipid peroxidation. N-acetyl-cysteine (Sigma-Aldrich, St. Louis, MO, USA) was used as an intracellular scavenger of reactive oxygen species and Diphenyl-phenyl-enediamine (DPPD, Sigma-Aldrich, St. Louis, MO, USA), a lipophilic synthetic alternative to Vitamin E was used as a lipid peroxidation inhibitor. Malondialdehyde-thiobarbituric acid (MDA-TBA) levels were used as a measure of lipid peroxidation after non-thermal plasma treatment of
mammalian cells. Cells were treated either directly or separately for 15 s at a dose of 1.95 J/cm². Cells were held after plasma treatment for either 1 min or 10 min before adding butylated hydroxylamine (BHT). Whole lysates were used to measure the level of MDA following the manufacturer’s protocol (OxiSelect™ TBARS Assay kit, Cell BioLabs, San Diego, CA, USA).

**Western Blot**

Protein expression and modification were analyzed by Immunoblotting technique. Total cell lysates were prepared by direct lysis of washed cells in 2X SDS sample buffer containing β-mercaptoethanol. Samples were electrophoresed at 150 V in Tris-glycine SDS running buffer. Following electrophoresis, proteins were transferred onto PVDF (Millipore, MA, USA) membrane for two hours in Tris-glycine transfer buffer. Immunoblotting was carried out by using the technique described by Kalghatgi, et al.¹⁶

### 3. Results and Discussion

To determine whether DBD plasma treatment of cells induced DNA damage, we looked at phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA damage. Western blot with an antibody that detects phosphorylated H2AX (γ-H2AX) revealed that plasma treatment of cells induces a dose-dependent increase in the level of γ-H2AX (Fig. 1). These data are consistent with a dose-dependent increase in DNA damage after non-thermal plasma treatment of mammalian cells.

![Representative blot with antibodies towards γ-H2AX (top panel) and α-tubulin (bottom panel, loading control) showing non-thermal DBD plasma induces dose-dependent DNA damage in mammalian cells.](image)

We next sought to directly test whether the damage induced by DBD plasma is due to ROS (e.g. H₂O₂, OH⁻, singlet oxygen, atomic oxygen, superoxide radical etc.) generated in the media and/or cells by plasma treatment. Cells were pre-treated with the ROS scavenger, N-acetyl cysteine, which was found to block induction of γ-H2AX even at high doses of DBD (Fig. 2), suggesting that the effects are mediated by ROS.

To determine whether the effects of DBD plasma are due to modification of the cell medium by plasma treatment, the medium was treated in the same way without cells and then added to cells (separated treatment). As shown in Fig. 3, damage induced by the treatment of the medium separately from cells was not significantly less than that produced by direct treatment. This suggests that ROS generated in the medium by plasma treatment are responsible for the induction of DNA damage. These ROS species must survive long enough to remain active while being transferred to the cells. In order to determine how long living are these ROS species, cell medium was separately treated as described above and then held for increasing times before being added to cells. Induction of DNA damage by the medium treated with DBD plasma was not significantly reduced by holding media up to one hour prior to adding it to cells, suggesting that neutral species react with organic components in the cell medium to produce long living organic peroxydes which are known to have a half-life on the order of 12-24 h.

![Fig. 2. MCF10A cells were incubated for 1 hour with 4 mM N-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. γ-H2AX (upper panel) or α-tubulin (lower panel) was detected by immunoblot of cell lysates prepared one hour after plasma treatment.](image)

To determine whether the effects of DBD plasma are mediated by long living organic peroxydes produced in medium, we compared the effect of separated treatment of medium vs. phosphate buffered saline (PBS), which is comprised of inorganic salts. We observed no DNA damage in cells exposed to separately treated PBS (Fig. 4), whereas separately treated medium induced DNA damage as shown in previous experiments. Taken together, these data suggest that DNA damage is induced by organic peroxydes formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium.

