Cytoprotective Effects of Polyamines Against Oxidative Stress

Seoni Ahn[1], Ji Young Lee[2], Hae Young Chung[2,3], Mi-Ae Yoo[2,4], Jong-Min Kim[5] and Byeong Gee Kim[1,2]*

[1]Department of Biology, [2]Institute of Genetic Engineering, [3]Department of Pharmacy, [4]Department of Molecular Biology, Pusan National University, Busan, 609-735, [5]Department of Anatomy and Cell Biology, College of Medicine, Dong-A University, Busan, 602-714 Korea

The polyamines are essential components of all eukaryotic cells and absolutely necessary for cell growth. In the present study, the cytoprotective role of polyamine was characterized. When Ac_2F rat liver cells were treated with 1 mM 2,2'-azobis (2-amidinopropane) dehydrochloride (AAPH), a water soluble free radical initiator, viability of the cells was noticeably decreased due to the increase of reactive oxygen species (ROS). The cytotoxic effect of AAPH as well as ROS generation were significantly inhibited by the treatment of polyamines. Among polyamines, especially spermine at 20 µM concentration exerted over 45% inhibition of AAPH-induced ROS generation. Western blotting was performed to determine whether superoxide dismutase (SOD) or catalase (CAT) expression was involved in oxidative stress. The AAPH treatment blocked both SOD and CAT protein expressions. Spermine could recover those protein expressions to the untreated control levels. According to the result of cyclin E measurement, AAPH might block the entry of the cells into S phase of the cell cycle. The reduced expression of cyclin E protein could be fully recovered by the addition of spermine. The antioxidative effects of spermine was also further proved by the apopotitic morphological analysis using ethidium bromide and acridine orange.

Key words ─ antioxidant, apoptosis, polyamine, ROS, spermine

The polyamines including putrescine, spermidine and spermine are essential for cell growth and differentiation. Because of their polycationic nature and unique charge distribution, polyamines are also believed to be important to maintain the proper structure and stability of chromatin and protein[5]. Cell proliferation and high transformation induced by growth factor are characterized by increased polyamine biosynthesis and an enhanced uptake of polyamines. In general, rapidly growing cells or tumor cells have a higher level of polyamine than slowly growing, normal or quiescent cells. Polyamines have also been reported to be antioxidants and to be important for a DNA repair mechanism. Spermine, in particular, was recently reported to act as a potent antioxidant either by scavenging oxygen radicals[7] or through chelation of Fe^{2+} that catalyzes OH^- generation via Fenton reaction without the formation of toxic byproducts[14]. Lovaas *et al.*[8] suggested that spermine chelates transition metal inos, thus preventing the generation of ROS. However, spermine is able to block DNA strand breakage induced by radiation and O_2^-, processes which do not involve the transition metal ions. A decrease in polyamine levels, especially in spermidine and spermine, has been shown to be a common feature in apoptosis induced by a variety of stimuli. Apoptosis is a physiological cell death regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in their nuclei, including chromatin condensation and internucleosomal DNA fragmentation[18].

Aerobic organisms, which derive their energy by the reduction of oxygen, are susceptible to the damaging action of a small amount of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). They are inevitably formed during the metabolism of oxygen, especially in the reduction of oxygen by the electron transfer system of mitochondria[6]. These are referred to as reactive oxygen species (ROS). H_2O_2 is the substrate for two enzymes, catalase (CAT) and glutathion peroxidase (GPX), that catalyze the conversion of H_2O_2 to H_2O plus O_2, which is presumably a detoxification mechanism. The antioxidant enzyme system characterized by SOD, CAT and GPX enzymes is the primary defense system against highly reactive molecules called ROS. H_2O_2 is involved in damaging living systems because it can give rise to the formation of OH^- radicals. It is, therefore, biologically advantageous for the cell to control the amount of H_2O_2 that is allowed to accumulate. Exposure to

*Corresponding author
Tel : +82-51-510-2286, Fax : +82-51-581-2962
E-mail : bgkim@pnu.edu

elevated concentration of oxygen increases the intracellular prodution of O_2^- and H_2O_2. After exposure to hyperoxia, increases in the antioxidant enzymatic defense systems, such as SOD, CAT and GPX, have been characterized in cell culture systems. Furthermore, tolerance to O_2 injury in culture systems and in whole animals has been associated with increases in the activities of SOD, CAT and GPX[11]. Increases in the activities of antioxidant enzymes are thought to occur in response to increased production of substrate for the antioxidant enzymes[10].

