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Low Dose of β-Carotene Regulates Inflammation, Reduces Caspase Signaling, and Correlates with Autophagy Activation in Cardiomyoblast Cell Lines

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Excessive reactive oxygen species (ROS) stimulate mitochondrial damage that causes degenerative diseases such as cardiovascular disease (CVD). β-carotene (BC), a natural antioxidant able to counteract free radicals, acts as a cytoprotective agent. However, knowledge of the role of BC on cardiomyoblasts is limited. In this study, we explored its role on COX4, Tom20, Nfr1, Nrf2, Nf-κB, LC3, p62, caspase 3, and caspase 9 and its association with cardiomyoblast viability and survival.

Material/Methods: H9C2 cell lines were seeded, cultivated until 90% to 100% confluency, and treated with various doses of BC: 10 μM, 1 μM, 0.1 μM, and 0.01 μM. After 24 h, the cells were harvested, lyzed, and tested for specific related protein expressions from each dose.

Results: Low-dose BC induced autophagy most effectively at 1 μM, 0.1 μM, and 0.01 μM, as indicated by a decrease of LC3II and p62 levels. We observed that Nf-κB protein levels were suppressed; Nrf2 was stimulated, but Nrf1 was not altered significantly. Further, low-dose BC might stimulate cell viability by reducing apoptotic signals of caspase 3 and 9. Notably, low-dose BC also showed potential to increase Tom20 protein levels.

Conclusions: Low-dose BC supplementation shows beneficial effects, especially at 0.01 μM, by reducing inflammation through the suppression of Nf-κB and increase of Nrf2 level. Autophagy as a cellular maintenance mechanism was also stimulated, and the amount of the mitochondria marker Tom20 increased. Taken together, results showed that specific low-dose BC is effective and might improve cell viability by stimulating autophagy, inhibiting proinflammatory factors, and suppressing apoptosis.

MeSH Keywords: Antioxidants • Mitochondria • Myocytes, Cardiac

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Background

Aging is a process whereby the structural integrity and physiological function of every organ in the human body is lost over time. Aging is positively linked to cognitive and biological degeneration including physical weakening, frailty, psychological alteration, and cognitive decline. Among several theories that describe the process of aging, the free radical theory of aging is well known and states that aging is a result of accumulated failure of several defensive mechanisms as a consequence of damages induced by reactive oxygen species (ROS), specifically within mitochondria. Excessive ROS production leads to structural impairment in mitochondria, followed by the damage of biophysical properties of the membrane resulting from alteration in activities of electron transport chain (ETC) protein complexes, impaired fluidity, and mitochondrial DNA (mtDNA) changes, which result in mitochondrial failure due to cellular energy imbalance. These disruptions impair cellular homeostasis and increase cell vulnerability to oxidative stress that contributes to critical aspects of premature aging, increased incidence of chronic degenerative diseases, and even a shortened life span [1,2]. Vital organs, such as the heart and brain, with high rates of oxygen intake, are at a risk of this phenomenon, and this partly explains the high incidence of cardiovascular disease (CVD) and neurological disorders in the elderly population [3]. CVD includes diseases and conditions of blood vessels that affect blood flow to the heart, brain, and peripheral areas of the body such as coronary heart disease, stroke, and heart failure, mainly due to atherosclerosis [4].

The pathogenesis of atherosclerosis as a CVD depends on the balance among antiinflammatory, proinflammatory, and antioxidant defense mechanisms. During the formation and enlargement of plaque, smooth-muscle proliferation and depletion of endothelial nitric oxide synthase (NOS) stimulate ROS, which lead to premature and accelerated vascular aging and senescence [5,6]. ROS also lead to the oxidation of low-density lipoprotein (OxLDL) that accumulates within plaque, activates the inflammatory process of atherosclerosis, and plays a vital role in its pathogenesis [7]. Sustained OxLDL also leads to endothelial malfunction and progressive vasoconstriction and even results in cell death through apoptosis.

Elevated ROS induce nuclear respiratory factor 1 (Nrf1) and nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf1 plays a role in regulating endogenous antioxidant glutathione as a response to toxic agents and oxidative stress [8,9].. Nrf2 is a potent transcription factor that binds with the antioxidant response element (ARE) responsible for the activation of several endogenous antioxidant enzymes, including glutathione S-transferase, NADPH quinone oxidoreductase (NQO1), γ-glutamylcysteine synthetase, heme oxygenase-1, and ferritin. Meanwhile, ROS also activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1), and when there is persistent accumulation or extremely high levels of ROS in the cell, Nf-κB regulates various cellular responses to stress, including apoptosis and inflammation. It achieves this versatility through the regulation of host genes, including those involved in reactive species formation, and triggers proinflammatory cytokine and chemokine production and molecular adhesion that determine cell survival [10–13].

As stated earlier, the exposure of cells to high concentrations of ROS and long-term oxidative stress can lead to cellular damage and senescence. ROS are also known to induce autophagy, a mechanism pronounced by cells breaking down and devouring internal proteins and organelles within the lysosome and recycling the damage for cellular renewal as a cytoprotective and survival response. Cell response and the maintenance homeostasis of ROS levels potentially involves and promotes autophagy (or mitophagy) to eliminate the impairment that is responsible for harmful ROS production in the mitochondria [10]. Other important physiological roles of ROS include the regulation of cellular redox balance, cellular signal transduction, and activation of protein kinases that modulate gene and immune functions [14,15].

