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Caffeic Acid Phenethyl Ester (CAPE) Reduces LDH Release and Cell Cytotoxicity in Cardiomyocyte

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Abstract

Background: Ischemia cardiomyocyte undergo death or damage has been identified as essential process in the progression of heart failure. Under hypoxic conditions, mitochondria can represent a threat to the cell because of their capacity to generate toxic reactive oxygen species (ROS). **Aims:** As ROS appear to have a critical role in heart failure, there has been considerable interest in identifying the candidate component or compound to reduce cell death via oxidative stress inhibition. **Methods:** In this study, we used human cardiomyocyte and embryonic rat heart derived H9c2 cells as cell models to speculate the role of ROS in cardiomyocytes. **Results:** Results showed that hypoxia or hydrogen peroxide (H₂O₂) induced cells Lactate dehydrogenase (LDH) release and cytotoxicity. Interestingly, caffeic acid phenethyl ester (CAPE) reverses hypoxia-induced LDH release and cell death in human cardiomyocyte, as well as ROS scavenger, Tiron also prevents H₂O₂ induces LDH release and cytotoxicity. **Conclusion:** Results demonstrate that reduction of cell death in cardiomyocytes by CAPE is associated with a decrease in cellular LDH level and ROS production.

1. Introduction

Coronary artery disease (CAD) are major diseases causing heavy burden of many countries and people around the world [1]. It has been reported that the atherosclerosis, the main cause of CAD, is involved in endothelial dysfunction and inflammation [2-4]. Furthermore, Lavie et al. reported that exercise is a secondary prevention of CAD [5], and some reports indicated that exercise seems to be improved the endothelial function [6, 7]. Nitric oxide (NO) plays a critical role in regulation of endothelial function. Production of NO is either increased by endothelial nitric oxide synthase (eNOS) enzymes [8-10] or reduced by reactive oxygen species (ROS) [11]. ROS production is increased in mitochondria upon hypoxia, as well as, ischemic preconditioning (IPC) [12, 13]. Additionally, hypoxia-inducible factor transcription factors (HIF) is upregulated upon hypoxia [14], and triggers the expression of genes involved in oxygen transport, glycolytic metabolism, cell death, cell survival, and other processes that can affect cell survival in ischemia [13].

Caffeic acid phenethyl ester (CAPE) is the major active element of propolis and has an anti-proliferative effect on tumor cells [15, 16]. The antioxidative

activities of CAPE have been reported *in vitro* and in different biological systems [17, 18]. Moreover, it has been reported that administration of CAPE is useful in delaying age-related cellular damage in cardiovascular system *in vivo* [19]. However, the effect of CAPE on human cardiomyocyte is still unclear. In this study, we investigated the role of CAPE on these events.

2. Materials and Methods

2.1. Cell Lines and Cell Culture

Human cardiomyocyte (HCM) (PromoCell GmbH, Heidelberg, Germany) were cultured at 37°C in T-25 flasks (Corning Glassworks, Corning, N.Y., USA) in Myocyte Growth Medium (PromoCell GmbH, Heidelberg, Germany) supplemented with 0.05 ml/ml fetal calf serum, 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 µg/ml insulin in a 5% CO₂/95% air atmosphere. The culture medium was replaced every 2 days. Once the cells reached 70–80% confluence, they were trypsinized and seeded on 6-well plastic dishes for the following experiments. Passage 3–9 HCMs were used in the experiment. In addition, the embryonic rat heart derived H9c2 cells were cultured in cultured at 37°C in T-25 flasks (Corning Glassworks, Corning, N.Y., USA) in DMEM (Gibco, New York, N.Y., USA) supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 U/ml, Sigma, St. Louis, Mo., USA) in a 5% CO₂ /95% air atmosphere. The culture medium was replaced every alternate day. Once the cells reached 70–80% confluence, they were trypsinized and seeded on 6- or 24-well plastic dishes for the following experiments.

2.2. Lactate Dehydrogenase (LDH) Release and Cytotoxicity Assay

The analysis was performed using the LDH Cytotoxicity Assay Kit (Pierce) according to the manufacturer's instructions. Statistical significance for all the experiments was determined by performing the t test. Error bars are used to indicate the standard errors of the means and p values of < 0.01 were considered significant.

