

Effects of nitrogen on the apoptosis of and changes in gene expression in human lymphoma U937 cells exposed to argon-based cold atmospheric pressure plasma

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Abstract. Cold atmospheric pressure plasma (CAP) is known as a source of biologically active agents, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). In the present study, we examined the effects of nitrogen (N₂) on the apoptosis of and changes in gene expression in human lymphoma U937 cells exposed to argon (Ar)-CAP. Enormous amounts of hydroxyl (OH) radicals in aqueous solution

were produced using Ar-CAP generated using a 20 kHz low frequency at 18 kV with a flow rate of 2 l/min. The increase in the levels of OH radicals was significantly attenuated by the addition of N₂ to Ar gas. On the other hand, the level of total nitrate/nitrite in the supernatant was significantly elevated in the Ar + N₂-CAP-exposed U937 cells. When the cells were exposed to Ar-CAP, a significant increase in apoptosis was observed, whereas apoptosis was markedly decreased in the cells exposed to Ar + N₂-CAP. Microarray and pathway analyses revealed that a newly identified gene network containing a number of heat shock proteins (HSPs), anti-apoptotic genes, was mainly associated with the biological function of the prevention of apoptosis. Quantitative PCR revealed that the expression levels of HSPs were significantly elevated in the cells exposed to Ar + N₂-CAP than those exposed to Ar-CAP. These results indicate that N₂ gas in Ar-CAP modifies the ratio of ROS to RNS, and suppresses the apoptosis induced by Ar-CAP. The modulation of gaseous conditions in CAP may thus prove to be useful for future clinical applications, such as for switching from a sterilizing mode to cytotoxic effect for cancer cells.

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Abbreviations: ADM, adrenomedullin; AHR, aryl hydrocarbon receptor; ANXA1, annexin A1; ATF3, activating transcription factor 3; BAG3, BCL2-associated athanogene 3; BCL6, B-cell CLL/lymphoma 6; bZIP, basic-region leucine zipper; CAP, cold atmospheric plasma; DNAJB1, DnaJ (HSP40) homolog, subfamily B, member 1; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; ERG1, early growth response 1; FITC, fluorescein isothiocyanate; FOS, FBJ murine osteosarcoma viral oncogene homolog; FTH1, ferritin, heavy polypeptide 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMOX1, heme oxygenase (decycling) 1; HSF1, heat shock transcription factor 1; HSPs, heat shock proteins; HSPA1A/B, heat shock 70 kDa protein 1A/B; HSPA6, heat shock 70 kDa protein 6; ID2, inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; JUN, jun proto-oncogene; JUND, jun D proto-oncogene; KLF, Kruppel-like factor; MXD1, MAX dimerization protein 1; NR4A2, nuclear receptor subfamily 4 group A member 2; PDCD4, programmed cell death 4; PTGS2, prostaglandin-endoperoxide synthase 2; qPCR, quantitative polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDs, standard deviations; SGK1, serum/glucocorticoid regulated kinase 1; TSC22D3, TSC22 domain family member 3; VIM, vimentin

Key words: cold atmospheric pressure plasma, human lymphoma U937 cell, apoptosis, gene network

Introduction

The therapeutic use of cold atmospheric plasma (CAP) constitutes an emerging interdisciplinary field that capitalizes on the rapidly evolving technology of low-temperature plasma (1-4). Plasma is an at least partially ionized gas and is defined as the fourth state of matter. It is considered to be a mixture of electrons, negative and positive ions, excited gas species, free radicals and electromagnetic radiation. The properties of CAP can be modified by changing various experimental conditions, such as the types of set-ups, the voltage applied, the type of feed gas and the gas flow rate (3-6). It has been demonstrated that CAP is useful in potential applications, such as sterilization (7), wound healing (8), dentistry (9) and tissue regeneration (10). In addition, a recent medical investigation focused on applying CAP to the treatment of cancer (11).

CAP has been reported to effectively suppress cancer cell growth in *in vivo* experimental models (12-14). There is also

growing evidence that the exposure of cancer cells to CAP or CAP-activated medium induces apoptosis, and reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are considered to be effective agents for CAP-induced apoptosis (12–22). In a previous study, CAP produced dose-dependent effects, ranging from increased cell proliferation to apoptosis; these effects were the result of the production of ROS (15). Air-CAP generates a variety of ROS and RNS, and these radicals induce an apoptotic signaling cascade by inducing mitogen-activated protein kinase (MAPK) signaling in cancer cells (17). Adachi *et al.* indicated that hydrogen peroxide (H₂O₂) and other reactive agents in plasma-activated medium induce cell injury via the mitochondria-nuclear apoptotic cascade in cancer cells (21). More recently, we demonstrated that much higher levels of hydroxyl (OH) radicals were produced using argon (Ar)-CAP in an aqueous solution than by using X-irradiation, based on the analysis and quantification of electron paramagnetic resonance (EPR) spectra. Under this condition, Ar-CAP produced intracellular ROS, OH radicals, H₂O₂, which is the recombination product of OH, and hypochlorite ion (OCl⁻) in human lymphoma U937 cells (22). Although the generation of ROS induced by CAP and the biological effects of the thus generated ROS have been clarified gradually, the mechanisms of CAP-mediated RNS-cell interactions are not yet fully understood. In this study, we investigated the additional effects of nitrogen (N₂) in Ar gas on the apoptosis of and changes in gene expression in U937 cells exposed to CAP.