Under normal conditions, the amount and generation of free radicals are controlled by antioxidant defenses within our body, so that homeostasis is maintained. The source of antioxidant defenses includes endogenous and exogenous, or diet-derived, compounds, which can be grouped into 3 main categories: antioxidant enzymes, chain-breaking antioxidants, and metal-binding proteins. Further, chain-breaking antioxidants can be classified into 2 broad categories: water and fat soluble. One example of a fat-soluble antioxidant is β-carotene (BC). BC is the most common form of carotenoid, and is provitamin A. In vertebrates, BC is converted into retinol by β-carotene 15150-dioxygenase (BCDO), which is exhibited especially in the liver and intestinal epithelium. Unlike vitamin A, whose high dose can cause toxicity, BC is not toxic as a vitamin A precursor. BC can be found in deep yellow-orange-red pigmented fruits and vegetables and has natural antioxidant and antiinflammatory properties.

BC is a potent chemical and physical quencher of singlet oxygen and acts as an ROS scavenger [16]. In addition, BC is inversely correlated with oxLDL levels, suggesting that BC offers protection against the progression of atherosclerosis [1]. Another study revealed that BC may not only reduce the risk of CVD but may also prevent several age-related cancers, such as breast cancer, by modulating Nrf2/ARE as a response to oxidative stress, and that it improves cellular endogenous antioxidants. Nrf2 also increases the activity of mitochondrial ETC, protecting against toxicity and maintaining mitochondrial homeostasis, possibly through the ablation of Akt2 signaling [17].

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Table 1. Primary antibodies.

No.	Antibody name	Source	Catalog number	Company	Dilution
1	COX4	Rabbit	PAS-17511	Thermo Scientific	1: 1000
2	Tom20	Rabbit	#42406	Cell Signaling	1: 1000
3	Nrf1	Mouse	MAB5306-SP	RnD System	1: 1000
4	Nrf2	Mouse	MAB3925-SP	RnD System	1: 1000
5	Nf-κB	Rabbit	13586	Cell Signaling	1: 1000
6	LC3II	Rabbit	2775	Cell Signaling	1: 1000
7	p62	Rabbit	Cst 5114	Cell Signaling	1: 500
8	Caspase 3	Goat	AF-605-NA	RnD System	1: 250
9	Caspase 9	Goat	AF8301	RnD System	1: 250
10	GAPDH	Mouse	AM4300	Thermo Scientific	1: 1000

The connection between the risk of CVD or atherosclerosis and circulating serum carotenoids is apparently beneficial, marked by the reduction of inflammation, oxidative stress, and endothelial dysfunction, which are known to accompany the pathophysiology of CVD [18,19]. Therefore, BC can be a promising marker of dietary or lifestyle factors associated with a reduced risk of CVD [20,21].

Clinical trials have thus far focused only on the effects of BC among the carotenoids. In addition, doses of BC and participant compliance varied and the risk-factor profiles of participants were different among studies. The effect of BC itself on cardiomyoblasts has not been sufficiently studied. In particular, BC has not been studied as an antioxidant that may prevent the formation of atherosclerosis in relation to the aging process at the cellular and biomolecular levels, which are closely related to organelle dysfunction, particularly mitochondrial damage, which triggers protein misfolding, oxidative injury, and cell death. Thus, in the present study, we explored the role of BC as a source of an exogenous antioxidant in maintaining mitochondrial function and inflammatory responses caused by excessive ROS. Potentially, understanding BC as cytoprotective agent could be a new strategy of preventing CVD and other age-related diseases.

Material and Methods

β-carotene

In this study, we used type II, synthetic, ≥95% (by high-performance liquid chromatography) crystalline 5 mg BC (cat. No. 7235-40-7, purchased from Sigma-Aldrich, USA). Trypsin-EDTA (cat. No. BE17-16IF, Lonza, Verviers, Belgium). Phosphate-buffered saline (PBS, cat. No. P4417) and penicillin-streptomycin (cat. No. P4333) was purchased from Sigma-Aldrich. Fetal bovine serum (cat. No. 10499-044, Gibco) was purchased from Life Technologies (Grand Island, NY, USA).

Cell culture

This study used a cardiomyoblast cell culture (H9C2 cells with register number ATCC® CRL-1446™). H9C2 is a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue by Kimes and Brandt, which exhibits many of the properties of skeletal muscle [22]. H9C2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, cat. No. D6546) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, maintained at 37°C in a humidified incubator with 5% CO_2. Next, the H9C2 cells were seeded and cultivated until they reached 90% to 100% confluency. This condition pushed cells into an overpopulated state, thereby triggering stress and apoptotic signals.

Cell treatment

The H9C2 cell lines (1.7×10^2 cells/well) were seeded in 20-well plates in a volume of 100 µL. After 24 h, 4 well groups were treated with various concentrations of BC (10 µM, 1 µM, 0.1 µM, and 0.01 µM) and H9C2 in a normal medium as a control group.

Protein extraction and Western blot

The cells were harvested and processed through *in vitro* extraction. An equal amount of cells was added to 120 µL/well of a lysis solution into 20-well plates. The lysis solution consisted of RIPA lysis buffer and a sample buffer in a ratio of 1: 1. The protein inhibitor and DTT were added later in a ratio of 1: 100. All samples were heated at 96°C for 5 min and snap frozen in ice for 2 to 3 min. An amount of 10 µL of protein was separated on SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen) by electrophoresis for 3.5 h.