The major goal of the present work is to demonstrate the importance of polyamines on cell proliferation and the cell cycle, especially, the importance of spermine as an antioxidant agent that scavenges oxygen radicals. In this study, the cytoprotective effect of spermine was also studied at the regulation of antioxidant enzymes, SOD and CAT. To estimate the gene expression of SOD and CAT, the protein levels were measured by using Western blotting. The antiapoptotic effect of spermine was also examined by morphological analysis.

MATERIALS AND METHODS

Chemicals and cell culture

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium (MTT), putrescine (tetracethylenediamine), spermidine (N-[3- aminopropyl]-1,4-butanediamine), spermine (N,N'-bis[3-aminopropyl]-1,4-butanediamine), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide (etidium bromide), methylglyoxal bis-[guanylhydrazone] (MGBG) and Dulbecco's modified Eagle's medium (DMEM, with L-glutamine and 1,000 mg/L Glucose) were purchased from Sigma Chemical Co (St. Louis, Mo, USA). Fetal bovine serum (FBS) was purchased from GIBCO (New York, USA). 2,2'-azobis(2-amidinopropane)dihyrochloride (AAPH) was purchased from Wako Pure Chemical Co. (Japan). All other chemicals were purchased from standard commercial sources. The rat liver cell line Ac$_2$F was provided by the Japanese Cancer Research Resources Bank and maintained in DMEM containing phenol red and 10% fetal bovine serum. Culture media were changed every 2 or 3 days.

Measurement of cell viability

Cell viability was determined by using the MTT assay as follows. Cells were plated at the density of 2×10^4 cells/ml in 48 multi-wells with 400 µl DMEM per well. Cells were treated with each different drug or with the same volume of vehicle (0.1% of ethanol). After each treatment, media were replaced with 400 µl of MTT (0.5 mg/ml) in DMEM and the cells were incubated at 37℃ under 5% CO_2 for 1 hr. The MTT medium was then aspirated and replaced with 400 µl solublization (dimethyl sulfoxide 200 µl + 99.9% EtOH 200 µl). The color intensity was measured by the ELISA reader (Hitachi, Japan) with a 540 nm filter.

Measurement for reactive oxygen species (ROS)

Cells were grown in 96 multi-well plates at a density of 2×10^4 cells/well. After each treatment, cells were incubated for 2 hr in phenol red-free DMEM with DCFH-DA (25 µM/ ml) in a final volume of 200 µl/well at 37℃. The fluorescence intensity was measured at the wavelength of 485 (excitation) and 540 (emission) nm with Fluorescence Plate Reader (Bio-Tec Instruments, Inc., Winooski, USA).

Western blot analysis

Cells were collected by centrifugation at 4,000 rpm for 5 min at 4℃. Proteins were extracted by washing cells twice with ice-cold PBS and incubating them for 60 min on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 2 µg/ml leupeptin. For Western blotting, an aliquot of 20~40 µg of protein was separated by sodium dodecylsulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose transfer membrane. Blotting was performed using the primary antibodies of anti-SOD (1:1000 dilution), anti-CAT (1:1000 dilution), anti-cyclin E (1:1000 dilution) and then with the secondary antibody. The immuno complexes were detected using the ECL detection kit (Amersham USA).

Microscopy of apoptosis

Cells grown on a cover glass in the bottom of 6 multi-well plates were pre-incubated for 24 hr. Then the cells were treated with a vehicle (0.1% ethanol) or each drug for 3 days. After the incubation period, cells were stained with 2 µl of 1:1 mixture of ethidium bromide (100 µl) and acridine orange (100 µg/ml).