Materials and methods

Cell culture. Human myelomonocytic lymphoma U937 cells were obtained from the Human Science Research Resources Bank of the Japan Health Sciences Foundation (Tokyo, Japan). The cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum, and maintained at 37°C in humidified air with 5% CO₂ and 95% air.

Exposure to CAP. CAP was produced using our previously described method (22). The inert gases, Ar and N₂, were purchased from Hokusan Co., Ltd. (Toyama, Japan). All gases were of pure grade (≥99.9%). The U937 cells in a well of a 24-well plate were exposed to Ar-CAP or Ar containing 2.5% of N₂ (Ar + N₂-CAP) generated using a 20 kHz low frequency at 18 kV with a flow rate of 2 l/min for 0 to 3 min at room temperature. Following exposure to CAP, the cells were cultured for 0–18 h at 37°C in a CO₂ incubator. Non-treated cells served as controls.

Optical emissions from CAP. Optical emissions from Ar-CAP were collected using an optic fiber and a lens directed to a position at 8 mm ahead of CAP. To examine the effects of N₂ on the optical emissions of Ar-CAP, N₂ was added to the Ar gas at flow rates from 0 to 50 (standard cubic centimeter per minute). The emission was observed using a spectrometer (Shamrock SR-561-B1) and an intensified charge-coupled-device camera (iStar DH734-25F-03) (both from Andor Technology, Ltd., Belfast, UK), as described in a previous study of ours (22).

EPR-spin trapping for the detection of hydroxyl radicals. The detection of OH radicals following exposure to Ar-CAP or Ar + N₂-CAP was carried out using the EPR-spin trapping with

5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Labotec Co., Ltd., Tokyo, Japan). An aqueous solution containing a spin trap at a concentration of 10 mM was exposed to CAP for up to 120 sec. Immediately following exposure, a sample was transferred to a glass capillary tube (VC-HO75P; Terumo, Tokyo, Japan) which was then inserted into a special quartz tube in the cavity of an EPR spectrometer (RFR-30; Radical Research Inc., Tokyo, Japan). The EPR settings were as follows: microwave power, 4 mW; frequency, 9.425 GHz; center magnetic field, 329.5 mT; and modulation width, 0.1 mT. The yields of spin adducts were determined using the stable nitroxide radical 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrroline-1-oxide as a standard at room temperature. The peak heights of the EPR signals were expressed in relative units compared with those of the Mn²⁺ internal standard, with one unit being equivalent to approximately 7.7 × 10⁶ M nitroxide radicals (22).

Measurement of nitrite (NO₂)/nitrate (NO₃). The U937 cells were exposed to Ar-CAP or Ar + N₂-CAP for 3 min and then the supernatant of the cells was collected by centrifugation. The concentration of total NO₂/NO₃ in the supernatant of the cells was measured using an NO₂/NO₃ assay kit-C II (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Measurements of cell viability and apoptosis. For measuring cell viability, we used a water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2H-tetrazolium, monosodium salt]-based assay (Cell Counting kit-8; Dojindo Laboratories). In brief, the cells were incubated in 110 µl RPMI-1640 medium containing 9.1% (v/v) of WST-8 reagent in a 96-well cell culture plate at 37°C. Two hours later, the produced formazan dye concentration was determined from the absorbance at 450 nm using a microplate reader (23). The level of apoptosis was determined using an Annexin V-FITC kit (Immunotech, Marseille, France). Fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide were added to the cell suspension. Following incubation for 20 min in the dark, the cells were analyzed using a flow cytometer (Epics XL; Beckman Coulter K.K., Tokyo, Japan). Apoptosis was expressed as the sum of early apoptotic and secondary necrotic fractions.

RNA isolation. For global-scale gene expression and quantitative polymerase chain reaction (qPCR) analyses, total RNA was extracted from the cells using a NucleoSpin® RNA isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany) along with on-column DNase I treatment. RNA quality was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA).

Global-scale gene expression analysis. Global-scale gene expression analysis was carried out using a GeneChip® microarray system with a Human Genome U133-plus 2.0 array, which was spotted with 54,675 probe sets (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The obtained hybridization intensity data were analyzed using GeneSpring® GX (Agilent Technologies, Inc.) to extract the significant genes. To examine gene ontology, including biological processes, cellular components, molecular functions