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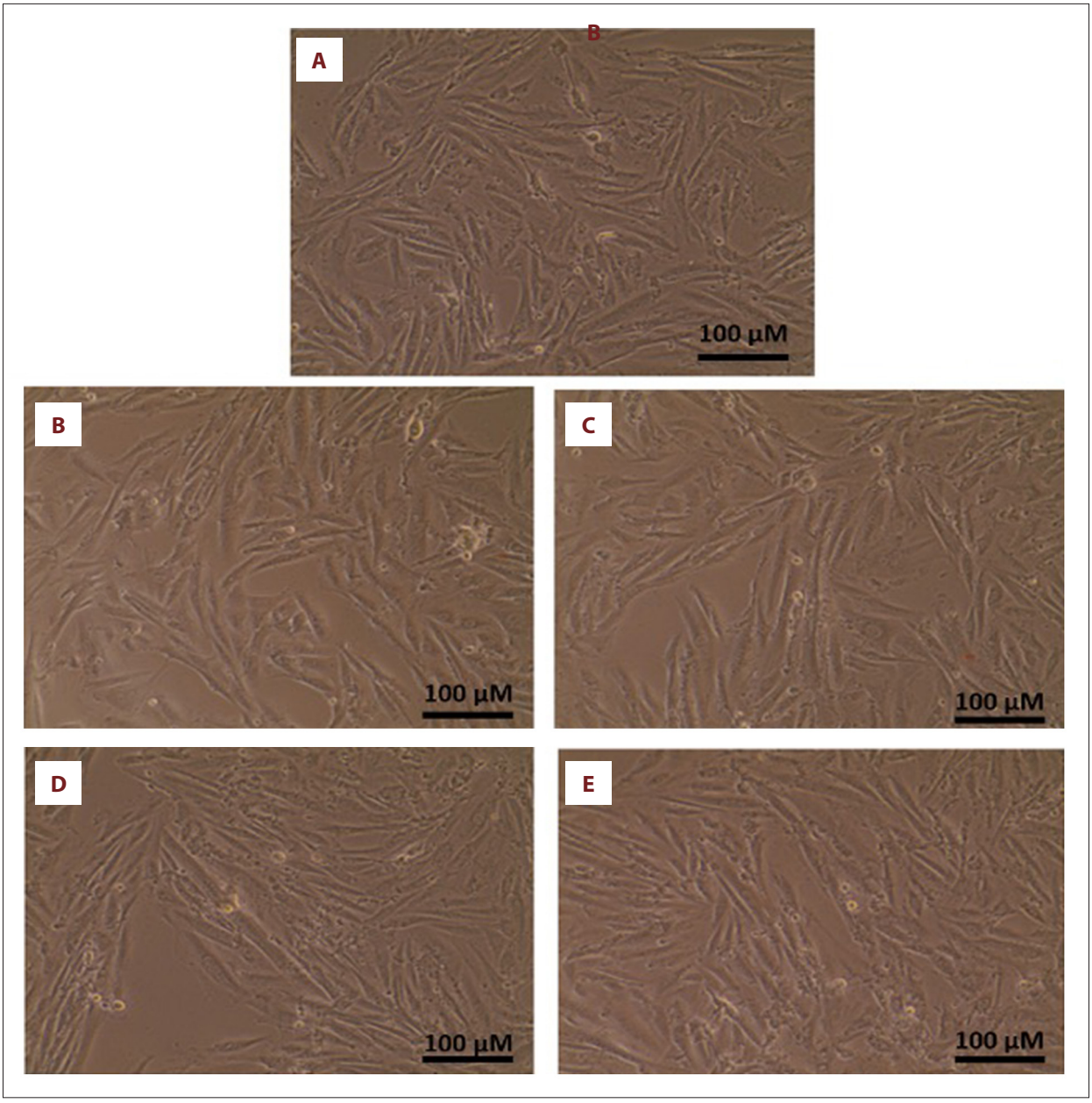

Figure 1. H9C2 cells were exposed to different concentrations of β-carotene (BC) after 24 h incubation (magnification 20×). (**A**) H9C2 cells in normal medium as a control group, BC was given as follows (**B**) 10 µM, (**C**) 1 µM, (**D**) 0.1 µM, and (**E**) 0.01 µM.

A 2% blocking reagent (Invitrogen) that consisted of phosphate-buffered saline with 0.1% Tween 20 (PBST) was added and incubated at 4°C overnight. Membrane immunoblotting was conducted using primary antibodies. Specific primer antibodies of COX4, Tom20, Nrf1, Nrf2, Nf-κB, LC3, p62, caspase 3, and caspase 9 with 1: 1000 dilution in PBST were incubated for 24 h (Table 1). Blots were stripped by a stripping buffer (Thermo Fisher Scientific) and reprobed using GAPDH as an internal control for monitoring protein levels. The signals were visualized by using a secondary antibody infrared system with dilution 1: 15 000, then were imaged using LI-COR Odyssey (USA). Protein band thicknesses were analyzed using LI-COR software.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) test using SPSS version 23 software and GraphPad Prism version 7 software for Windows. Data were presented as mean±standard error of mean. *P* values <0.05 indicated statistical significance. The experiments were performed in triplicate.

