Non-thermal gas plasma-induced endoplasmic reticulum stress mediates apoptosis in human colon cancer cells

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Received March 25, 2016; Accepted August 5, 2016

DOI: 10.3892/or.2016.5038

Abstract. Colorectal cancer is a common type of tumor among both men and women worldwide. Conventional remedies such as chemotherapies pose the risk of side-effects, and in many cases cancer cells develop chemoresistance to these treatments. Non-thermal gas plasma (NTGP) was recently identified as a potential tool for cancer treatment. In this study, we investigated the potential use of NTGP to control SNUC5 human colon carcinoma cells. We hypothesized that NTGP would generate reactive oxygen species (ROS) in these cells, resulting in induction of endoplasmic reticulum (ER) stress. ROS generation, expression of ER stress-related proteins and mitochondrial calcium levels were analyzed. Our results confirmed that plasma-generated ROS induce apoptosis in SNUC5 cells. Furthermore, we found that plasma exposure resulted in mitochondrial calcium accumulation and expression of unfolded protein response (UPR) proteins such as glucose-related protein 78 (GRP78), protein kinase R (PKR)-like ER kinase (PERK), and inositol-requiring enzyme 1 (IRE1). Elevated expression of spliced X-box binding protein 1 (XBP1) and CCAAT/enhancer-binding protein homologous protein (CHOP) further confirmed that ROS generated by NTGP induces apoptosis through the ER stress signaling pathway.

Introduction

Colorectal cancer is the third most common cancer in males and the second in females, and is thus a severe health threat worldwide (1). Several factors including genetics, gender, ethnic origin, geographical region, and environmental conditions influence the incidence of colorectal cancer (2). Moreover, lifestyle factors such as consumption of processed meat products, high-fat and low-fiber diet, lack of physical activity, and obesity increase the risk of this disease (3,4). Key obstacles to long-term survival of colorectal cancer patients include resistance of the tumors to chemotherapeutic agents and the side-effects of prolonged chemotherapeutic treatments (5).

Non-thermal gas plasma (NTGP), a novel tool successfully used in wound healing and surface sterilization, promotes cell proliferation and increases transfection efficiency (6,7). In addition, this method has recently emerged as a promising approach for treating cancer. The anticancer activity of NTGP has been demonstrated in both *in vivo* and *in vitro* models including skin, liver, lung, and colon cancers (8,9). Plasma, considered to be the fourth stage of matter, consists of charged particles (electrons, ions), excited atoms, and reactive oxygen species (ROS) (10). Several lines of evidence suggest that plasma ROS induce apoptosis via oxidative stress (9). Induction of selective cell death in colorectal cancer cells by NTGP treatment represents a promising approach to colorectal cancer therapy that would both avoid the deleterious side-effects of chemotherapy and circumvent chemoresistance.

Cells can undergo apoptosis via three different pathways, respectively mediated by death receptors, mitochondria, or the endoplasmic reticulum (ER) (11-13). The ER is the primary site for synthesis and folding of secreted and membrane-bound proteins, as well as some organelle-targeted proteins. This organelle is highly sensitive to stresses that perturb cellular energy level, redox state, or Ca^{2+} concentration (14). Protein chaperones such as glucose-related protein 78 (GRP78)/BiP and GRP94 maintain the correct folding of newly synthesized proteins in the ER (15). High ROS levels disturb ER function, leading to accumulation of unfolded proteins and a state referred to as ER stress. In response to ER stress, the cell activates signaling pathways including the unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (16,17). The UPR, the primary defense

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Key words: non-thermal gas plasma, endoplasmic reticulum stress, unfolded protein response, CCAAT/enhancer-binding protein homologous protein, apoptosis

mechanism of the ER, restores cellular function by halting protein synthesis and bolstering protein folding capacity, thereby improving the cell's likelihood of survival (17). However, when ER stress is so severe that these tactics cannot restore cellular homeostasis, the UPR triggers apoptosis (16).

Because plasma generates ROS, resulting in oxidative stress, we predicted that NTGP would induce ER stress in SNUC5 human colon cancer cells and thereby cause apoptosis. The effects of the plasma exposure depend on the plasma source (e.g., plasma jet or needle, surface or volume of plasma), exposure time, and process gas (e.g., air, argon or helium) (18). For this study, we used a non-thermal dielecteic barrier discharge (DBD) plasma source with a gas consisting of 70% oxygen and 30% argon at atmospheric pressure. Previously, we showed that a DBD plasma system can generate ROS and induce apoptosis in human keratinocytes (19), and we used the same experimental settings for this study.