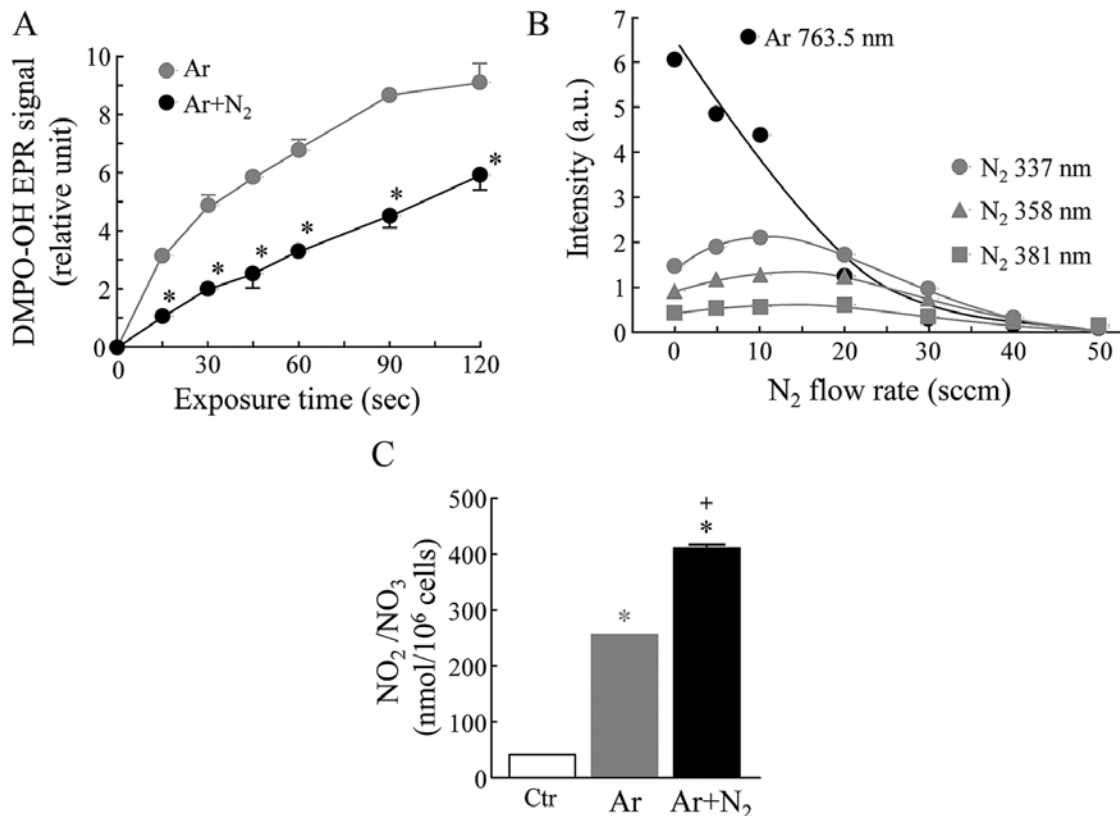


Figure 1. (A) Hydroxyl radical formation induced by cold atmospheric plasma (CAP). Argon (Ar)-CAP or Ar + Nitrogen (N₂) (2.5%)-CAP generated by using a 20 kHz low frequency at 18 kV with a flow rate of 2 l/min was applied to aqueous solutions for 0 to 120 sec. Electron paramagnetic resonance (EPR)-spin trapping experiments were performed with DMPO as a spin trap for detection of [•]OH in aqueous solution. Data are presented as the means \pm SDs (n=3). *P<0.05 vs. each group of Ar-treated cells (Student's t-test). (B) The effects of N₂ on the intensity of optical emissions generated by Ar-CAP. N₂ at flow rates from 0 to 50 [standard cubic centimeter per minute (sccm)] were added to Ar. Ar 763.5 nm, emission spectra from excited states of Ar; N₂ 337, 358 and 381 nm, emission spectra from excited N₂. (C) Nitrite/nitrate formation induced by Ar-CAP or Ar + N₂-CAP. The supernatant was collected from the U937 cells exposed to Ar-CAP (Ar) or Ar + N₂ (2.5%)-CAP (Ar + N₂) for 3 min. The concentration of total NO₂/NO₃ was measured by using a commercial colorimetric assay kit. Non-treated cells served as controls (Ctrl). Data are presented as the means \pm SDs (n=3). *P<0.05 vs. Ctrl; +P<0.05 vs. Ar-CAP-exposed cells (Student's t-test).

and gene networks, the obtained data were analyzed using Ingenuity® Pathway Analysis tools (Ingenuity Systems Inc., Mountain View, CA, USA), as previously described (23,24).

Measurement of mRNA levels by qPCR. The mRNA levels in the cells were determined following exposure to CAP for 2 min followed by culture at 37°C for 3 h using an Mx3005P real-time PCR system (Agilent Technologies, Inc.) with using SYBR Premix Ex Taq or Premix Ex Taq (for the use of TaqMan probes) (both from Takara Bio Inc., Shiga, Japan). The specific primers and probes for BCL2-associated athanogene 3 (BAG3), DnaJ [heat shock protein (HSP)40] homolog, subfamily B, member 1 (DNAJB1), early growth response 1 (ERG1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), heme oxygenase (decycling) 1 (HMOX1), heat shock 70 kDa protein 1A/B (HSPA1A/B) and heat shock 70 kDa protein 6 (HSPA6 or HSP70B') were designed based on the database. GAPDH was used as a control for normalization, as previously described (24-26).

Statistical analysis. Data are presented as the means \pm standard deviations (SDs). Differences between pairs of data sets were analyzed using Student's t-test, with values of P<0.05 considered to indicate statistically significant differences.