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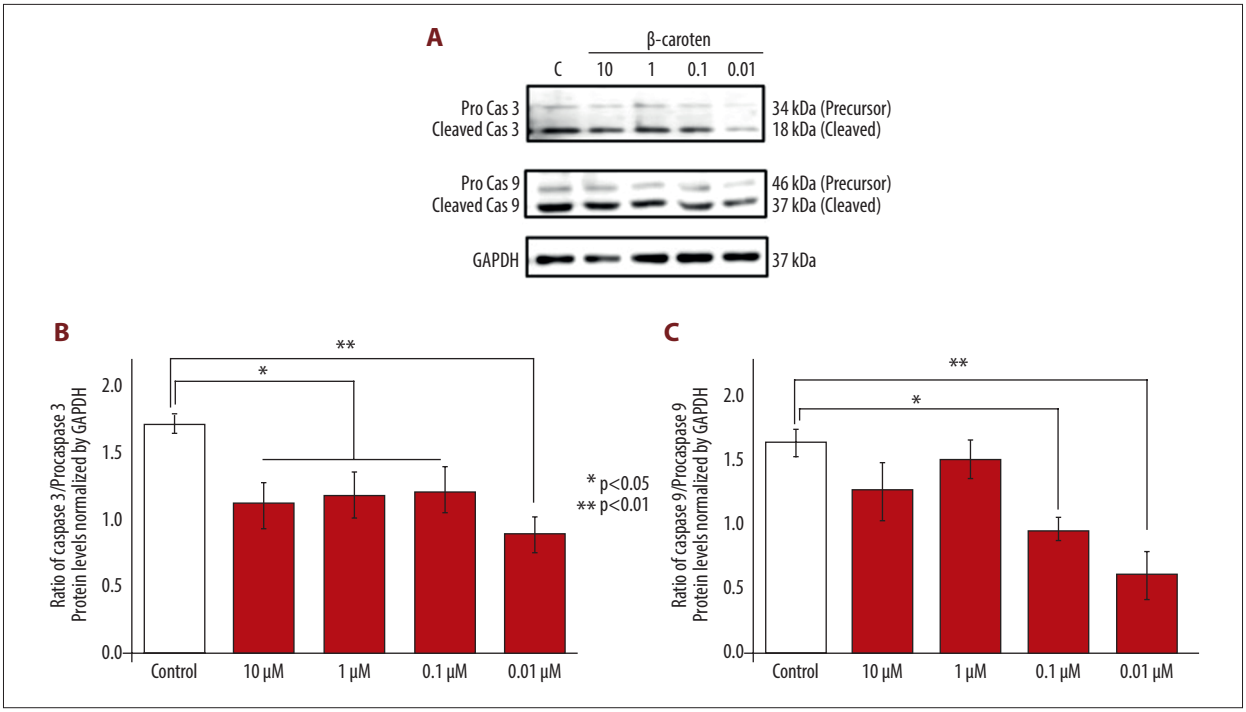

Figure 2. Evaluation of apoptosis markers with various concentrations of β-carotene (BC) for 24 h; representative blot of caspase 3, caspase 9, and GAPD, as internal control. (**A**) Caspase 3 was suppressed by BC at 10 μM, 1 μM, 0.1 μM, and 0.01 μM. (**B**) Caspase 9 was suppressed by low doses of BC at 0.1 μM, and 0.01 μM. (**C**) Data are presented as average mean±standard error of mean (SEM) with *P*<0.05 considered significant (*) and *P*<0.01 considered very significant (**).

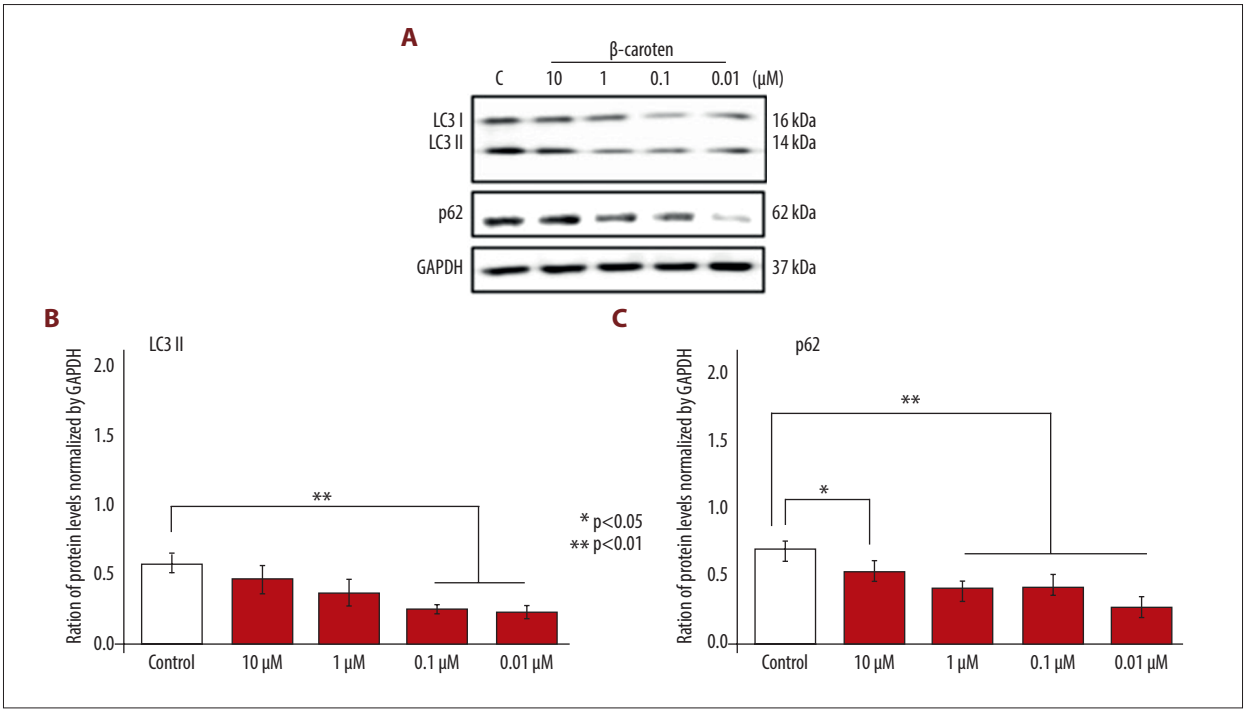

Figure 3. Representative blot of LC3II, p62, and GAPD, as internal control, after the cells were treated with various concentrations of β-carotene (BC) for 24 h. (**A**) LC3II was suppressed by BC at various doses of 1 μM, 0.1 μM, and 0.01 μM. (**B**) p62 was suppressed sat 10 μM, 1 μM, 0.1 μM, and 0.01 μM. (**C**) Data are presented as average mean ± standard error of mean (SEM) with *P*<0.05 considered significant (*) and *P*<0.01 considered very significant (**).