Statistical analysis

All the experiments were carried out at least three times. Statistical significance between the control and the treated groups was determined by one-way analysis of variance and followed by the Duncan's multiple range test. In all cases, a P value of less than 0.05 was considered significant.

RESULTS

Effect of AAPH on cell proliferation

In this study, AAPH was used as an inducer of oxidative stress in cultured rat liver cell. AAPH is a water-soluble radical initiator, which generates a peroxyl radical molecule. When the cells were treated with 0.5~1.0 mM of AAPH for 3 day, viability of the cells was significantly decreased in a dose-dependent manner (Fig. 1). Cell viability was not changed after 2 days of AAPH treatment. But when the cells were treated for 3 days with 0.5, 0.7 and 1.0 mM AAPH, the cell viability was reduced to 12%, 23% and 44% of the control, respectively.

Effect of polyamines on cell proliferation

In order to study the effect of exogenous polyamines on the liver cell growth, cells were grown for 3 days in the presence of each polyamine; 0.5~20 µM range of putrescine, spermidine and spermine. Putrescine had no significant effect on the cell growth throughout the whole concentration range tested. Both spermidine and spermine, however, increased the cell growth up to 130% and 121% of control at 20 µM, in the liver cell (Fig. 2). The increased viability was not much changed up to 100 µM of spermidine or spermine, but at the concentration higher than 100 µM, cell viability began to decrease (data not shown).

Inhibitory effects of polyamines on AAPH - induced ROS generation

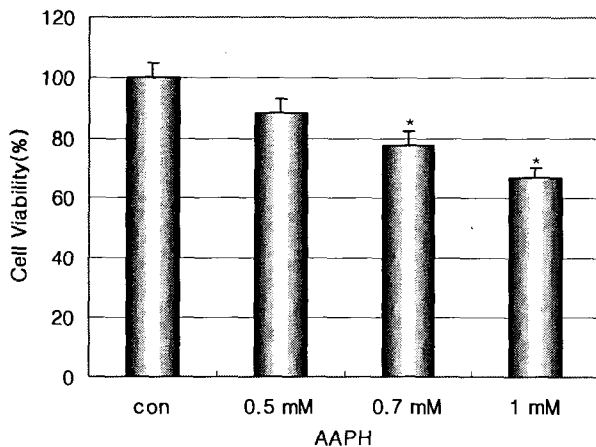Fig. 3 shows the effect of polyamines on AAPH-induced

Fig. 1. Effect of AAPH on cell proliferation. Cells were plated in 48-wells at 2×10^4 cells per ml in DMEM. After 3 days of AAPH treatment, the cell viability was measured by using the MTT assay. $*p<0.05$ vs. control
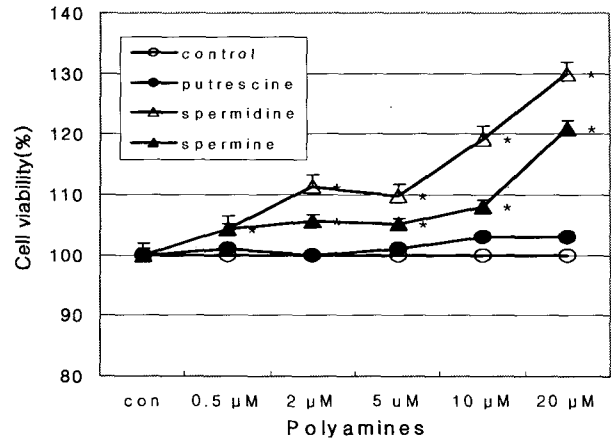