Materials and methods

Reagents. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), *N*-acetylcysteine (NAC), and anti-actin primary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). ER-Tracker™ Blue-White DPX and Rhod-2 AM dyes were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Primary antibodies against GRP78, phosphorylated eukaryotic initiation factor 2α (p-eIF2α), phosphorylated PERK (p-PERK), and X-box binding protein 1 (XBP1) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and anti-CHOP antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Primary antibody against phosphorylated IRE1 (p-IRE1) was purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA).

Cell culture. The SNUC5 colon cancer cell line was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Cells were cultured at 37˚C in an incubator containing humidified air (95%) and carbon dioxide (5%). The culture medium was RPMI-1640 (Invitrogen, Grand Island, NY, USA) containing 10% heat-inactivated FBS (Sigma-Aldrich), streptomycin (100 μg/ml), and penicillin (100 U/ml).

Plasma treatment. Non-thermal DBD was used as the plasma source, as previously described (19). For DBD plasma treatment, cells were trypsinized and counted to adjust the density to $2x10^5$ cells/ml, and 11 ml of cell suspension was placed in a 60-mm cell culture dish. After exposure to DBD plasma, cell suspensions were transferred to new cell culture dishes or wells for subsequent experiments. Control samples were subjected to all steps except plasma exposure.

Detection of intracellular ROS. Plasma-treated cells were seeded at a density of $1x10^5$ cells/plate and incubated for 24 h at 37˚C. Cells were then harvested, washed, and re-suspended in PBS containing 25 μM DCFH-DA. After 15 min at 37˚C, the cells were washed, re-suspended in PBS, and analyzed by flow cytometry (Becton Dickinson, Mountain View, CA, USA). For image analysis, cells were loaded with DCFH-DA and incubated for 30 min at 37˚C. The stained cells were washed and mounted on microscope slides in mounting medium (Dako, Carpinteria, CA, USA). Microscopic images were obtained on a confocal laser-scanning microscope and analyzed using LSM 5 PASCAL software (Carl Zeiss Jena GmbH, Jena, Germany).

Cell viability. Cell viability was assayed by MTT test. Cells were suspended in 11 ml of media at a density of $2x10^5$ cells/ml, and the suspensions were placed in 60-mm cell culture dishes. With the lids removed from the dishes, the cells were exposed to plasma for 2 min. Plasma-treated cells were transferred to 24-well plates at a density of $1x10^5$ cells/well. Twenty-four hours later, 50 μl of MTT stock solution (2 mg/ml) were added to each well to yield a total reaction volume of 200 μl. After incubation for 4 h, the plate was centrifuged at 800 x g for 5 min, and the supernatants were aspirated. Formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and A_{540} was measured using a scanning multi-well spectrophotometer (20).

Nuclear staining with Hoechst 33342. Cells were transferred into 24-well plates following plasma exposure and incubated at 37˚C for 24 h. The DNA-specific fluorescent dye Hoechst 33342 was added to each well, and the cells were incubated for 10 min at 37˚C. The stained cells were visualized under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera. The degree of nuclear condensation was evaluated by counting apoptotic cells in randomly selected equally sized areas in each well.

ER staining. Plasma-exposed cells were seeded in chamber slides (Nalge Nunc International Corp., Rochester, NY, USA) at a density of $1x10^5$ cells/ml and incubated for 24 h at 37˚C. ER-Tracker™ Blue-White DPX dye was added to the cells, and the samples were incubated for an additional 30 min. The cells were washed twice with PBS before the addition of mounting medium. Microscopic images were collected using LSM 5 PASCAL software.

Western blotting. Harvested cells were lysed by incubation on ice for 30 min in 150 μl of lysis buffer (iNtRON Biotechnology, Seoul, Republic of Korea). The resultant cell lysates were centrifuged at 13,000 rpm for 5 min. Supernatants were collected, and protein concentrations were determined. Aliquots were boiled for 5 min and electrophoresed on 12% SDS-polyacrylamide gels. Protein blots of the gels were transferred onto nitrocellulose membranes. The membranes were incubated with the appropriate primary antibodies (1:1,000) followed by horseradish peroxidase-conjugated anti-IgG secondary antibodies (1:5,000) (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, UK).