Results

Hydroxyl radical and NO₂/NO₃ formations induced by exposure to CAP. EPR-spin trapping experiments were carried out with DMPO as a spin trap to detect [•]OH radicals in aqueous solution exposed to Ar-CAP or Ar + N₂ (2.5%)-CAP generated by using a 20 kHz low frequency at 18 kV with a flow rate of 2 l/min at room temperature. The formations of [•]OH radicals as measured by the amount of DMPO-OH adducts were markedly increased in the aqueous DMPO solutions exposed to Ar-CAP in an exposure time-dependent manner. When the aqueous DMPO solutions were exposed to Ar + N₂-CAP, the levels of DMPO-OH adducts were significantly reduced to approximately 50% of the levels induced by Ar-CAP (Fig. 1A). The effects of N₂ on the intensity of optical emissions generated by Ar-CAP were also investigated. In our previous study, we already confirmed that the emission lines and bands from excited Ar and N₂ were mainly observed at 696.5-852.1 and 316.0-416.0 nm, respectively (22). The intensity of emission spectra from excited Ar at 763.5 nm was readily decreased depending on the increase in the amount of N₂ in Ar gas. On the other hand, a slight and transient increase in the intensity of emission spectra from excited N₂ at 337.0, 358.0 and 381 nm was observed in the N₂-exposed samples (Fig. 1B). A much

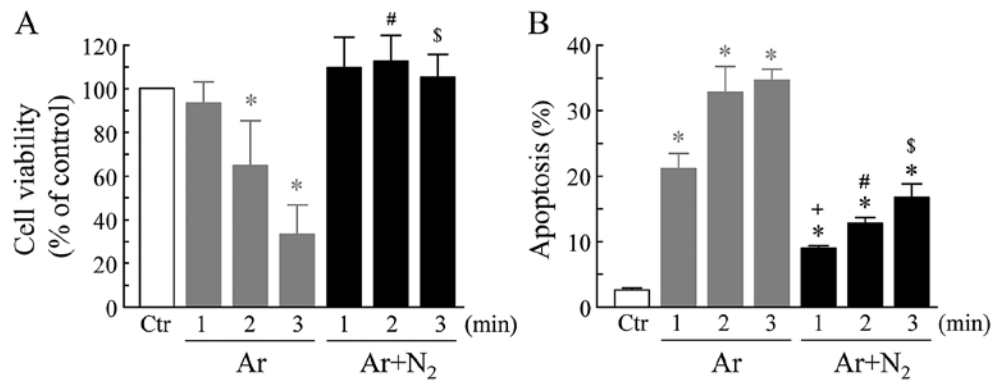


Figure 2. Effects of argon cold atmospheric plasma (Ar-CAP) and Ar + Nitrogen (N₂)-CAP on (A) cell viability and (B) apoptosis. The cells were exposed to Ar-CAP (Ar) or Ar + N₂ (2.5%)-CAP (Ar + N₂) for 1-3 min. Eighteen or six hours later, (A) cell viability or (B) apoptosis were measured using a cell counting kit-8 or an Annexin V-fluorescein isothiocyanate (FITC) kit, respectively. Non-treated cells served as controls (Ctr). Ctr=100%. Data are presented as the means \pm SDs (n=3-4). *P<0.05 vs. Ctr; #P<0.05 vs. Ar for 1 min; \$P<0.05 vs. Ar for 2 min; *P<0.05 vs. Ar for 3 min (Student's t-test).

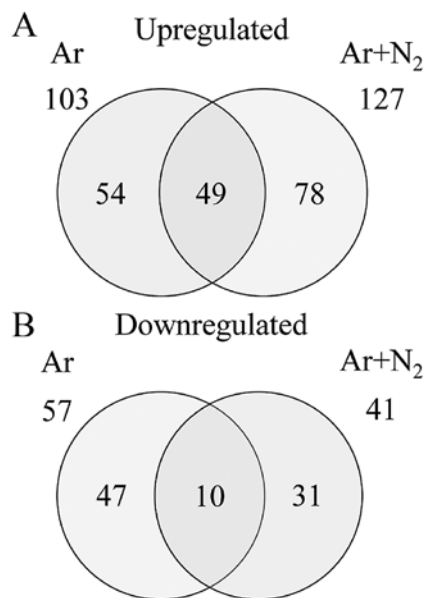


Figure 3. Venn diagram of genes that were differentially expressed. The cells were exposed to argon cold atmospheric plasma (Ar-CAP) (Ar) or Ar + Nitrogen (N₂) (2.5%)-CAP (Ar + N₂) for 2 min, followed by culture at 37°C for 3 h. Gene expression analysis of the probe sets that were upregulated and downregulated by a factor of ≥ 2.0 was conducted using GeneSpring® GX software. The diagram shows the number of specifically and commonly expressed genes affected by CAP. (A) Upregulated genes; (B) downregulated genes. The experiments were repeated twice.

greater amount of total NO₂/NO₃ (255.0 \pm 0.87 nmol/10⁶ cells, mean \pm SD) in the supernatant was detected in the human lymphoma U937 cells exposed to Ar-CAP compared to the control cells (41.7 \pm 0.49) (Fig. 1C). Moreover, a further elevation in the amount of total NO₂/NO₃ (410.3 \pm 4.0 nmol/10⁶ cells, mean \pm SD) was observed in the Ar + N₂-CAP-exposed cells.

Effects of Ar- and Ar + N₂-CAP on cell viability and apoptosis. The viability or apoptosis of the cells following exposure to CAP for 1 to 3 min followed by culture at 37°C for 18 or 6 h, respectively, was investigated. As demonstrated in Fig. 2, Ar-CAP significantly decreased cell viability in an exposure-time dependent manner. By contrast, cell viability was not affected by the exposure of the cells to Ar + N₂-CAP (Fig. 2A).

