□ 원 저 □

Astaxanthin Suppresses H_2O_2-induced Apoptosis in H9c2 Cardiac Myoblast Cells

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Astaxanthin is one of carotenoids, which has powerful antioxidant activity and is found in many human foods. We investigated whether astaxanthin would prevent H_2O_2-induced apoptotic cell death of H9c2 myoblast cells. To examine the protective effects of astaxanthin, cytotoxicity assay, flowcytometric analysis, and Western blot analyses were performed. Treatment of H_2O_2 was shown to induce apoptosis in a dose dependent manner and astaxanthin attenuated the caspase-3 dependent apoptotic cell death in H9c2 myoblast cells. In Western blot analyses for cytochrome c release, astaxanthin suppressed the release of cytochrome c. These results suggest that the antiapoptotic effect of astaxanthin is associated with caspase-3 inhibition and inhibition of cytochrome c release into the cytosol. **(Korean J Str Res 2008;16:357～362)**

Key Words: Astaxanthin, Apoptosis, H_2O_2, H9c2 cardiac myoblast cells

INTRODUCTION

Carotenoids (carotenes and xanthophylls) are antioxidant pigments found in vegetables, fruits and crustaceans. Astaxanthin is one of the carotenoids (carotenoids and xanthophylls) and found primarily in crustaceans (Tanaka *et al.,* 1976).

Many reports regarding the antioxidant properties of astaxanthin were published recent years (Kurashige *et al.,* 1990; Palozza *et al.,* 1992; O'Connor *et al.,* 1998). Lawlor *et al.* (1995) reported that astaxanthin has antioxidant activity against paraquat-induced oxidative stress in primary cultures of chicken embryo fibroblasts. In their report, astaxanthin attenuated paraquat-induced cellular damages using various cellular antioxidant enzyme systems.

Recent studies indicated that oxidative stress has important roles in pathophysiology of various neurodegenerative diseases, ageing, and cancer (Beal, 2002; Rao *et al.,* 2002). Reactive oxygen species (ROS) including superoxide and H_2O_2, formed during the various physiological and pathological processes, may have major roles in cell loss in oxidative stress and in apoptosis (Bulkley, 1983; Markesbery, 1997). Apoptotic cell death is not only a physiological process in normal cell function but also has been occurred at pathological conditions. Although there are many stimuli, which induce apoptotic cell death, caspase-3 is known to most common final pathway to apoptosis (Budihardjo *et al.,* 1999; Porter *et al.,* 1999). In cardiovascular disorders,

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oxidative stress is an important contributing factor in congestive heart, atherosclerosis, and ischemic heart disease (Singal *et al.,* 1998). It is well known that apoptosis may exert important roles in pathogenesis and progression of cardiovascular disorders (Valen, 2003).

Although astaxanthin, as a member of carotenoids, has been known to have antioxidant effects against various oxidative stresses and has been used in various food additives, it is little known about mechanisms of antioxidant effect on the heart, especially caspase-3 mediated apoptosis pathway.

The purpose of this study was to determine whether astaxanthin could prevent H_2O_2 induced apoptotic cell death in the H9c2 cells through the inhibition of caspase-3.

MATERIALS AND METHODS

1. Cell culture

H9c2 cardiac myoblast cells were purchased from ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Gaithersburg MD) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL, Gaithersburg, MD) at 37°C in 5% CO_2, 95% air in a humidified cell incubator (Kimes *et al.,* 1976).

2. Treatment with H_2O_2 and astaxanthin

To examine the cytotoxic effects of H_2O_2, the cells were treated with varying concentrations of H_2O_2 for 24h. According to cytotoxicity data, 75 μM of H_2O_2 was treated and various concentrations of astaxanthin was added shortly after the treatment of H_2O_2.