Fig. 2. Effect of polyamines on cell proliferation. Cells were plated in 48-wells at 2×10^4 cells per ml in DMEM and treated with each polyamine for 3 days. The cell viability was analyzed by using the MTT assay. $*p<0.05$ vs. control.
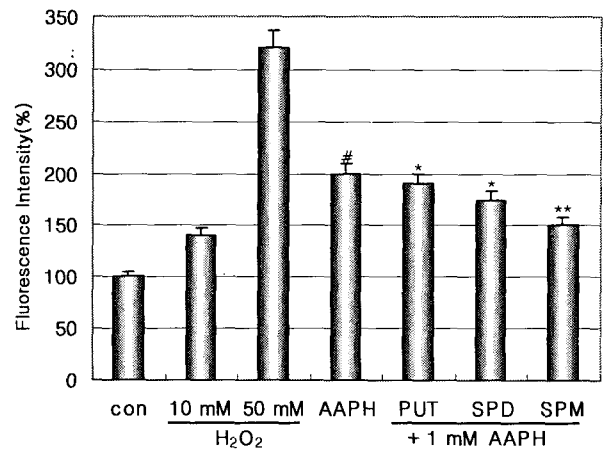

Fig. 3. Effect of polyamines on AAPH-induced ROS generation. AAPH treated cells were incubated for 2 hr in DMEM media containing 25 µM/ml DCFH-DA, in final volume of 200 µl/well at 37℃. After incubation, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. $*p<0.05$, $**p<0.01$ vs. 1mM AAPH. $\#p<0.05$ vs. control.

ROS production. Cells were treated with 1 mM AAPH and 20 µM polyamines simultaneously. In the co-treatment with each polyamine, 20 µM putrescine showed only minor inhibitory effects on the ROS generation. But spermidine and spermine at the same concentrations exerted 21% and 45% inhibition of AAPH-induced ROS generation. This result indicates that spermine acted as a good antioxidant in AAPH-induced stress. These findings also explain the differential effects of each polyamine on the cell viability shown in Fig. 2.

Inhibitory effect of spermine on AAPH-induced intracellular ROS prodution

The intracellular ROS concentration was determined by measuring the intensity of fluorescence. Intracellular redox state levels were measured using a fluorescent dye, DCFH-DA. DCFH-DA is a nonpolar compound which is converted into DCFH by cellular esterases after incorporation into the cells. DCFH is membrane-impermeable and is rapidly oxidized to highly fluorescent 2'7'-dichlorofluorescein in the presence of intracellular hydrogen peroxide and peroxidase. Twenty µM spermine and 1 mM AAPH were applied simultaneously for 3 days. After each treatment, the medium was replaced with PBS containing 10 µM DCFH-DA, and fluoroscence intensity was measured 30 min later with a confocal laser-scanning microscope. Fig. 4 shows that spermine inhibited AAPH-inducedintracellular ROS production in a dose dependent manner. In 1 mM AAPH treatment, the intensity of the fluorescence was very high. But as the concentration of spermine increased, the fluorescence intensity decreased rapidly.

Western blot analysis for SOD, CAT, and cyclin E expression

To determine whether SOD or CAT expression was induced via oxidative stress or not, its protein level was detected by Western blotting. A discernable difference in both SOD and CAT protein expression was detected in each AAPH and/or spermine-treated cells. As shown in Fig. 5,
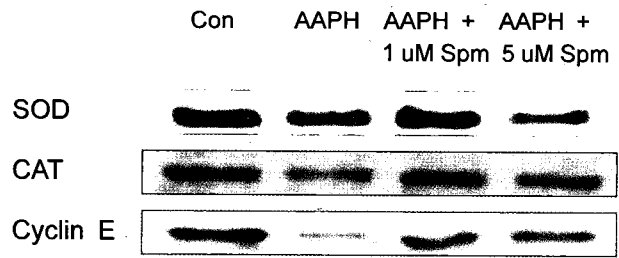

Fig. 5. Effect of AAPH and spermine on the superoxide dismutase, catalase and cyclin E protein expression. To determine the protein level of SOD, CAT and cyclin E. Western blotting was performed by using each specific antibody. See M&M for details.

1 mM AAPH blocked SOD and CAT gene expression, but spermine recovered the gene expressions to the untreated control level. To determine whether spermine prevention of growth-arrest of cells exposed to oxidative stress was associated with changes in the activities of cyclin E gene expression, Western blotting was performed to see its protein level. The results clearly indicated that AAPH blocked the expression of cyclin E but the addition of spermine recovered that.