A significant induction of apoptosis was observed in the cells exposed to Ar-CAP in an exposure-time dependent manner. The addition of N₂ to the Ar gas markedly suppressed Ar-CAP-induced apoptosis, and the percentage of suppression was approximately 50% (Fig. 2B).

Gene expression analysis. The gene expression patterns in the cells following exposure to CAP for 2 min followed by culture at 37°C for 3 h was monitored using a GeneChip® microarray system. The complete lists of probe sets from all samples are deposited at the Gene Expression Omnibus, a public data base (accession no. GSE76022). Gene expression analysis using GeneSpring® software revealed that a number of genes were differentially expressed by a factor of ≥ 2.0 between the cells exposed to Ar- or Ar + N₂-CAP and the control cells. The numbers of genes expressed in either group or commonly in both groups are shown in the Venn diagram in Fig. 3. The total numbers of genes that were found to be differentially expressed were 160 (103 up- and 57 downregulated genes) and 168 (127 up- and 41 downregulated genes) in the Ar- and Ar + N₂-CAP groups, respectively. In addition, the numbers of commonly up- and downregulated genes were 49 and 10, respectively (Fig. 3).

Identification of gene networks associated with apoptosis. To identify gene networks associated with CAP-induced apoptosis, functional category and pathway analyses were conducted by using Ingenuity® Pathway Analysis tools. A number of functionally annotated genes were identified among both the upregulated and downregulated genes of the Ar- and Ar + N₂-CAP groups. We identified two gene networks, designated as the pro-apoptosis gene network and the anti-apoptosis gene network, in the functionally annotated and upregulated genes of the Ar-CAP- and Ar + N₂-CAP-exposed groups, and showed that these were mainly associated with the biological functions of the induction and prevention of apoptosis, respectively. The pro-apoptosis gene network included 8 genes, namely Annexin A1 (ANXA1), activating transcription factor 3 (ATF3), FBJ murine osteosarcoma viral oncogene homolog (FOS), inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2), jun proto-oncogene (JUN), Kruppel-like factor 4 (KLF4), programmed

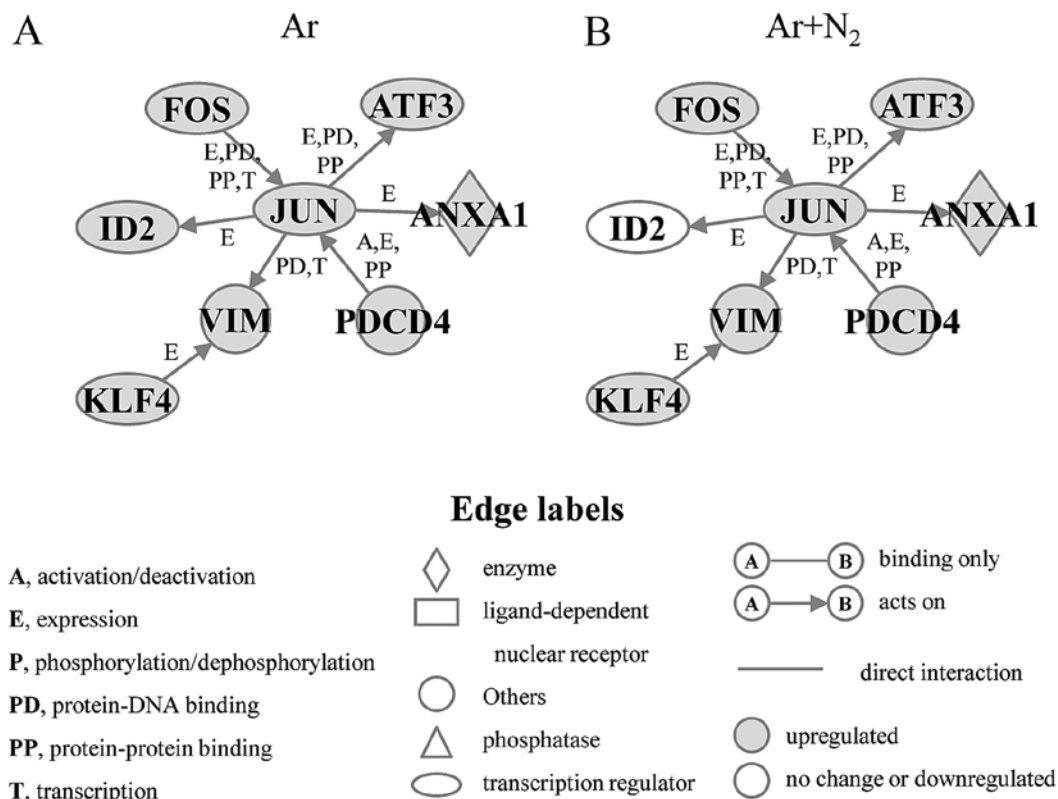


Figure 4. The pro-apoptosis gene network. Upregulated genes in argon cold atmospheric plasma (Ar-CAP)-exposed cells were analyzed by Ingenuity® Pathways Analysis tools. The expression levels of genes in Ar- or Ar + Nitrogen (N₂)-CAP (Ar + N₂)-exposed cells are shown in the (A) gene network Ar or (B) gene network Ar + N₂, respectively. In the graphical representation of the networks, nodes refer to genes and edges refer to the biological relationships between nodes. The node color indicates the expression level of genes. Nodes and edges are displayed using various shapes and labels reflecting the functional class of each gene and the nature of the relationships involved, respectively.