3. MTT-based cytotoxicity assay

To examine the effects of astaxanthin on the H_2O_2-induced cytotoxicity in H9c2 cardiac myoblasts, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described (Yim SV *et al.,* 2001). H9c2 cells were cultured in each well of 96-well plate (Corning Inc., Corning, NY) at a density of 1×10^4 per well. The cells were treated with H_2O_2 (75 μM) and with or without varying concentrations of astaxanthin (0, 5, 10, 25, 50 μM). After 24 h, the cells were washed and treated with MTT. The plate was incubated in the dark for 4 h, and absorbances at 570 nm were measured using a microtiter plate reader (Bio-Tek, Winooski, VT). For determination of cell viability, percent viability was calculated as (absorbance of H_2O_2 and/or astaxanthin treated sample / control absorbance)×100.

4. Flowcytometry

Flowcytometric analyses were performed as previously described using ANNEXIN V-FITC APOPTOSIS DETECTION KIT I by BD PharMingen Corp (Vermes *et al.,* 2000). Cells were treated with 75 μM of H_2O_2 and with various concentrations of astaxanthin. After 24 hr, cells were washed twice with ice cold PBS and then resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. 1×10^5 cells (100 μl) were transferred to culture tube and 5 μl of Annexin V-FITC and PI solution was added. Then cells were incubated for 15 min at room temperature (25°C) in the dark. After the incubation period, 400 μl of 1X binding buffer was added. Stained cells were analyzed using a FACSCalibur (Becton Dickinson, san Jose, CA) within an hour and data were analyzed using CELLQUEST software.

5. Western blot analysis

Cells were harvested, washed twice with ice-cold PBS, and resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin). Whole cell lysates were prepared for immunoblot analysis of caspase-3, and PARP by sonication, and of cytochrome c by a Dounce homogenizer with 20 strokes, and the lysates were centrifuged at 13,000 g for 20 min at 4°C. Proteins (40 μg) were separated on an 8% SDS-PAGE gel for analysis of PARP or a 12% SDS-PAGE gel for analysis of caspase-3 and cytochrome c and transferred on a nitrocellulose membrane. The membrane was hybridized with PARP antibody (PharMingen, San Diego, CA) (1 : 1,000), caspase-3 antibody (Transduction Laboratories, Lexington, KY) (1 : 500), or cytochrome c antibody (PharMingen, San Diego, CA) (1 : 1,000). Protein bands were visualized by exposing to x-ray film.

RESULTS

1. Effects of astaxanthin on the H_2O_2-induced cell death

Fig. 1 illustrates the dose-dependent pattern of cell death in H9c2 cells treated with various concentrations of H_2O_2. Compared to the untreated control (100%), viabilities of cells treated with H_2O_2 of concentrations 10, 20, 50, 75, 100 μM were about 81.2±6.8, 67.6±10.5, 65.1±0.6, 53.5±2.7, 45.7±0.3%, respectively. These results are indications of H_2O_2-induced cell death in H9c2 cells and are well correspondence with other reports (DiPietrantonio *et al.,* 1999).

To find out whether astaxanthin affects the cell viability of H_2O_2-induced cell death, cells were treated with 75 μM of H_2O_2 (50% of cell death) and varying concentrations of astaxanthin. Compared to the untreated control (100%), viabilities of cells treated with astaxanthin of concentrations 0, 5, 10, 25, 50 μM were about 54.6±1.3, 59.9±0.4, 70.3±0.7, 80.0±0.7, 88.2±3.1%, respectively. These results show that astaxanthin has protective effects on the H_2O_2-induced cytotoxicity in H9c2 cells (Fig. 2).

2. Flowcytometric analyses

In flowcytometric analysis, percentage of vital (AV-/PI-, left lower quadrant) cells in control culture was 94.93% (Fig. 3A). H_2O_2-treated cells (75 μM) showed only 10% of vital cells. In astaxanthin treated cells showed 13.36%, 65.64%, 73.50%, and 84.72% of vital cells according to their concentration (5, 10, 25, 50 μM), respectively (Fig. 3).