Measurement of mitochondrial Ca^{2+} levels. Mitochondrial Ca^{2+} levels were measured using Rhod-2 AM (21). Plasma-treated cells were seeded at a density of $1x10^5$ cells/plate and incubated for 24 h at 37˚C. Cells were harvested, washed, and re-suspended in PBS containing Rhod-2 AM. After 15 min at 37˚C, the cells were washed, re-suspended in PBS,

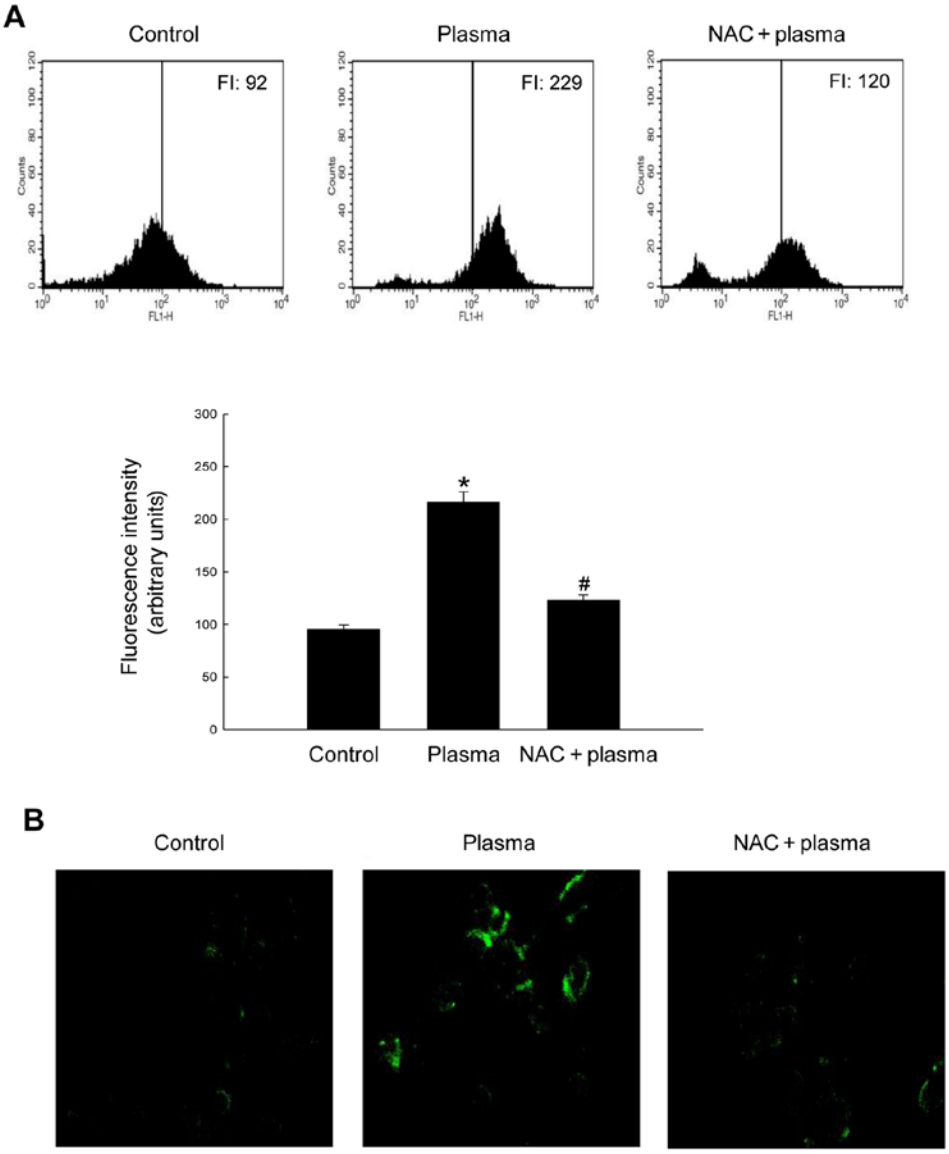

Figure 1. Effect of NTGP on ROS generation. NAC was used as a ROS scavenger. SNUC5 cells were placed in 60-mm cell culture dishes in 11 ml of media at a density of $2x10^5$ cells/ml. With the lids removed from the dishes, the cells were exposed to plasma for 2 min. Plasma-treated cells were stained with DCFH-DA fluorescent dye, and intracellular ROS levels were analyzed using (A) flow cytometry and (B) confocal microscopy. *Significantly different from control cells ($p<0.05$); #significantly different from plasma-exposed cells ($p<0.05$). NTGP, non-thermal gas plasma; ROS, reactive oxygen species; NAC, *N*-acetylcysteine.

and analyzed by flow cytometry. For image analysis, cells were loaded with Rhod-2 AM and incubated for 30 min at 37˚C. The stained cells were washed and mounted on microscope slides in mounting medium. Microscopic images were obtained under a confocal laser-scanning microscope and analyzed using LSM 5 PASCAL software.

Statistical analysis. All measurements were made in triplicates, and all values are expressed as means ± standard error of the mean (SEM). The results were subjected to analysis of variance (ANOVA) using the Tukey's test to analyze differences. P<0.05 was considered statistically significant.

Results

NTGP induces ROS level in SNUC5 cells. Plasma generates ROS and induces oxidative stress in cells (9). Therefore, we assessed intracellular ROS generation in plasma-treated cells using DCFH-DA, a ROS-sensitive fluorogenic dye. Flow cytometry revealed that 2 min of plasma exposure increased DCFH-DA fluorescence to 229 (FI: 229) vs. 92 in control cells not exposed to plasma (Fig. 1A). Samples pre-treated with NAC, a well-known free radical scavenger, yielded an FI value of 120. The flow cytometry results were consistent with those obtained by confocal microscopy analysis of DCFH-DA-stained cells (Fig. 1B). These results indicate that NTGP generates ROS in SNUC5 human colon carcinoma cells.

NTGP induces apoptosis in SNUC5 cells. MTT assay results revealed that plasma treatment markedly decreased cell viability (55% relative to untreated controls). This reduction in viability was rescued by treatment of NAC prior to plasma exposure (Fig. 2A). Next, we investigated whether cells would undergo apoptosis following plasma exposure using the nuclear staining dye Hoechst 33342. As shown in Fig. 2B,