cell death 4 (PDCD4), and vimentin (VIM). The expression levels of 7 of these 8 genes, with ID2 being the exception, were increased under both the Ar-CAP- and Ar + N₂-CAP exposure conditions. A significant elevation in the ID2 levels was only observed in the cells exposed to Ar-CAP (Fig. 4). The anti-apoptosis gene network contained 17 genes, including HSPs, DNAJB1, HMOX1, HSPA1A/B and HSPA6, and adrenomedullin (ADM), aryl hydrocarbon receptor (AHR), BAG3, B-cell CLL/lymphoma 6 (BCL6), EGR1, ferritin, heavy polypeptide 1 (FTH1), jun D proto-oncogene (JUND), KLF2, MAX dimerization protein 1 (MXD1), nuclear receptor subfamily 4 group A member 2 (NR4A2), prostaglandin-endoperoxide synthase 2 (PTGS2), serum/glucocorticoid regulated kinase 1 (SGK1) and TSC22 domain family member 3 (TSC22D3). In this network, the expression levels of 7 genes, AHR, BCL6, FTH1, JUND, MXD1, NR4A2 and TSC22D3, were significantly lower in the Ar-CAP-exposed cells than in the Ar + N₂-CAP-exposed cells. However, the other 10 genes remained significantly elevated under both conditions (Fig. 5).

Effects of Ar- and Ar + N₂-CAP on gene expression, as shown by qPCR. The mRNA levels in the cells following exposure to CAP for 2 min followed by culture at 37°C for 3 h was monitored by using real-time qPCR. We selected 6 genes, BAG3, DNAJB1, EGR1, HMOX1, HSPA1A/B and HSPA6, from the anti-apoptosis gene network. The expression levels of these 6 genes were significantly elevated in the Ar-CAP-exposed cells

compared to the control cells (Fig. 6). Under the Ar + N₂-CAP exposure conditions, a further increase in the expression levels of these genes was observed in comparison with those under the Ar-CAP exposure conditions.

Discussion

CAP is well known as a source of biologically active agents, such as ROS and RNS (1-4), and its characteristics can be modified by altering the experimental conditions (3-6). In the present study, enormous amounts of 'OH radicals in aqueous solution were produced using an Ar-CAP-generating system as reported previously (22). Moreover, Ar gas modification by the addition of N₂ resulted in decreased levels of 'OH radicals in the aqueous solutions and increased levels of NO₂/NO₃ in the supernatant of human lymphoma U937 cells, indicating that N₂ gas in Ar-CAP modified the ratio of ROS to RNS. In the system used in this study and in our previous study, a certain amount of NO₂/NO₃ was detected even if Ar-CAP was used, suggesting that the effluent Ar gas mixed with atmospheric air in remote regions can influence the CAP characteristics (22).

There is accumulating experimental evidence that CAP is an effective agent to suppress cancer cell growth and to induce apoptosis under both *in vivo* and *in vitro* models, and CAP-produced ROS and/or RNS play a principal role in its anticancer effects (12-22). In general, relatively low levels of ROS are essential to maintain the physiological homeostasis of the cell. On the other hand, increased levels of ROS may