3. Western blot analysis

It has been reported previously that hydrogen peroxide induces apoptotic cell death by activating caspase-3 (DiPietrantonio *et al.,* 1999), therefore, changes in H_2O_2-induced caspase-3 activity following astaxanthin treatment (0.1 mM) in H9c2 cardiac myoblast cells was examined. H_2O_2 was shown to activate caspase-3 in a dose-dependent manner and astaxanthin attenuated caspase-3 activation (Fig. 4). The active fragment (17 kDa) of caspase-3 appeared with H_2O_2 treatment, and astaxanthin treatment abolished this cleavage of caspase-3, as seen from the Western blot analysis. PARP, one of the substrates for caspase-3, is cleaved from an intact 116-kDa protein into 85- and 31-kDa fragments

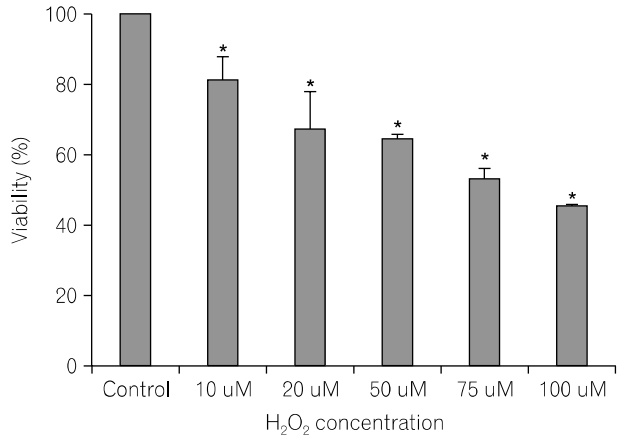

Fig. 1. Viability of rat H9c2 cardiac myoblast cells treated with various concentration of H_2O_2. Cell viability was determined by MTT assay. Cell viability at various concentrations of H_2O_2 was evaluated using cell viability index (%), which was determined by the formula: (mean absorbance in the test group/mean absorbance in the control group)×100. Results are mean±S.E. Values significantly different from those of the corresponding controls at $p<0.01$ are indicated with asterisks.

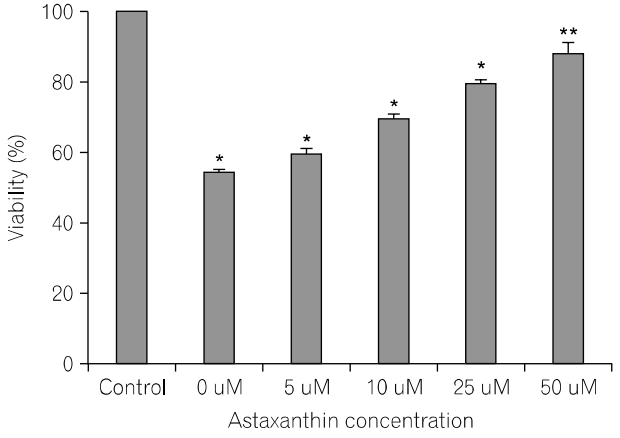

Fig. 2. Effect of astaxanthin on the H_2O_2-induced cell death of H9c2 cardiac myoblast cells. Various concentration of astaxanthin was added simultaneously with 75 μM hydrogen peroxide in the H9c2 cardiac myoblast cells and viability was determined by MTT assay. Cell viability was evaluated using cell viability index (%), which was determined by the formula: (mean absorbance in the test group/mean absorbance in the control group)×100. Results are mean±S.E. Values significantly different from those of the corresponding controls at $p<0.01$ and $p<0.05$ are indicated with single and double asterisks, respectively.