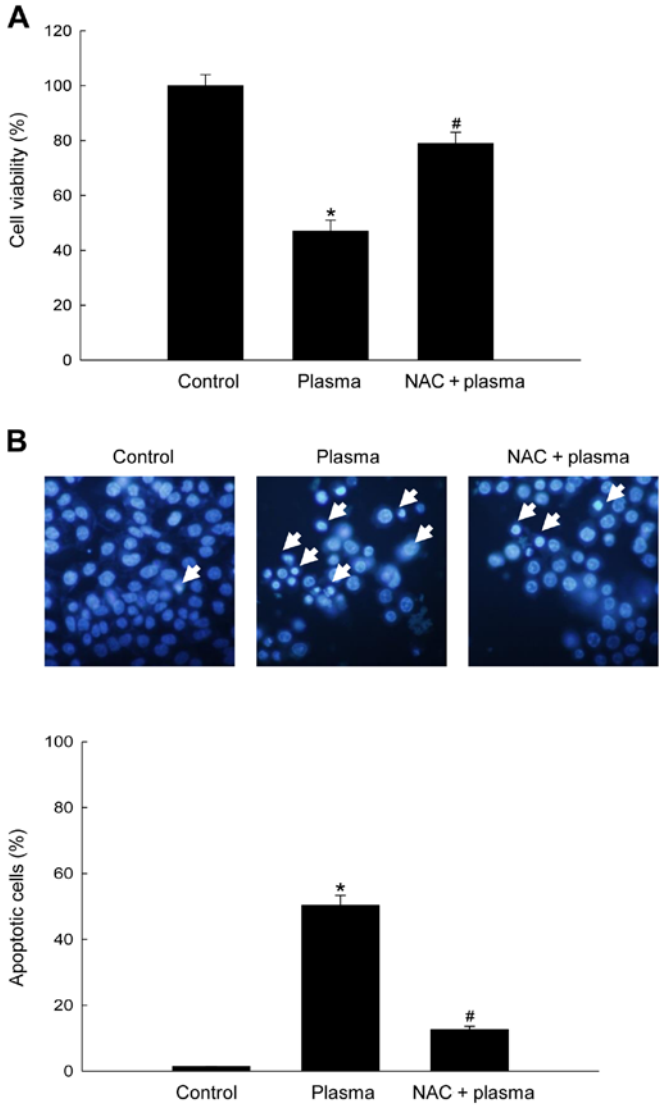

Figure 2. Effect of NTGP on apoptosis. (A) SNUC5 cells were plated in 60-mm cell culture dishes in 11 ml of media at a density of $2x10^5$ cells/ml. With the lids removed from the dishes, cells were exposed to plasma for 2 min. Cells exposed to plasma were transferred to 24-well plates at a density of $1x10^5$ cells/well. Cell viability of control, plasma-treated, and NAC-pre-treated/plasma-treated cells were measured by MTT assay. (B) Cells were transferred into a 24-well plate followed by plasma exposure and incubated at 37˚C for 24 h. The DNA-specific fluorescent dye Hoechst 33342 was added to each well, and cells were incubated for 10 min at 37˚C. Stained cells were visualized by fluorescence microscopy, and apoptotic bodies were counted in randomly selected areas. *Significantly different from control cells ($p<0.05$); #significantly different from plasma-exposed cells ($p<0.05$). NTGP, non-thermal gas plasma; NAC, *N*-acetylcysteine.

>50% of plasma-exposed cells underwent apoptosis, as indicated by the formation of apoptotic bodies. Again, NAC pre-treatment diminished the formation of apoptotic bodies. These results suggest that NTGP induces apoptosis via ROS generation in SNUC5 cells.

Plasma-induced apoptosis in SNUC5 cells is mediated by ER stress. Overwhelming ROS levels cause ER stress (16). Therefore, we next investigated whether plasma exposure can induce ER stress. To this end, plasma-exposed cells were stained with ER-Tracker™ Blue-White DPX dye, and were observed by confocal microscopy. Under these conditions, plasma-exposed cells stained bright blue, indicative of ER stress (Fig. 3A); as with the loss of viability and apoptosis described above, pre-treatment with NAC prior to plasma exposure decreased the brightness of staining, demonstrating that ER stress in plasma-treated cells arises due to high levels of ROS.

