The effects of Caffeic Acid Phenethyl Ester (CAPE) on hydrogen peroxide-induced oxidative stress in rat H9c2 myoblasts compared to common antioxidants

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Introduction

Cellular injury from oxidative stress, the result of increased reactive oxygen species (ROS) formation and/or decreased antioxidant reserves, is a common feature in many cardiovascular diseases.[1] High levels of ROS have a harmful effect on the functional and structural integrity of the tissue, damaging proteins and lipids leading to loss of organelle function and cell death/apoptosis.[2] However, common antioxidants (e.g. vitamin E) failed to show any benefits for cardiovascular disease patients in clinical studies. Therefore, development of a novel compound to mitigate oxidative stress is clinically necessary. Caffeic Acid Phenethyl Ester (CAPE, Figure 1), a natural compound derived from honeybee propolis, exhibits not only anti-oxidant but also anti-proliferative and anti-inflammatory effects.[3] Our lab has shown that CAPE given during reperfusion significantly reduces hydrogen peroxide (H_2O_2) levels in an I/R model.[4] We have also reported that the cardioprotective effects of CAPE may be mediated by increasing the bioavailability of heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme degradation.[5] HO-1 has shown upregulation in treatment with CAPE under oxidative stress in HUVEC cells.[6] However, no studies have explored if CAPE pretreatment in H9c2 myoblasts following H_2O_2-induced injury can be cardioprotective by inducing HO-1. In this study, we compared the effects of CAPE to several known antioxidants (caffeic acid, vitamin C, and a vitamin E analog trolox) in H9c2 cells under oxidative stress induced by H_2O_2. We also investigated CAPE's effects on intracellular reactive oxygen species (ROS) and heme oxygenase-1 (HO-1) induction in H_2O_2-induced H9c2 cells.

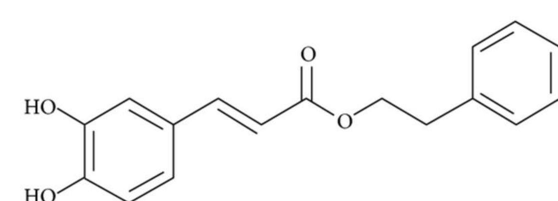

Figure 1. CAPE chemical structure.

Hypothesis

We hypothesized that H9c2 rat myoblasts pretreated with CAPE would provide cardioprotection compared to common antioxidants under H_2O_2-induced oxidative stress. Given CAPE's ability to limit cell death, we also hypothesized that CAPE pretreatment would reduce intracellular ROS production in H_2O_2-induced H9c2 cells. Further, we hypothesized that increasing CAPE dosage would lead to increased HO-1 induction.

Methods

Measurement of H9c2 cell viability after hydrogen peroxide and test compound incubation: H9c2 rat myoblasts (American Type Culture Collection) were cultured, maintained, and seeded 24 hours prior to experiments. Cells were treated with various doses of H_2O_2 (100-700 μM) for 24 hours to evaluate H_2O_2 dose effects on cell viability. To determine each test compound's effect on H9c2 cells under oxidative stress, cells were pretreated with CAPE (1-40 μM), trolox (50-400 μM), caffeic acid (1-40 μM), or vitamin C (100-5000 μM) for 24 hours, then incubated with H_2O_2 (500 μM) for an additional 24 hours. Cell viability was determined by measuring absorbance at 450 nm using a CCK-8 assay (Dojindo Molecular Technologies). Cellular morphology was observed using microscopy.

Measurement of H9c2 intracellular ROS production following hydrogen peroxide and CAPE pretreatment incubation: H9c2 rat myoblasts were incubated with 25 μM non-fluorescent and cell permeable dichlorofluorescein diacetate (DCFDA, Abcam) for 45 minutes. After washing of DCFDA with incubation buffer, cells were treated with various doses of CAPE (1-40μM) and H_2O_2 500 μM. After incubation, the fluorescence, excited at 480 nm, was recorded at 520 nm using a Fluroskan Ascent CF scanner (Thermo Scientific) at 1 hour and 24 hours post-treatment. The fluorescent signals were normalized to the initial control at time zero. The relative fluorescence served as an index to the production of reactive oxygen species.