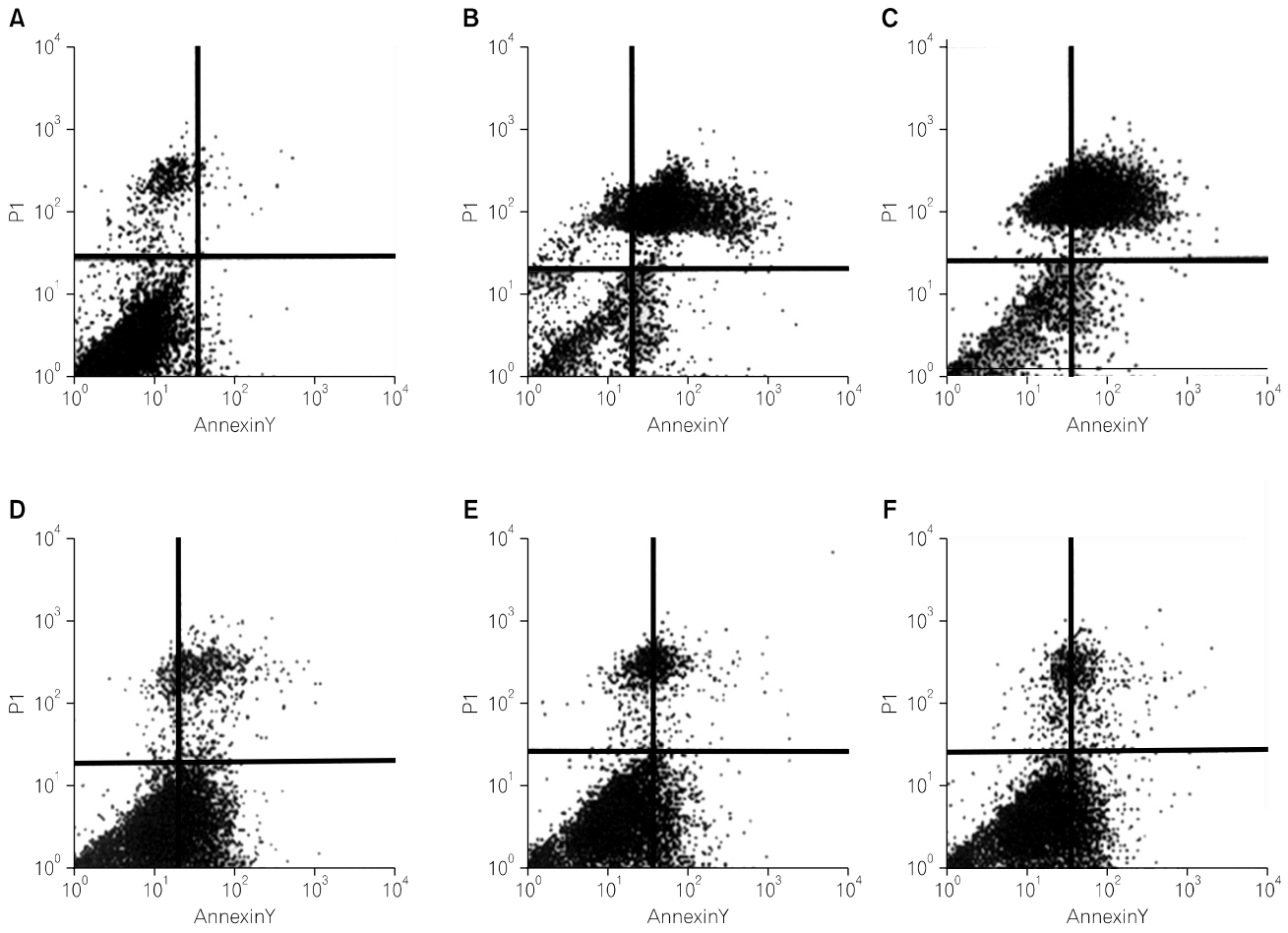

Fig. 3. Flowcytometric analysis. The cells were treated with Annexin V/PI and analyzed by flow cytometry. (A) Control: (B) H_2O_2 75 M: (C) H_2O_2 75 M+astaxanthin 5 M: (D) H_2O_2 75 M+ astaxanthin 10 M: (E) H_2O_2 75 M+ astaxanthin 25 M: (F) H_2O_2 75 M+ astaxanthin 50 M.

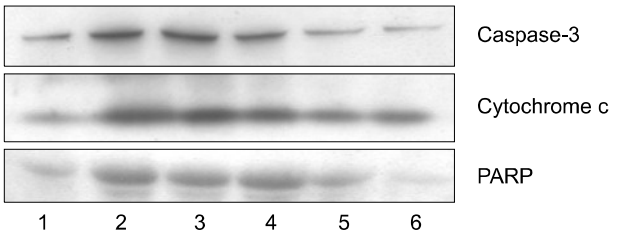

Fig. 4. Western blot analyses. H9c2 cells were treated with 75 μM H_2O_2 and with various concentrations of astaxanthin. Western blotting for caspase-3, PARP, and cytochrome c release was performed as described in Materials and methods. 1. Control: 2. H_2O_2 75 M: 3. H_2O_2 75 M+astaxanthin 5 M: 4. H_2O_2 75 M+astaxanthin 10 M: 5. H_2O_2 75 M+astaxanthin 25 M: 6. H_2O_2 75 M+astaxanthin 50 M.