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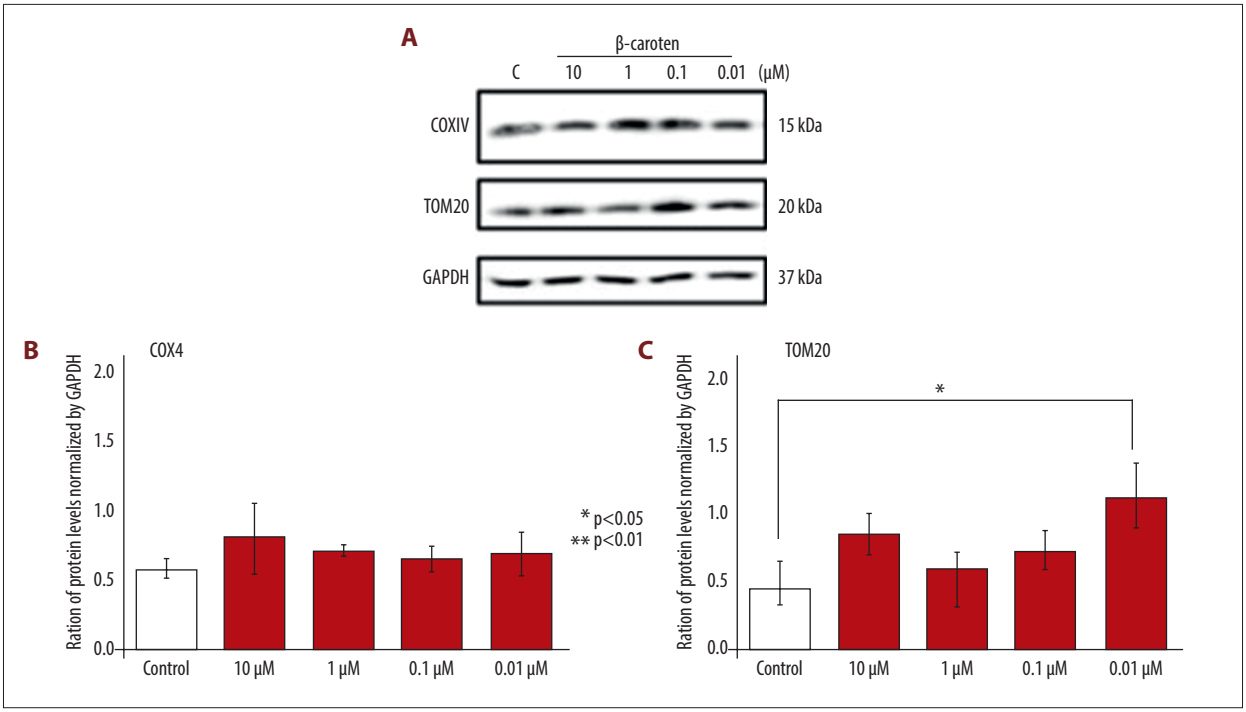

Figure 4. Representative blot of COX4, Tom20 as mitochondrial markers, and GAPD, as internal control, after the cells were treated with various concentrations of β-carotene (BC) for 24 h. (**A**) Various concentrations of BC did not interfere the expression of COX4 significantly. (**B**) Meanwhile, various concentrations of BC tended to improve Tom20 starting at 0.1 μM, and 0.01 μM. (**C**) Data are presented as average mean±standard error of mean (SEM) with *P*<0.05 considered significant (*) and *P*<0.01 considered very significant (**).

Results

Cell morphology after BC treatment

H9C2 cell morphology was evaluated using a light microscope with 20× magnification after 24-h treatment with various concentrations of BC in 10 μM (Figure 1B), 1 μM (Figure 1C), 0.1 μM (Figure 1D), and 0.01 μM (Figure 1E) BC and H9C2 in a normal medium without BC as a control group (Figure 1A). After 24 h of treatment, there was no significant change in the cell count, and cellular morphology defects were not noticed, indicating that BC administration can be considered safe and non-toxic.

Lower doses of BC reduced Caspase 3 and Caspase 9 activation

To investigate the molecular pathways involved in apoptosis induction by BC, we examined the expression of caspase 3 and caspase 9 as apoptosis-promoter proteins. BC treatment decreased the activity of caspase 3 and caspase 9 as shown in representatif blot (Figure 2A), which was significant at 0.01 μM in caspase 3 (Figure 2B) and which had a slight variation at 0.1 μM and 0.01 μM and a significant variation at 1 μM in caspase 9 (Figure 2C). Activation of caspase 3 levels was suppressed 0.77-fold at a dose of 10 μM, 0.69-fold at 1 μM, 0.71-fold at 0.1 μM, and 0.52-fold at 0.01 μM. The caspase 9 level was suppressed 0.59-fold at 0.1 μM and 0.38-fold at 0.01 μM.