Apoptotic Morphological analysis

Fig. 6 shows the influence of AAPH on cell morphology and the antioxidative effect of spermine. The basic morphology of the dying cells was observed by the ethidium bromide and acridine orange staining under the fluorescence microscope. Normal cells should appeare bright green both
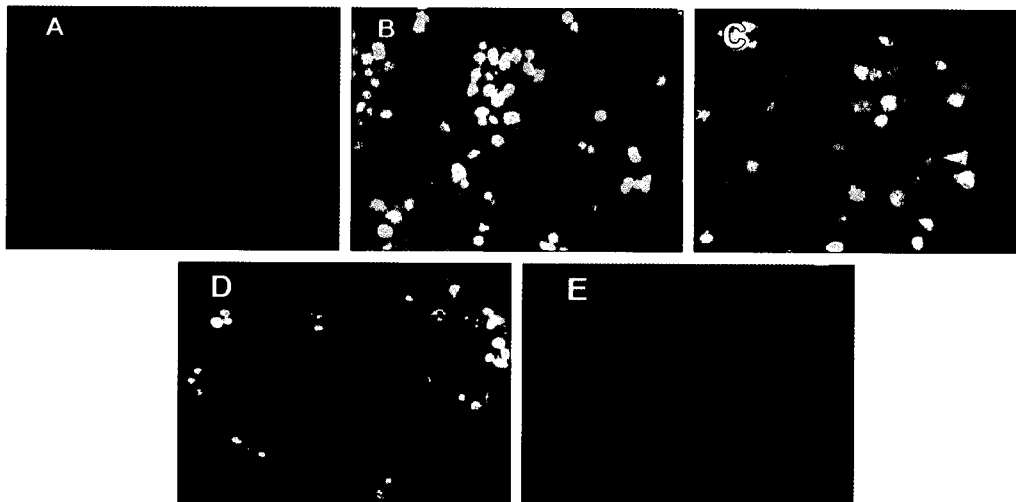

Fig. 4. Effects of spermine on intracellar ROS generated by AAPH. Cells grown in 6-wells containing glass slides, were treated with spermine and AAPH for 3 days. After each treatment, DCFAH-DA was added into each medium. The fluorescent intensity was measured 30 min later with a confocal laser-scanning microscope. A, control; B, 1 mM AAPH; C-E 1 mM AAPH + spermine (1, 5, 20 µM spermine, respectively).
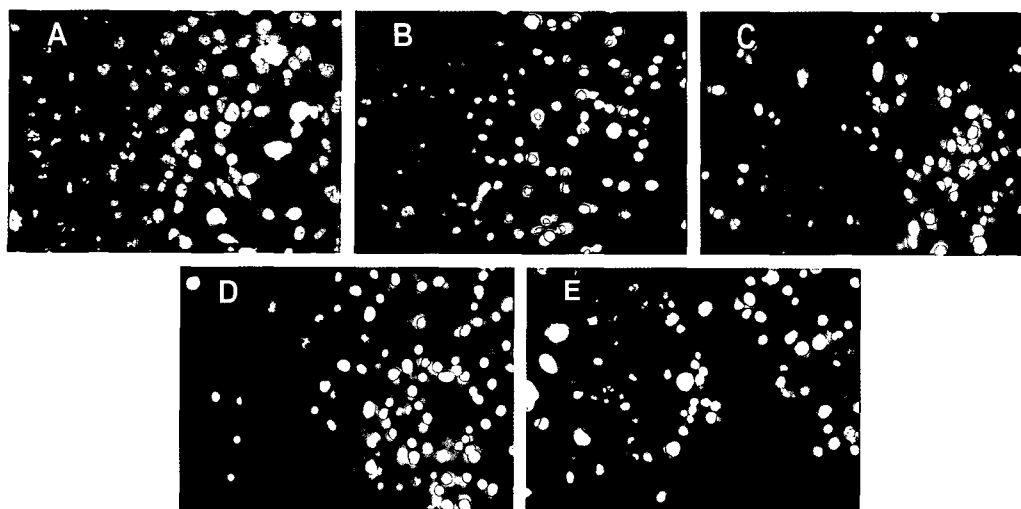
Fig. 6. Fluorescence micrographs showing the effect of spermine on AAPH-treated cells. Cells grown on a glass slide in 6 multi-wells were treated in the same way as in Fig. 4, and visualized by double staining with acridine orange and ethidium bromide. See M&M for details. A, control; B, 1 mM AAPH; C-E, 1 mM AAPH + spermine (1, 5, 20 μM spermine, respectively).