![Fig. 3. Cells were subjected to DBD plasma as described earlier (direct, D) or media (100 µl) was exposed to DBD plasma separately and then transferred to the cells (separate, S). Representative immunoblot with γ-H2AX (upper panel) or α-tubulin (lower panel) are shown. The graph below the immunoblot show quantification using Odyssey of triplicate samples in three separate experiments. The γH2AX signal was normalized to the amount of α-tubulin. Data (+S.D.) are expressed relative to lowest dose of direct treatment that was set at 1.0.](image)

Non-thermal plasma produces a large ROS concentration in the extracellular medium during treatment. However, it is unclear how these ROS go inside cells. N-acetylcysteine, an intracellular ROS scavenger completely blocked
phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells, which indicates that, ROS produced by plasma extracellularly may move across the cell membrane through lipid peroxidation, opening transient cell membrane pores, or signaling pathways which modify the concentration of ROS inside cells. Active species produced by plasma may also modify the cell medium, which in turn interacts with cells.

Since many of active species have a short life span, they may immediately interact with medium components including amino acids and proteins, leading to production of long lived reactive organic hydroperoxides. These hydroperoxides may then induce lipid peroxidation and the by-products of lipid peroxidation, like MDA may lead to DNA damage, or they may bind to cell membrane receptors and activate intracellular signaling pathways leading to subsequent DNA damage.

Fig. 4. Cells overlaid with 100 µl of PBS were treated directly with DBD plasma (direct, D) or were treated with PBS that was separately exposed to DBD plasma prior to addition to cells (S). Separately treated PBS does not induce DNA damage in mammalian cells.

As shown in Fig. 5, we see that plasma indeed induces lipid peroxidation in cells immediately after treatment, when treated under medium either directly or separately. In contrast, non-thermal plasma does not induce lipid peroxidation in mammalian cells treated under PBS, either directly or separately. This suggests that long living organic hydroperoxides, produced as a result of interaction of neutral active species produced by non-thermal plasma in medium with organic components of the medium like proteins and amino, may induce lipid peroxidation. These data are consistent with the fact that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium.

We next sought to test whether plasma induced lipid peroxidation led to the observed DNA damage. In order to

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Fig. 5. Non-Thermal plasma treatment of MCF10A cells leads to release of malondialdehyde (MDA), a commonly used marker for measuring lipid peroxidation in mammalian cells. Plot shows MDA equivalent for untreated cells and cells treated at indicated plasma dose or with 1 mM BrCCl₃. Data from triplicate samples (± S.D.) are plotted

Fig. 6. MCF10A cells were incubated for 1 hour with 10 mM N-diphenylphenyl-enediamine (DPPD) (+DPPD) or cell culture medium (-DPPD), followed by treatment at the indicated dose of DBD plasma or with 1 mM bromotrichloromethane (BrCCl₃). Lipid peroxidation was measured via release of malondialdehyde (MDA). Plot shows MDA equivalent for untreated cells (control) and cells treated at indicated plasma dose or with 1 mM BrCCl₃. Data from triplicate samples (± S.D.) are plotted.
block lipid peroxidation in MCF10A cells after plasma treatment, we used DPPD, a synthetic lipophilic antioxidant and bromotrichloromethane (BrCCL) a known inducer of lipid peroxidation as a pro-oxidant. DPPD is frequently used in cell culture and in in-vivo studies to inhibit lipid peroxidation by various chemical agents. 

MCF10A cells were pre-incubated with DPPD for 15 min at 37°C before plasma treatment or addition of 1 mM BrCCL. As shown in Fig. 6, DPPD significantly inhibits lipid peroxidation in MCF10A cells, both after plasma treatment and after incubation of cells with BrCCL for 2 hours. Thus, DPPD is a potent inhibitor of non-thermal plasma induced lipid peroxidation.

DPPD proving to be a potent blocker of lipid peroxidation, we next sought to investigate the role of non-thermal plasma induced lipid peroxidation in the observed DNA damage. As earlier, MCF10A cells were preincubated with 10 mM DPPD for 15 min before exposing the cells to non-thermal plasma at a dose of 1.95 J/cm² (15 s at low frequency) or to 1 mM BrCCL for 2 h. After plasma treatment cells were incubated for 1h at 37°C prior to lysing for analyzing DNA damage. DNA damage was analyzed as earlier by western blot technique for measuring γ-H2AX. As shown in Fig. 7, DNA damage after plasma treatment of MCF10A cells with or without pre-incubation of DPPD was same while DNA damage induced by BrCCL was significantly reduced by DPPD. Non-thermal plasma induced lipid peroxidation does not lead to non-thermal plasma induced DNA damage. Plasma produces significant concentration of reactive oxygen species in the medium covering the cells during treatment that may lead to lipid peroxidation and DNA damage simultaneously, but DNA damage is not induced as a result of lipid peroxidation.

**References**

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