To confirm the induction of ER stress upon plasma exposure, we analyzed ER stress-related proteins. The UPR is a suite of signaling pathways that cells activate to restore cellular homeostasis once ER stress has occurred. Cells that are unable to achieve homeostasis undergo apoptosis (22). The ER-resident transmembrane receptors PERK (pancreatic eIF2-α kinase or protein kinase R (PKR)-like ER kinase), inositol-requiring enzyme 1 (IRE1), and ATF6 are maintained in their inactive forms through association with GRP78, which dissociates upon ER stress. Upon GRP78 dissociation, the UPR receptors are activated by phosphorylation (15). Western blotting revealed that the expression of GRP78, which is itself a transcriptional target of the UPR, increased dramatically following plasma treatment (Fig. 3B). Furthermore, levels of p-IRE1 and p-PERK were significantly elevated following plasma exposure, suggesting that plasma exposure induces the UPR in SNUC5 cells. In accordance with the data presented above, NAC pre-treatment attenuated upregulation of GRP78 and phosphorylation of both IRE1 and PERK. Activated PERK phosphorylates eIF2α, leading to inhibition of general protein translation, a hallmark of the UPR (23). Plasma exposure increased the level of p-eIF2α, confirming the activation of the UPR; as with the other indicators of UPR activity, the p-eIF2α level decreased in NAC-pre-treated cells (Fig. 3B).

Next, we investigated whether the UPR restores ER function or initiates apoptosis in SNUC5 cells. After non-conventional splicing of the XBP1 mRNA by IRE1, XBP1 protein translocates to the nucleus, where it induces transcription of genes involved in protein degradation and inhibition of the PERK-mediated translational block (24). At this stage, if the UPR has restored homeostasis, the cell will survive; otherwise, it will be driven to apoptosis. Expression of CCAAT/enhancer-binding protein homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is activated by the UPR; CHOP downregulates the anti-apoptotic mitochondrial protein Bcl-2 (25), fostering a pro-apoptotic environment and stimulating the mitochondria to release cytochrome *c* and activate caspase-3. Our data revealed that plasma exposure increased the expression of both XBP1 and CHOP, indicating that ER stress exerted by NTGP causes apoptosis in SNUC5 cells. All of the aforementioned effects were suppressed by the pre-treatment with NAC prior to plasma exposure.