Low-dose BC induced autophagy activity

To explore and analyze the effects of BC in regulating cellular renewal and viability, we measured the expression of LC3II, a marker that is responsible for forming an isolation membrane and autophagosomes during the initial stage of cellular autophagic response along with p62, a protein, which expressed as a monitor of autophagic activity, directly binds to LC3 and is selectively degraded during autophagy, as shown in representatif blot (Figure 3A). BC modulated the autophagy activity in relatively lower doses, especially at 1 μM, 0.1 μM, and 0.01 μM (Figure 3C), marked by decreases of p62 by 0.58-fold at 1 μM, 0.62-fold at 0.1 μM, and 0.39-fold at 0.01 μM, which were significant compared to that of the control treatment. Paired with the p62 results, the LC3II level was suppressed significantly by 0.42-fold at 0.1 μM and 0.39-fold at 0.01 μM, compared to the control treatment, indicating that a super-autophagic flux occurred (Figure 3B).

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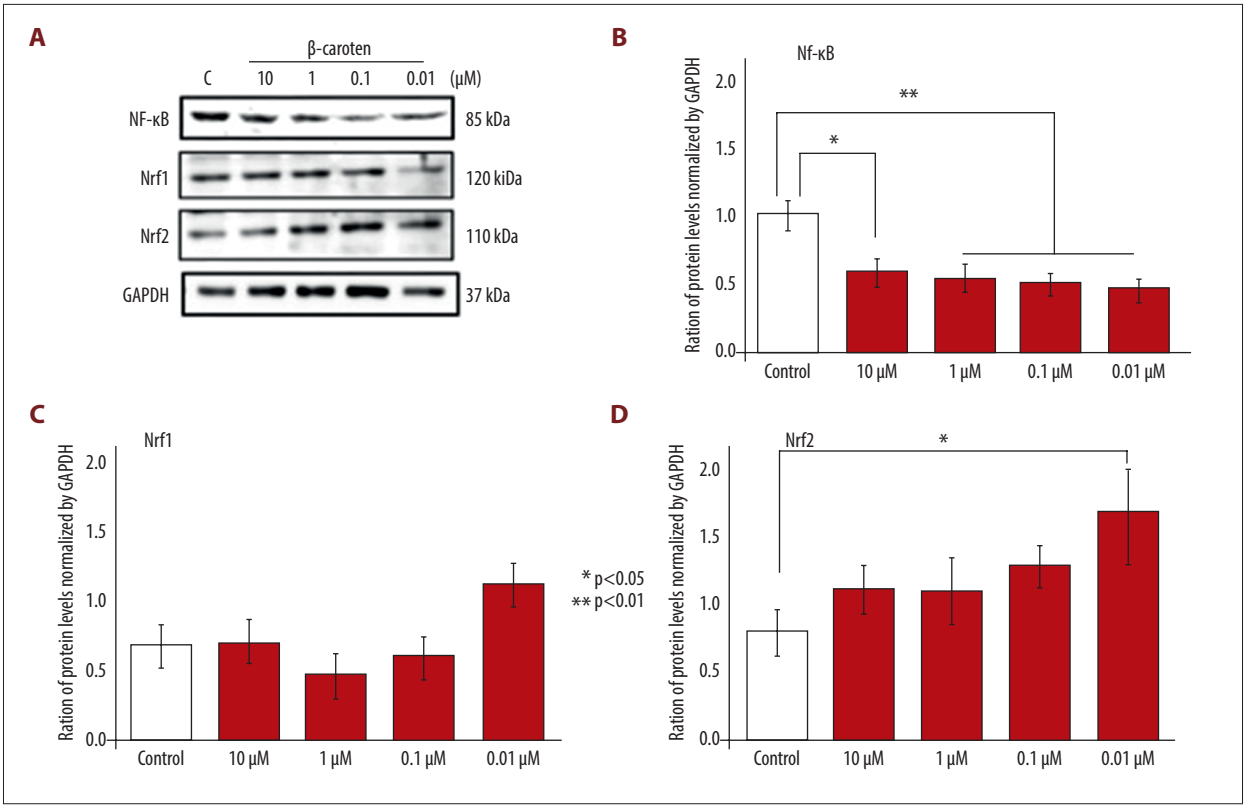

Figure 5. Representative blot of Nf-κB, Nrf1, Nrf2, and GAPDH, as internal control, and evaluation of proinflammatory and antioxidant markers after 24-h administration of various doses of β-carotene (BC). (**A**) Nf-κB was suppressed starting at 10 μM, followed by 1 μM, 0.1 μM, and 0.01 μM. (**B**) Meanwhile, there were no significant effects in Nrf1 at any concentration. (**C**) Nrf2 increased at 0.1 μM and increased significantly at 0.01 μM. (**D**) Data were presented as average mean±standard error of mean (SEM) with $P<0.05$ considered significant (*) and $P<0.01$ considered very significant (**).

BC did not entirely stimulate mitochondria marker levels

To study the pathway of cellular renewal and survival, we analyzed several expressions of mitochondrial markers such as Tom20 and COX4, which indicate mitochondrial viability modulated by BC as shown in representatif blot (Figure 4A). However, in this study, we found that no doses of BC stimulated COX4 (Figure 4B), although the mechanism of autophagy was elevated, and the suppression of cellular apoptosis occurred. Notably, Tom20 levels were elevated significantly in low-dose BC by 2.44-fold at 0.01 μM, which indicated that mitochondrial renewal might have occurred (Figure 4C).

Low-dose BC suppressed Nf-κB levels and increased Nrf2 but not Nrf1

To explore the signaling pathway that is closely related to the regulation of various cellular responses to stress such as apoptosis and inflammation as shown in representatif blot (Figure 5A), we also analyzed the expression of NF-κB. Nf-kB was suppressed significantly by 0.57-fold at 10 μM, 0.54-fold at 1 μM, 0.49-fold at 0.1 μM, and 0.45-fold at 0.01 μM (Figure 5B).