during apoptosis (Zhang *et al.,* 1994). One of the products of PARP cleavage (85 kDa) was detected in the lysate of H_2O_2-treated cells and astaxanthin treatment almost completely inhibited PARP cleavage (Fig. 4). These results suggest that the antiapoptotic effect of melatonin involves the inhibition of the caspase-3- mediated pathway.

To elucidate the mechanism of the astaxanthin's protective effect in H_2O_2-induced cell death, Western blot analyses were performed to address involvement of expression of Bcl-2 and the release of cytochrome c. Treatment of astaxanthin was shown to suppress the release of cytochrome c from the mitochondria (Fig. 4) but Bcl-2 expression was not detected (data not shown).

DISCUSSION

Astaxanthin is a powerful antioxidant and is found in many human foods mainly in crustaceans (Katsuyama *et al.,* 1987; Nakano *et al.,* 1999). Astaxanthin's antioxidant and antitumor

effects are well known (Nakano *et al.,* 1999; Jyonouchi *et al.,* 2000). Astaxanthin is commonly added to various foods in order to make red color and for its anticancer and antioxidant properties (Jyonouchi *et al.,* 2000).

Apoptosis is physiological processes, which play important roles in many pathologic conditions. Although there are many stimuli, which induce apoptosis, common pathway to apoptosis is caspase-3 (Budihardjo *et al.,* 1999; Porter *et al.,* 1999). In cardiovascular disorders, oxidative stress is one of the most important noxious stimuli which cause pathologic changes (Ferrari *et al.,* 1998).

In this study, we examined that astaxanthin could prevent the H_2O_2-induced apoptotic cell death of H9c2 cardiac myoblast cells. Reactive oxygen species (ROS) are formed in association with variety of disorders and may play an important role in apoptosis. Because treatment with H_2O_2 induces oxidative stress in cultured cells and causes apoptotic cell death, H_2O_2-treated system is often used as in vitro model of oxidative stress (Dipietrantonio *et al.,* 1999). Treatment of H_2O_2 showed dose-dependent cell death and astaxanthin attenuated cell death in MTT assay. In flowcytometric analysis, astaxanthin showed dose-dependent anti-apoptotic effect. In order to elucidate the mechanism of anti-appoptotic cell death of astaxanthin, Western blot analyses of caspase-3, PARP, Bcl-2 expression, and cytochrome c release were examined. As a result, H_2O_2 induced cell death via caspase-3 dependent manner and astaxanthin blocks apoptosis by suppression of cytochrome c release. But there is no involvement in Bcl-2 pathway (data not shown).

These results showed that astaxanthin could prevent caspase-3 dependent H_2O_2-induced apoptosis and astaxanthin might be used as a dietary supplement because of its ability to prevent oxidative stress in heart cells.

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= 국문초록 =

Astaxanthin은 carotenoids의 하나로 강력한 항산화 효과를 갖는 것으로 알려지고 있으며 많은 음식물에 함유되어 있다. 저자들은 astaxanthin이 H_2O_2에 의해 유도된 H9c2 심근세포주의 세포사멸을 억제할 수 있는지에 대하여 연구하였다. Astaxanthin의 세포 보호 효과를 알아보기 위하여 세포독성, flowcytometry, Western blot 분석이 수행되었다. H_2O_2의 처리는 농도의존적으로 H9c2 심근세포주에서 세포사멸을 유도하였고 astaxanthin은 caspase-3에 의한 세포사멸을 억제하였다. Western blot 분석에서는 astaxanthin이 cytochrome c의 방출을 억제하였다. 이러한 결과는 astaxanthin의 항세포사멸 효과가 caspase-3의 활성화와 cytochrome c의 세포질 내로의 분비를 억제하여 일어나는 것을 나타낸다.

중심단어: Astaxanthin, 세포사멸, H_2O_2, H9c2 심근세포주