in the nucleus and cytoplasm since ethidium bromide could not enter the cell. In the present result, the cells without any treatment did not show any membrane blebbing or change in cell size (Fig. 6A). However, early apoptotic cells, whose membranes are still intact but have started to frag-ment their DNA, will show green nuclei since ethidium bromide cannot enter the cell yet. Chromatin condensation became visible as orange patches in the nuclei. As the cell progressed the apoptotic pathway, membrane blebbing started to occur, and ethidium bromide entered the cell staining them orange. Late apoptotic cells showed bright orange areas of condensed chromatin in the nucleus and dark red in cytoplasm that distinguished them from necrotic cells, which have a uniform orange color. As shown in Fig. 6B, the cells exposed to AAPH for 3 days showed morpho-logical characteristics of apoptosis, including cell shrinkage, cell volume loss and surface blebbing relative to the control cells. However, the treatment with spermine suppressed the AAPH-induced apoptosis in a dose-dependent manner (Fig. 6C-E).

DISCUSSION

The purpose of the present experiment is to clarify the roles of polyamines in the response to oxidative stress in Ac2F rat liver cell line. We also studied the effect of poly-amines on the growth and viability of the cells.

Polyamines, namely putrescine, spermidine and spermine are required for cell proliferation and development. Spermine

and spermidine, in particular, stabilize chromatin and many nuclear enzymes because of their ability to form complex with organic polyanions such as negatively charged groups in chromatin and DNA structure[13]. It is now becoming progressively clear that polyamine depletion brings about significant changes in chromatin and DNA structure. Also, a decrease in intracellular polyamine levels is associated with apoptosis[15].

Among polyamines, spermine has been reported as an antioxidant with high potency[7]. Although its precise bio-logical function is still unclear, it plays a part in protection against radiation damages and cell death. It was suggested that an important function of spermine is to prevent oxida-tive decay of living organisms, and protects oxidizable cell components, including proteins, nucleic acids, carbohydrates and lipids against oxidative modifications. In the present work, polyamines, especially spermine, increased the rate of proliferation of Ac2F cells. In the cells co-treated with spermine and AAPH, which induces peroxyl radical mole-cules, the viability was recovered from the toxic effect of AAPH. The recovery effect was due to the inhibition of ROS generation.

In normal conditions, there is a steady state balance be-tween the prodution of oxygen-derived free radicals and their destruction by the cellular antioxidant systems[4]. However, the balance can be broken experimentally either by increasing the ROS production or by decreasing the de-fense system. Some studies have indicated that after ex-posure to hyperoxia, increased antioxidant enzymatic de-

fense systems, such as SOD, CAT and GPX, have been chara-terized in cell culture system[17]. Successful antioxidant defense against ROS requires a balanced increase in anti-oxidant enzymes, not only coping with the initial radicals but also the resultant and potentially more toxic products of the catalyzed and spontaneous reduction reactions[16]. Therefore, to elucidate the capability of polyamines scav-enging ROS, the SOD and CAT were measured. In the pres-ent study, both SOD and CAT noticeably decreased with AAPH treatment. However, spermine again recovered the SOD and CAT protein to the level of the untreated control.