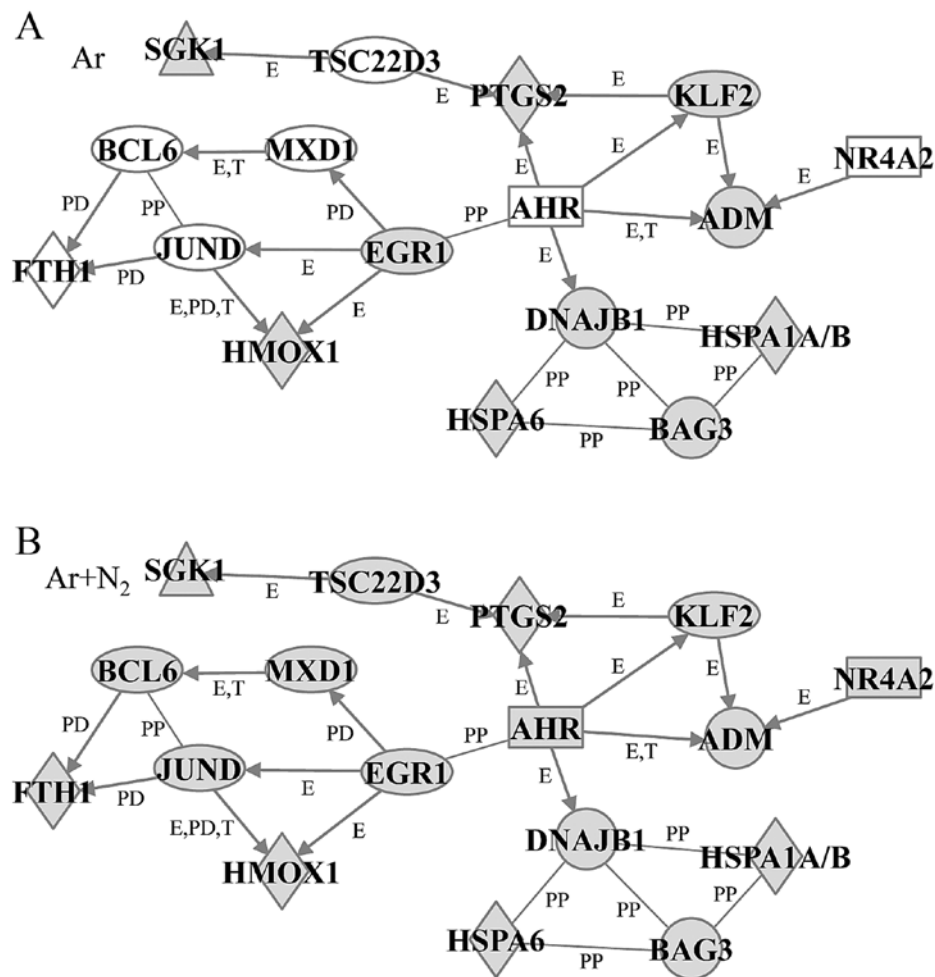


Figure 5. The anti-apoptosis gene network. Upregulated genes in Ar + Nitrogen (N₂) (2.5%)-CAP (Ar + N₂)-exposed cells were analyzed by Ingenuity® Pathways Analysis tools. The expression levels of genes in argon cold atmospheric plasma (Ar-CAP)- or Ar + N₂-exposed cells are shown in the (A) gene network Ar or (B) gene network Ar + N₂, respectively. For an explanation of the symbols and letters, see Fig. 4.

be detrimental and lead to cell death, including apoptosis (27). Kalghatgi *et al* (15) suggested that the dose-dependent effects of CAP, which range from increased cell proliferation to apoptosis, are related to the amount of ROS (15). In our previous studies using U937 cells, the degree of apoptosis was well associated with the degree of produced ROS under a variety of experimental conditions (28-30). In the present study, a marked induction of apoptosis was observed in the U937 cells exposed to Ar-CAP, whereas the addition of N₂ to the Ar gas significantly suppressed the Ar-CAP-induced increase in apoptosis. These results suggest that these inhibitory effects of N₂ may be due to a decrease in ROS and/or increase in RNS levels.

To elucidate the molecular mechanisms underlying CAP-induced apoptosis, gene expression patterns were investigated using a combination of high-density oligonucleotide microarray and computational gene expression analysis tools. In the present study, we identified a number of genes that exhibited a ≥ 2.0 -fold difference in expression between the cells exposed to Ar- or Ar + N₂-CAP and the control cells. In addition, by using Ingenuity® pathway analysis, we discovered a significant gene network, herein designated as the pro-apoptosis gene network, that was associated with the biological function of the induction of apoptosis. In this network, ANXA1 (31), ATF3 (32), FOS (33), JUN (34) and

KLF4 (35) have been reported to function as pro-apoptotic molecules in a wide variety of cell types, including cancer cells. JUN, ATF3 and FOS belong to the basic-region leucine zipper (bZIP) transcription factor family, and the homo- and heterodimeric bZIP protein complexes act as activators and suppressors of transcription. For example, the JUN and FOS proteins form the heterodimer of the activating protein-1 (AP-1) complex (36). These three transcription factors have been shown to be markedly upregulated in U937 cells undergoing heat stress-induced apoptosis (24). Previous findings have suggested that the overexpression of JUN in combination with FOS enhances the sensitivity of keratinocytes to apoptosis (37). In this network, almost all genes were upregulated under both the Ar- and Ar + N₂-CAP conditions, with the single exception being ID2, whose expression was observed only under the Ar-CAP conditions (Fig. 4). The elevation of gene expression in the pro-apoptosis gene network may have been closely associated with the induction of apoptosis by CAP in U937 cells.

In addition, we successfully identified the anti-apoptosis gene network, which was associated with the biological function of the prevention of apoptosis from upregulated genes in Ar + N₂-CAP-exposed cells (Fig. 5). In this network, 7 genes, including AHR (38), FTH1 (39) and NR4A2 (40), which have