2.3. Western Blotting

Cells were pelleted and resuspended in ice-cold RIPA buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1% dilution of Sigma protease cocktail, and 1% Triton X-100). Samples were centrifuged at 14,000 g for 20 min at 4°C to yield cell lysates. Proteins were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed onto a nitrocellulose membrane. Immunoblotting was performed using specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling), and peroxidase activity was assessed using an enhanced chemiluminescence kit (Perkin-Elmer Life Science, Boston,

MA, USA). The intensities of the reactive bands were analyzed using UVP Biospectrum (UVP LLC, Upland, CA, USA).

2.4. Establishment of Hypoxic Culture Condition

Hypoxic conditions was assessed using a method previously described [20] with some modifications. Briefly, cells were grown on 6- or 24- well plastic dishes, in a hypoxia chamber and equilibrating for 30 minutes with humidified gas containing 1% oxygen, 5% CO₂ and 94% nitrogen (Hypoxic incubator APM-30D, Astec, Tokyo). The cell lines were maintained under hypoxic conditions for various time courses. Control cells were grown in normal oxygen conditions for the same duration.

3. Results

3.1. Effect of H₂O₂ on Cell LDH Release and Cytotoxicity

LDH is well known as a biomarker for cell cytotoxicity and cytolysis. In addition, loss of intracellular LDH and its release into the culture medium has been reported as an indicator of irreversible cell death via cell membrane damage [21]. To study the effect of oxidative stress on cell cytotoxicity, the cells were exposed to extracellular H₂O₂ as oxidative damage [22]. Results showed that H₂O₂ increased LDH release and caused cell cytotoxicity increasing in a dose-dependent manner (Fig. 1).

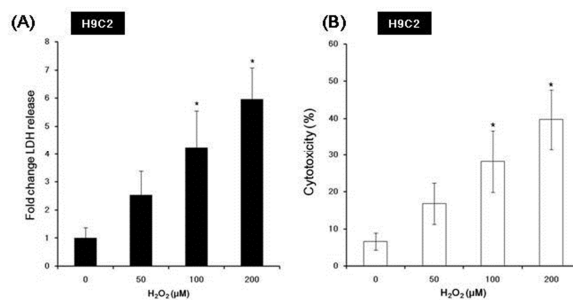


Fig. 1. Effect of different concentrations of H₂O₂ on (A) LDH release and (B) cytotoxicity.

3.2. ROS Accumulation is Required for Hypoxia-Induced Cell LDH Release or Cytotoxicity

It has been reported that ROS accumulation play an important role in the initiation of programmed cell death during myocardial infarction [23]. We examined the effect of ROS scavenger, Tiron on H₂O₂ -induced LDH release in H9c2 cardiomyocyte. Our data showed that under H₂O₂ treatment, LDH release and cytotoxicity were dramatically increased but diminished by the addition ROS scavenger, Tiron in a dose-dependent manner in H9c2 cells (Fig. 2 A and B). Interestingly, the similar results were observed in 30 µM

CAPE-treated human cardiomyocyte under hypoxia (Fig. 2C). These results indicated that CAPE may consider as a potential ROS scavenger to protect cell damage or death.

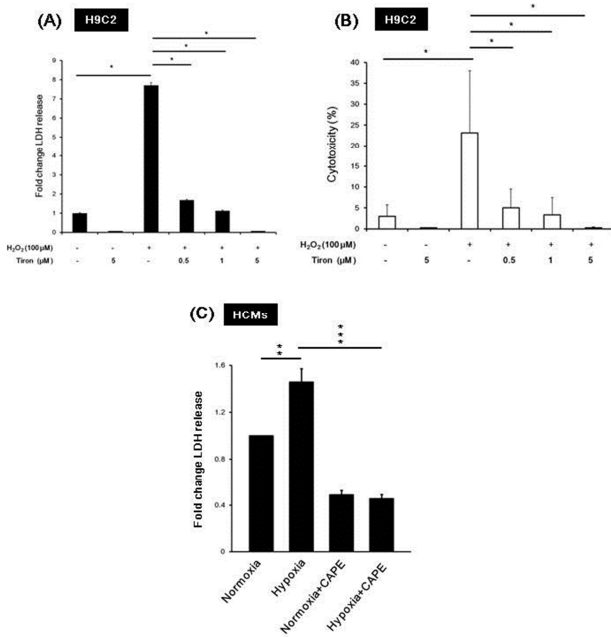


Fig. 2. ROS scavenger, Tiron decreased LDH release (A) and reversed cytotoxicity increasing (B) under H₂O₂-treatment in H9c2 cells. (C) CAPE attenuated hypoxia-induced LDH release in human cardiomyocytes (HCMs).