Mitochondrial Ca^{2+} overloading is involved in plasma-mediated ER stress. ER stress is characterized by various molecular markers; for example, depletion of Ca^{2+} from the ER and accumulation of Ca^{2+} in mitochondria are hallmarks of severe ER stress (26). Therefore, we detected mitochondrial Ca^{2+} overload in plasma-exposed cells using Rhod-2 AM fluorescent dye. Flow cytometry revealed a significant increase in the mitochondrial Ca^{2+} level in plasma-treated cells (FI: 148) relative to that in controls (FI: 97) (Fig. 4A), which was attenuated (FI: 128) by pre-treatment with NAC

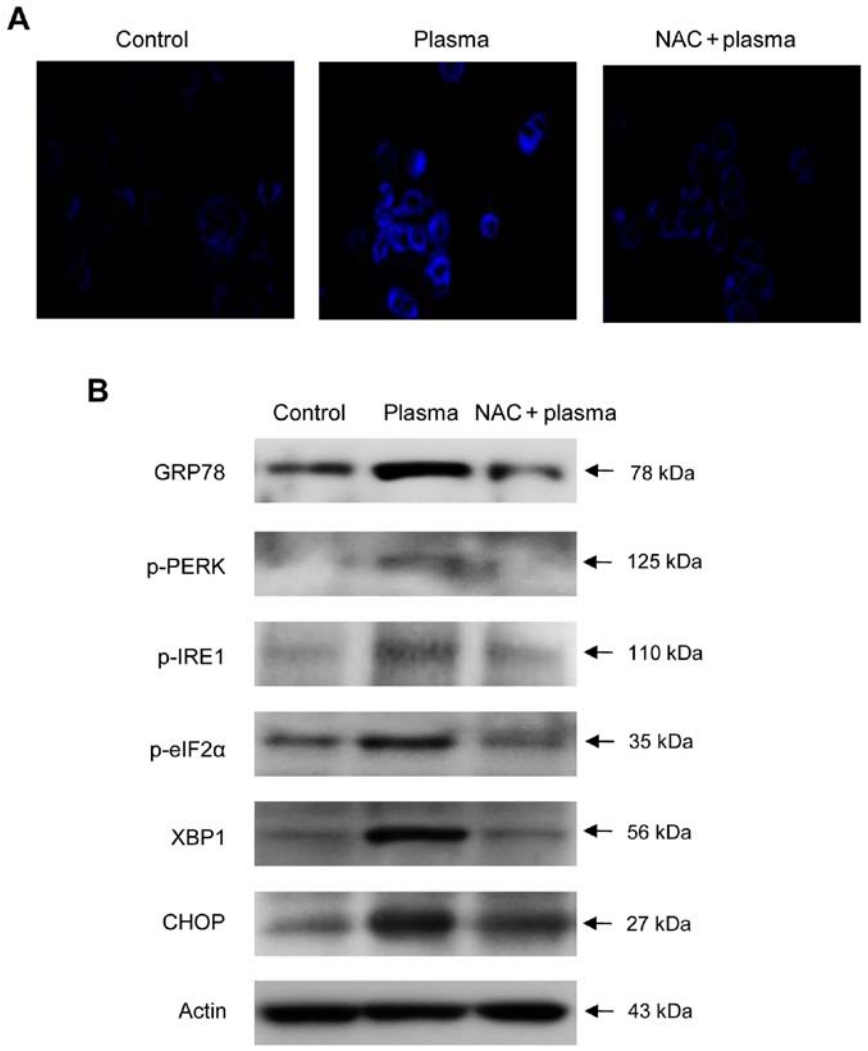

Figure 3. NTGP induces ER stress. (A) Cells were exposed to plasma and incubated for 24 h at 37˚C, and then ER-Tracker™ Blue-White DPX fluorescence staining dye was added and ER stress was analyzed by confocal microscopy. (B) SNUC5 cells were exposed to plasma and incubated for 24 h at 37˚C. Cells were harvested, and the proteins were extracted. Expression levels of GRP78, p-PERK, p-IRE1, p-eIF2α, XBP1, and CHOP proteins were evaluated using western blotting. Actin, a housekeeping protein, was used to confirm equal protein loading. NTGP, non-thermal gas plasma; ER, endoplasmic reticulum; GRP78, glucose-related protein 78; p-PERK, phosphorylated protein kinase R (PKR)-like ER kinase; p-IRE1, phosphorylated inositol-requiring enzyme 1; p-eIF2α, phosphorylated eukaryotic initiation factor 2α; XBP1, X-box binding protein 1; CHOP, CCAAT/enhancer-binding protein homologous protein.