At the same time, to verify the role of BC as an antioxidant, we measured the mechanism on the expression of Nrf2 as a master regulator of the endogenous antioxidant enzyme and on Nrf1, which regulates mitochondrial homeostasis and the cell cycle. We found that BC significantly promoted Nrf2 with the reduction in BC administration, specifically by 2.07-fold at 0.01 μM (Figure 5D). Unfortunately, in this case, BC did not have a significant effect on Nrf1 (Figure 5C).

Discussion

The increases in ROS levels, such as those that occur in atherosclerosis, are associated with premature biological aging, as atherosclerotic plaques show evidence of cellular senescence characterized by reduced cell proliferation, irreversible growth arrest and apoptosis, elevated DNA damage, epigenetic modifications, and telomere shortening and dysfunction [6]. ROS depend on the balance among proinflammatory, antiinflammatory, and antioxidative defense mechanisms to eventually promote cell survival and viability. ROS, as free radicals, are considered to play a causal role in this process and

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lead to OxLDL formation, contribute to the chronic inflammatory state of atherosclerosis, and play a key role in atherosclerosis pathogenesis [23]. An abnormal level of ROS may escape scavenging systems and compromise important cell organelle function, including mitochondrial function, leading to pathological responses, oxidative stress, and damage of important biomolecules such as DNA, then leading to the development of cellular senescence and chronic diseases including CVD [24,25]. Unfortunately, cell senescence, cell stress, and inflammatory response are not always shown on microscopic structure alterations at an early stage (Figure 1).

Cardiomyoblasts, which are functionally improved by a reduction of the proinflammatory level, activation of the antioxidant defense mechanism, mitochondrial renewal, and improved autophagic response, can result in the suppression of cellular death response induced by caspases. Our results suggest that low-dose BC might influence inflammatory signaling in H9C2 cell lines, which might be connected with the inhibition of caspase 3 and caspase 9 (Figure 2B, 2C). Notably, a low dose of BC administration seems more effective in inhibiting the apoptosic signal induced by cell overgrowth in our system. The apoptotic response mediated by caspase 3 appeared to be deprived with the decrease of the BC dose, although a variation was found at 0.1 µM (Figure 2B). Likewise, the apoptotic response activated by caspase 9 was consistent with a relatively low dose of BC, even though a variation in 1 µM appeared (Figure 2C). Beneficial effects of BC in the H9C2 cell line were suppressed by a more prooxidant cardiac tissue environment at higher doses of BC marked by heme oxygenase-1 production as an expected consequence of signif¬icantly higher levels of oxidative stress present in ischemia-reperfusion (I/R) injured tissue. These results suggest that as BC dosage was increased, the cytoprotective effect of the compound was diminished and may include deleterious prop¬erties as a high dose of BC might decrease its quenching ability and increase the oxidative stress on cardiomyoblasts [16,26]. Several studies also reported that low-dose BC might prevent apoptosis by activating BCDO2 that is localized in mitochondria by reducing the mitochondrial membrane potential, activating caspase 3, leaking cytochrome c and Apaf-1 from the mitochondria, changing cellular morphology and eventually chromatin condensation so that cells could be protected against apoptotic responses [27]. Contrarily, high-dose BC induces apoptotic effects in breast cancer cell lines at 5 µM and 10 µM after 48 h because of toxic-dose therapeutic levels [28].

In response to cellular stress, autophagy plays a cytoprotective role [29]. BC induces autophagy, stimulates removal of damaged cellular structures, activates an antioxidant defense mechanism, inhibits a proinflammatory response, which plays critical role in cell survival, and might prevent cellular senescence to achieve a better quality of life [30]. Further, autophagy plays an important role in response to starvation adaptation, mitochondria renewal, cell death, invading pathogens, and cellular quality control [31,32]. Cardiomyoblasts seem to retain proliferation in response to stress, but it is inefficient [33]. Thus, in physiological stress such as aging, autophagy and mitophagy are involved in the regulation of cardiac homeostasis in response to stress, and their downregulation is commonly observed in senescent hearts [34]. In the present study, low-dose BC suppressed LC3II expression and decreased p62 levels, indicating that hyperflux autophagy was taking place (Figure 3B, 3C). Low-dose BC likely boosts autophagic signal fusion between autophagosomes and lysosomes, which can be visualized using microtubule-associated protein light chain 3 (LC3) [35]. LC3 is conjugated to phosphatidylethanolamine to form LC3II, which is localized to isolation membranes and autophagosomes (on both outer and inner membranes) [36]. However, since this process becomes more dynamic than usual, LC3II, which is supposed to be upregulated, becomes suppressed because it cannot maintain the hyperflux signal. Since autophagy is a highly dynamic, multistep process, the mere detection of the number of autophagosomes and the presence of LC3II processing is insufficient and sometimes interpreted inappropriately for an overall evaluation of the entire mechanism, since LC3-II itself may be degraded by autophagy [34,37–39]. Mature autophagosomes that have acquired syntaxin17 (STX17) fuse with the lysosome to degrade their contents and then degrade p62, suggesting that p62 reduction is mediated by autophagy. Thus, p62 is an alternative method for detecting the autophagic flux and is widely used to monitor autophagic activity because p62 directly binds to LC3 and is selectively degraded by autophagy [37,38,40].