3.3. Effect of CAPE on p53 Expression Under Hypoxia in Human Cardiomyocyte

It is well known that p53 activation is associated either with cell cycle arrest and DNA repair or with apoptosis [24]. Results showed that 30 μM CAPE treatment reversed hypoxia-induced p53 overexpression (Fig. 3). Therefore, we suggested that CAPE controls ROS accumulation and cell death in cardiomyocyte.

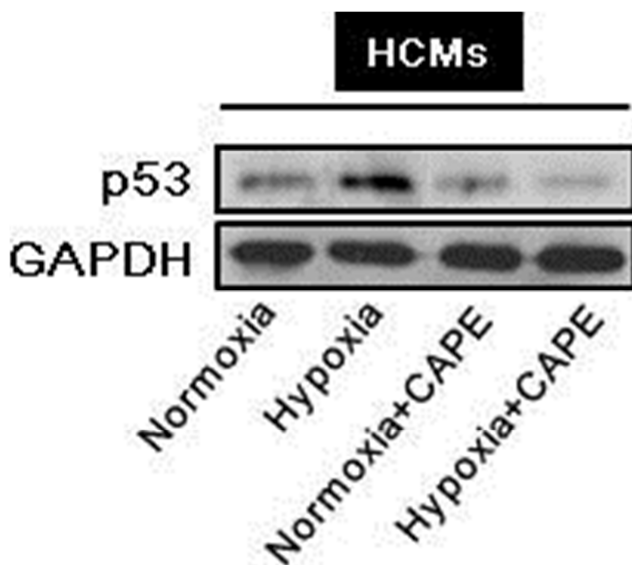


Fig. 3. CAPE inhibited p53 up-regulation under hypoxia in human cardiomyocytes (HCMs).

4. Discussion

The aim of tissues engineering is to apply the principles of engineering and life science toward the development of biological substitutes that maintain, restore, or improve tissue [25]. In clinical, new drug and vascular bypass have improved the quality of life for patients with cardiovascular disease, but have not necessarily decreased morbidity or mortality [26]. Furthermore, Tateishi-Yuyama et al. reported that autologous transplantation of bone-marrow-derived progenitor cells is a potential therapy of angiogenesis for patients with limb ischaemia [27]. Autologous cell therapies using bone marrow-derived or circulating blood-derived progenitor cells are safe and provide beneficial effects to therapeutic angiogenesis/vasculogenesis of ischemia diseases [28, 29]. Additionally, human embryonic stem cells (hESCs)-derived endothelial cell could be beneficial for potential applications such as engineering new blood vessels, endothelial cell transplantation into the heart for myocardial regeneration, and induction of angiogenesis for treatment of regional ischemia [30]. However, with regard to ethical issues of ESCs, epithelial progenitor cell (EPC)-derived from peripheral blood are more considerable as cell source for cell therapy [31]. EPC is a potential inexhaustible source of functional vascular cells that shows an important feature of mature EC for regenerative medicine. However, it is difficult to define the EPC generated from different source, because EPC lack a unifying phenotype [32]. Glaser et al. suggested that the categories of EPC include the colony-forming unit-Hill cells, circulating cells, and endothelial colony-forming cells (ECFC) [33].

Oxidative stress-induced apoptotic signaling can cause several pathological conditions, including the development and progression of heart disease, which are a consequence of the increases ROS or decreases in antioxidants, as well as a disruption in the intracellular redox homeostasis [34-36], however, there have been no reports on how CAPE regulates ROS production linked to effect of cardiomyocyte. It has been reported that H9c2 cells are considered as a cell model to study cardiac disease in response to oxidative stress conditions [37, 38]. Importantly, we also studied the role of CAPE on human cardiomyocyte.

5. Conclusion

In the present study, we show that CAPE decrease ROS accumulation and cell death in cardiomyocyte by LDH releasing and cytotoxicity analysis. Our pharmacological findings support further development of CAPE as a novel therapeutic agent for treating hypoxia or ischemia -related heart disease.

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