before plasma exposure. We also monitored mitochondrial Ca^{2+} overload by confocal microscopy in cells stained with Rhod-2 AM. As expected, the confocal microscopy results (Fig. 4B) were consistent with the flow cytometry data. These findings suggest that plasma treatment triggers ER stress and mitochondrial Ca^{2+} accumulation, which in turn promotes apoptosis, in SNUC5 colon carcinoma cells. Because NAC pre-treatment could ameliorate these effects, it is likely that the ER stress is induced by excess ROS.

Discussion

As a widespread type of cancer, colorectal cancer represents a serious health threat throughout the world. Chemoresistance and side-effects of prolonged chemical treatments are substantial challenges for the treatment of colorectal cancer (5). Therefore, novel non-chemical strategies for treating colorectal cancer are urgently needed. NTGP has been characterized in various clinical applications as a promising tool for wound healing (27), plasma sterilization (28), blood coagulation (29), cell detachment (30), induction of apoptosis (31), and cancer therapy (32). Therefore, we investigated whether NTGP induces apoptosis in SNUC5 human colon carcinoma cells by generating ER stress.

For this study, we exposed cells to NTGP for 2 min using a non-thermal DBD plasma source. First, we investigated whether NTGP induces ROS. To this end, we stained plasma-exposed cells with DCFH-DA and examined them by flow cytometry and confocal microscopy (Fig. 1A and B). Data obtained by both methods indicated that plasma treatment significantly increased intracellular ROS generation. Furthermore, NTGP decreased cell viability compared to the controls (Fig. 2A) by inducing apoptosis (Fig. 2B). Pre-treatment with NAC attenuated these and all other consequences of plasma treatment, strongly indicating that cell death and other effects of plasma exposure are mediated by ROS.

Overwhelming ROS levels can trigger ER stress. Staining of cells with ER-Tracker™ Blue-White DPX dye confirmed