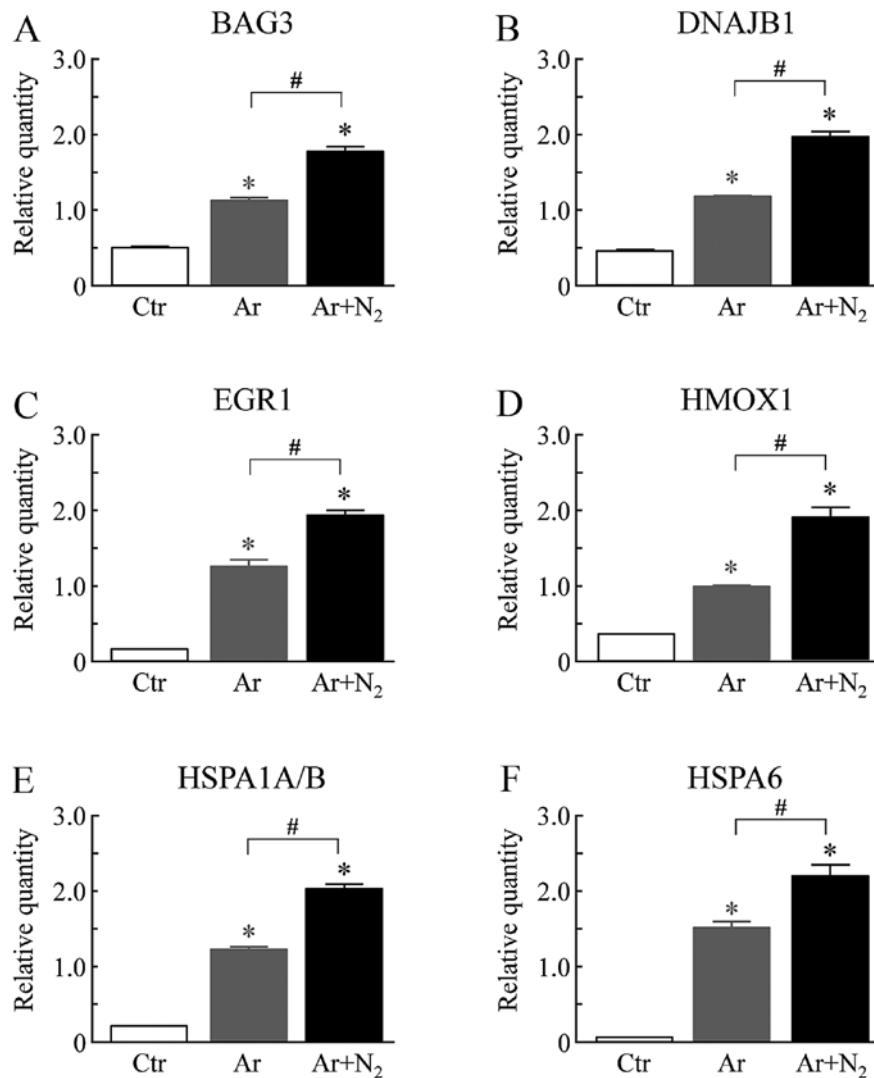


Figure 6. Effects of argon cold atmospheric plasma (Ar-CAP) and Ar + Nitrogen (N₂)-CAP on gene expression determined by qPCR. Following the exposure of the cells to Ar-CAP (Ar) or Ar + N₂ (2.5%)-CAP (Ar + N₂) for 2 min, the cells were cultured for 3 h at 37°C. qPCR was then performed. (A) BCL2-associated athanogene 3 (BAG3); (B) DnaJ (HSP40) homolog, subfamily B, member 1 (DNAJB1); (C) EGR1; (D) heme oxygenase (decycling) 1 (HMOX1); (E) heat shock 70 kDa protein 1A/B (HSPA1A/B); (F) heat shock 70 kDa protein 6 (HSPA6). Each expression level was normalized to the GAPDH expression level. Non-treated cells served as controls (Ctr). Data are presented as the means \pm SDs (n=4). *P<0.05 vs. Ctr; #P<0.05 vs. Ar-CAP-exposed cells (Student's t-test).

been reported to inhibit apoptosis, were expressed at lower levels under the Ar-CAP conditions compared to the Ar + N₂-CAP conditions. The downregulation of these genes may contribute to the ability to induce apoptosis induced by Ar-CAP. The anti-apoptosis gene network also contained several HSPs, DNAJB1, HMOX1, HSPA1A/B and HSPA6, and BAG3. Of note, qPCR clearly demonstrated that the expression levels of these genes were significantly higher under either the Ar- or Ar + N₂-CAP conditions than under the control conditions (Fig. 6). HSPs and BAG3, a co-chaperone of HSP70, are primarily regulated by heat shock transcription factor 1 (HSF1), and these proteins function as anti-apoptotic molecules against various types of stress, particularly heat (41,42). DNAJB1 (43), HMOX1 (44), HSPA1A/B (43), HSPA6 (45) and BAG3 (46) are known to participate in the prevention of apoptosis. Previous findings have indicated that very weak but nonetheless significant heat-inducible heat shock element-binding activity of HSF1 was observed when cells were incubated at 39°C for 20 min (47).

In the same CAP system as used herein and in our previous study, temperature increases of $1.7 \pm 0.2^\circ\text{C}$ (mean \pm SD) and $2.3 \pm 0.2^\circ\text{C}$ were observed following 2 and 5 min of exposure at room temperature, respectively (22). It is also known that HSF1 can be activated directly by oxidative stress (48). Therefore, we considered that the induction of HSP-related genes may have been due to the activation of HSF1 by ROS, rather than to an increase in temperature under both CAP conditions. In the present study, further elevations in the total amount of NO₂/NO₃ and the overexpression of HSP-related genes were detected in the cells exposed to Ar + N₂-CAP (Figs. 1C and 6). Previous studies have indicated that nitric oxide (NO) is generated by CAP with atmospheric N₂ and is then rapidly converted to other species including NOX (49). In previous studies, NO has been reported to induce Hsp70 expression and lead to cytoprotection in cells (50,51) and the induction of HSP70 expression by NO has shown to be regulated by HSF1 activation (52). Under our CAP conditions, the induction of HSPs via NO generation

may have participated in the suppression of Ar-CAP-induced apoptosis by N₂.

The findings of the present study provide insight toward the eventual elucidation of the molecular mechanisms underlying the CAP-induced apoptosis of cancer cells. The modulation of gaseous conditions in CAP may be useful for future clinical applications, such as when switching from a sterilizing mode to a cytotoxic effect for the treatment of cancer.

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