Little is known about cell-cycle checkpoint activation by oxidative stress in mammalian cells. The effects of hyperoxia on the cell-cycle progression were investigated in asynchro-nous human T47D-H3 cells, which contains mutated p53 and arrests at G1/S in response to DNA damage[1]. Not suprisingly, possible consequences of hyperoxic exposure include induction of DNA damage[2], activation of poly (ADP-ribose) polymerase[12], and induction of p53[9]. A major effect of hyperoxia on the cell proliferation is inter-ference of cell-cycle progression, apparently involving acti-vation of different checkpoints depending on cell types and genetic backgrounds[1]. For example, the proliferation arrest of lung type 2 epithelial cells exposed *in vitro* to hy-peroxia was shown to be associated with an inhibition of cyclin E/cyclin-dependent kinase-2 (Cdk2) complex activity and G1 arrest[3]. In the present work, cyclin E gene ex-pression was significantly decreased in 1mM AAPH-treat-ed cells. However, spermine blocked the inhibitory effect of AAPH. These results demonstrate that spermine as a free radical scavenger may regulate the G1 phase through cyclin E gene expression under oxidative stress. Furthermore, gene expressions of SOD and CAT was activated by spermine which blocked the oxidative stress.

The antioxidant effects of spermine were also confirmed by using the apoptotic morphological assay. The basic mor-phology of the dying cells was observed by ethidium bro-mide and acridine orange staining under the fluorescence microscope. After the treatments of AAPH, cytoplasm of liver cell was heavily stained with red, and cells showed chromatin condensation. However, spermine blocked the apoptotic effect of AAPH and the spermine-treated cells were as green as the normal cells.

In summary, polyamines, especially spermine, had an an-tioxidant effect on AAPH-induced oxidative stress. Spermine directly prevented the generation of ROS resulting in block-

ing apoptosis. These cytoprotective effects of spermine might be related to the enhanced expression of antioxidant enzymes, SOD and CAT. Although the clear mechanism in the mod-ulation of SOD and CAT expression was not elucidated in oxidative stress, spermine may increase gene expression in protein level by reducing the oxidative stress.

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초록 : 산화 스트레스에 대한 폴리아민의 세포보호 효과

안선이[1] · 이지영[2] · 정해영[2,3] · 유미애[2,4] · 김종민[5] · 김병기[1,2*]

([1]부산대학교 자연대 생물학과, [2]유전공학연구소, [3]약대 약학과, [4]자연대 분자생물학과, [5]동아대학교 의과대학 해부학교실)

폴리아민은 모든 진핵세포에서 발견되는 다가 양이온성의 저분자 물질이며 세포성장에 필수적인 것으로 알려져 있다. 본 논문에서는 폴리아민의 역할 중에서 산화적인 스트레스에 대한 세포보호 효과를 연구하였다. 쥐의 간세포주인 Ac2F에 산화 스트레스를 유발하기 위하여 2,2'-azobis(2-amidinopropane)dehydrochloride (AAPH)를 처리 하였을 때, 세포증식은 농도 의존적으로 감소하였다. 배지에 폴리아민을 첨가하였을 때 세포성장은 농도 의존적으로 증가 하였으며 ROS 발생은 현저히 감소하였다. 폴리아민 가운데 특히 spermidine과 spermine이 뚜렷한 세포증식효과를 보였다. Spermine의 경우, 20 µM 농도에서 AAPH에 의해 유도된 ROS 발생을 45%나 감소시켰다. 산화 스트레스에 관여하는 효소들 가운데 주된 효소인 superoxide dismutate (SOD)와 catalase (CAT)의 세포 내 단백질을 Western blotting으로 조사한 결과, AAPH는 이 두 가지 단백질의 생성을 억제한 것으로 나타났다. 그러나 spermine을 처리 하였을 때 두 단백질의 생산은 모두 정상적으로 회복이 되었다. 또한 세포주기의 중요한 조절 단백질인 cyclin E 역시 AAPH에 의하여 생성이 억제 되었다. 이는 AAPH에 의하여 생성된 ROS가 세포주기의 S phase의 진행을 억제한 것으로 생각된다. AAPH에 의한 cyclin E의 억제는 spermine에 의하여 정상적으로 회복되었다. 위와 같은 Spermine의 항산화 효과는 ethidium bromide와 acridine orange를 이용하여 형태학적으로도 증명되었다.