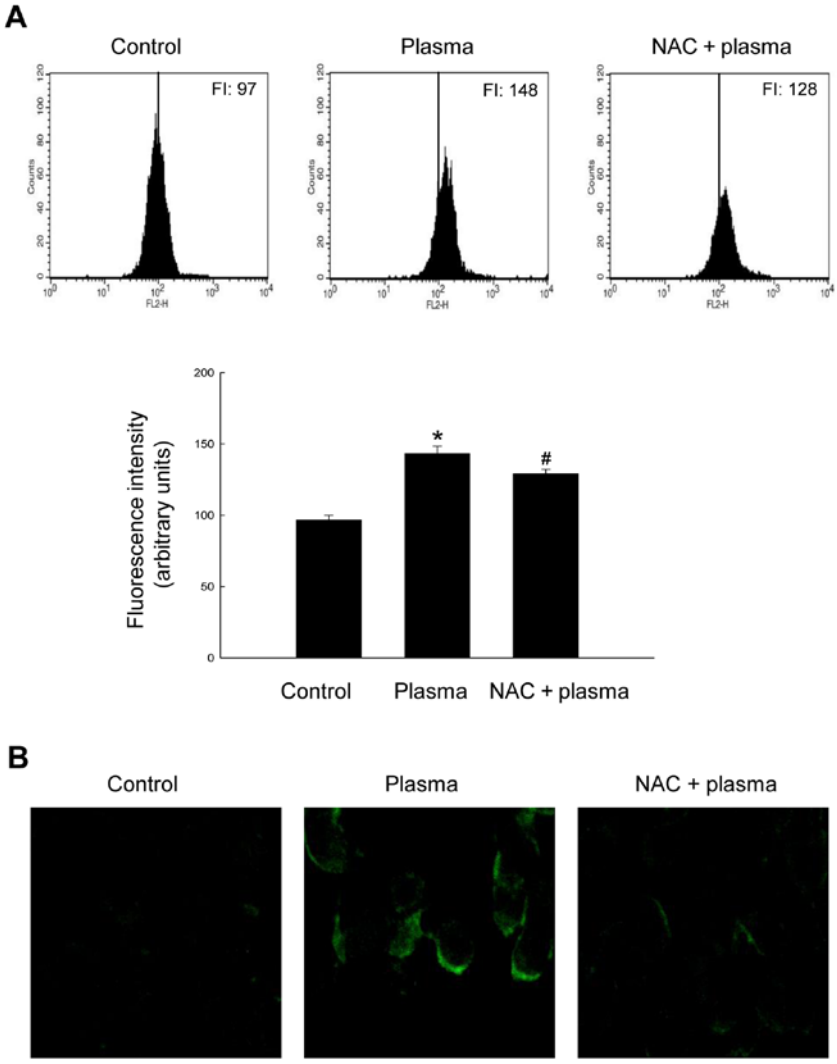

Figure 4. Influence of NTGP on mitochondrial Ca^{2+} overload. Control, plasma-treated, and NAC-pre-treated/plasma-treated cells were stained with Rhod-2 AM fluorescent dye, and mitochondrial Ca^{2+} overload was analyzed by (A) flow cytometry and (B) confocal microscopy. *Significantly different from control cells ($p<0.05$); #significantly different from plasma-exposed cells ($p<0.05$). NTGP, non-thermal gas plasma; NAC, *N*-acetylcysteine.

that plasma treatment induced ER stress (Fig. 3A). A mean of combating the detrimental effects of ER-stressed cells is the UPR, whose basic purpose is to halt protein synthesis and accumulation of misfolded proteins until proper ER function can be restored. If the UPR cannot restore cellular homeostasis, it triggers apoptosis (16). Under normal conditions, GRP78 is bound to the luminal domains of PERK and IRE1, maintaining them in their inactive states. Upon ER stress, GRP78 dissociates from the receptors, allowing them to be phosphorylated (33). Once activated, the UPR halts protein synthesis and upregulates production of ER-resident chaperones that promote protein folding and help the cell recover from stress. Plasma treatment increased the levels of GRP78, p-PERK, and p-IRE1, and also activated PERK-mediated phosphorylation of p-eIF2α, which imposes a translational block on ER protein synthesis (33) (Fig. 3B). These results confirmed that plasma exposure activates key proteins involved in the UPR.

Persistent ER stress inhibits PERK and halts the UPR, ultimately resulting in apoptosis (34). Cleaved ATF6 translocates to the nucleus and initiates transcription of XBP1 and CHOP. XBP1 mRNA is unconventionally spliced by p-IRE1, enabling translation of XBP1 protein (34), which translocates to the nucleus and induces the expression of P58IPK, which inhibits p-PERK and initiates protein degradation (23). Plasma exposure increased the levels of both XBP1 and CHOP (Fig. 3B). CHOP downregulates the anti-apoptotic mitochondrial protein Bcl-2, increasing mitochondrial membrane permeability and releasing cytochrome *c* into the cytoplasm to trigger apoptosis (35). Ca^{2+} accumulation in the mitochondria is a marker of the early and late stages of apoptosis (26). Because Bcl-2 expression is blocked under severe ER stress by activation of CHOP, Ca^{2+} can leak from the ER into the cytoplasm and mitochondria. Thus, mitochondrial Ca^{2+} overload is a hallmark of intensive ER stress (26). Consistent with this, plasma exposure strongly increased the mitochondrial Ca^{2+} level in SNUC5 cells (Fig. 4A and B).

These data confirm our hypothesis that NTGP induces apoptosis in SNUC5 human colon carcinoma cells via induction of ER stress. The attenuation of the UPR and related phenomena by NAC pre-treatment demonstrates that the effects of plasma exposure are mediated by generation of ROS.

Acknowledgements

This study was supported by the R&D Program of Plasma Advanced Technology for Agriculture and Food (Plasma Farming) through the National Fusion Research Institute (NFRI) of Korea funded by the Government.

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