We know that mitochondria are the main target organelles experiencing the negative effects of ROS accumulation within certain cells (cardiomyocytes in the present case) and are closely associated with incidences of myocardial I/R injury. Mitochondrial oxidative dysfunction and damage is likely due to the reduction of the counterbalance of ROS capability of mitochondria. These oxy-radicals are short-lived species, are highly reactive, and are known to initiate damage at or near the site of their formation in a cardiomyoblast. Low-dose BC is expected to quench free radicals by improving mitochondrial functions. Even though BC does not stimulate COX4 in the present study (Figure 4B), low-dose BC tended to increase Tom20 mitochondria marker levels (Figure 4C), indicating that BC may support mitochondrial function and cell viability, although the exact mechanism is not yet known. BC has been shown to act at least *in vitro* as an antioxidant, with high potential as a liposoluble radical quencher and a singlet oxygen [41]. Kaulmann et al. reported that as lipid-soluble molecules, BC scavenges radicals in the lipid phase. Thus, within cells, BC is easily affiliated with various membranes including the outer cell membrane, the outer layer of mitochondria

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expressed by Tom20, and the nucleus. Consequently, BC protects cellular membranes and lipoproteins against damage by peroxyl radicals [42,43]. Impairment of Tom20 from oxidative stress could lead to disruptions of cell homeostasis and signaling [24,44–46]. Also, COX4 deficiency is associated with mutations in COX4I1 DNA as a result of direct damage to mtDNA induced by I/R injury, where COX subunits are encoded [47], which alters the expression of OXPHOS complexes, leads to mitochondrial dysfunction, and triggers abnormal cell death through apoptosis [44,48]. Therefore, Tom20 and COX4 play important roles as markers of the mitochondrial renewal involved in cellular viability. Still, this requires further research of these markers' exact pathways in relation to BC.

β-carotene-oxygenase 1 is an enzyme that localizes in cytoplasm and converts a number of carotenoids, including BC, into retinoids and asymmetric apocarotenoids β-carotene-di-oxygenase 2, a mitochondrial carotenoid-oxygenase with broad substrate specificity. Their activity depends on the dosage that appears to be bioactive and genetic factors, including interactions with cellular signaling cascade proteins, such as NF-κB, mitogen-activated protein kinase, or Nrf2 [49–51]. Low-dose BC increases the translocation of Nrf2, a master transcription factor responsible for the expression of mitochondrial antioxidant active enzymes such as GPx, CAT, and SOD (Figure 5D) to the nucleus, contributing to the lowering of the peroxidation or oxidative stress of polyunsaturated fatty acids [52], protecting against cellular toxicity, and maintaining mitochondrial homeostasis, possibly through stimulation of the Nrf2/ARE pathway [17,42,53]. This process is parallel with the suppression of Nf-κB levels (Figure 5B), which is involved in the inflammatory response that leads to decreased cytokines levels, such as TNF-α, IL-1β, IL-6, and cyclooxygenase 2, cellular stress responses [42], downregulated expression of intercellular cell-adhesion molecules, vascular cell-adhesion molecules, and E-selectin in response to endothelial inflammatory and inducible nitric oxide synthase, chemokines, and prostaglandins. Therefore, BC might provide a cytoprotective effect as an antioxidant, reducing cellular inflammation due to oxidative stress, which is very beneficial. However, BC does not appear to influence the production or the activation of Nrf1 (Figure 5C). Nrf1 and Nrf2 express similar binding specificities and profiles; these 2 factors have been suggested to play overlapping roles in regulating basal expression of ARE-containing antioxidant genes, whereas inducible expression largely depends on Nrf2. Although Nrf1 and Nrf2 bind in equal affinity to the ARE in the metallothionein-1 (MT-1) gene promoter, unlike Nrf2, which has been shown to cause immediate activation of genes regulated by ARE, upregulation of Nrf1 gene expression is driven by the MT-1 promoter. Nrf1 has also been

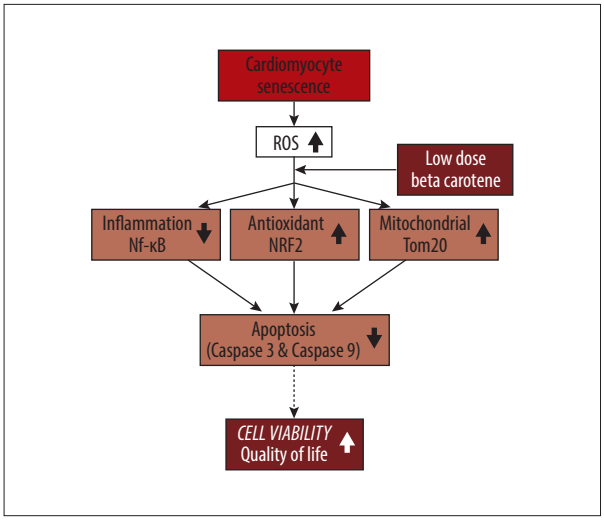

Figure 6. Proposed schematic mechanism.

reported to be glycosylated. This glycosylation may also play a role in managing the expression and localization of Nrf1 in the cell, which is primarily in the endoplasmic reticulum as an integral membrane protein. These findings raise the issues of how Nrf1 gets access into the nucleus and whether Nrf1 has any functional role in the mitochondria of cardiomyocytes [53]. Further studies on potential oxidative stress markers such as malondialdehyde, protein carbonylation, or several genes related to the formation of antioxidant defense such as GPx, SOD, and CAT might be useful [10,41].

Conclusions

A low dose of BC supplementation was most effective and beneficial for cardiomyoblasts by countering oxidative stress (Figure 6). BC increased the antioxidant response via the Nrf2 pathway, inhibiting proinflammatory factors, autophagic modulation, and suppression of apoptosis, thus increasing cellular survival and viability rates in a relatively safe range of BC doses. However, the mechanism in mitochondria is not yet clearly known.

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Conflict of interest

None.

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