Methods

Measuring HO-1 expression in CAPE-pretreated H9c2 rat myoblasts: Confluent H9c2 cell plates were treated with various CAPE concentrations (1-40 μM) and incubated for 24 hours. Cells were lysed and protein lysates collected using RIPA buffer and centrifugation. 50μg of protein was loaded into 4-16% Bis-Tris Gel (Novex) and electrophoresis run at constant pressure and 100V for one hour. Gel was transferred to Nitrocellulose membrane, blocked in Fast Western Antibody Diluent (Thermo Fisher) for one hour, and incubated overnight with HO-1 polyclonal antibody (Enzo) at 4°C. Blots were then washed, incubated with fast western optimized HRP reagent, and proteins detected using detection reagents from the fast western kit according to the manufacturer's instructions. Membranes were then stripped and re-probed for the internal control, β-actin.

Statistical Analysis: All experiments were performed in triplicate and repeated at least three times. All values are presented as a mean ± SE. Data were analyzed using ANOVA by a Fisher's PLSD post hoc test. Values of $p < 0.05$ were considered statistically significant.

Results

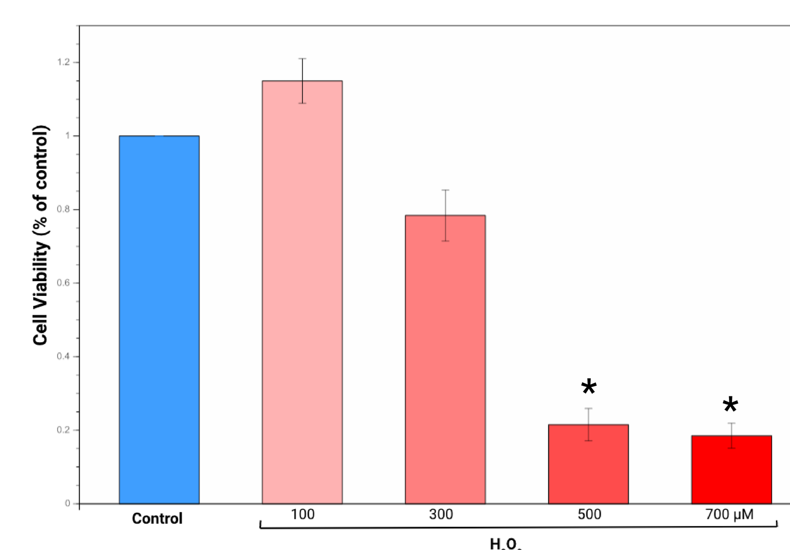

Figure 2. Dose-dependent effects of 24 hour pretreatment on H_2O_2 (100-700 μM) on H9c2 myoblasts (n=4, *: $p < 0.05$ vs. control).

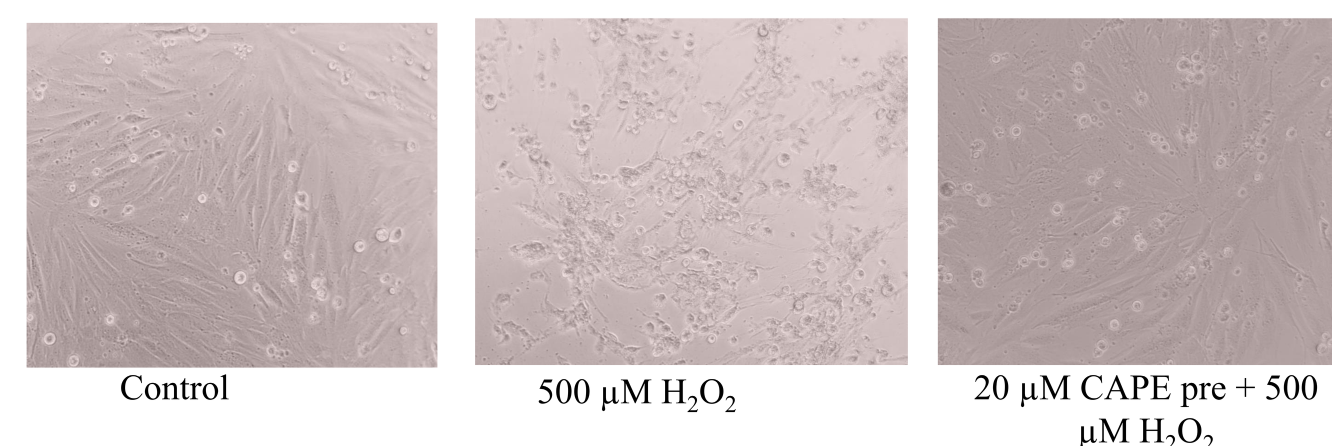

Figure 3. Representative microscopy of H9C2 cells (10X) under control, 500 μM H_2O_2, and 20 μM CAPE pretreatment with 500 μM H_2O_2.

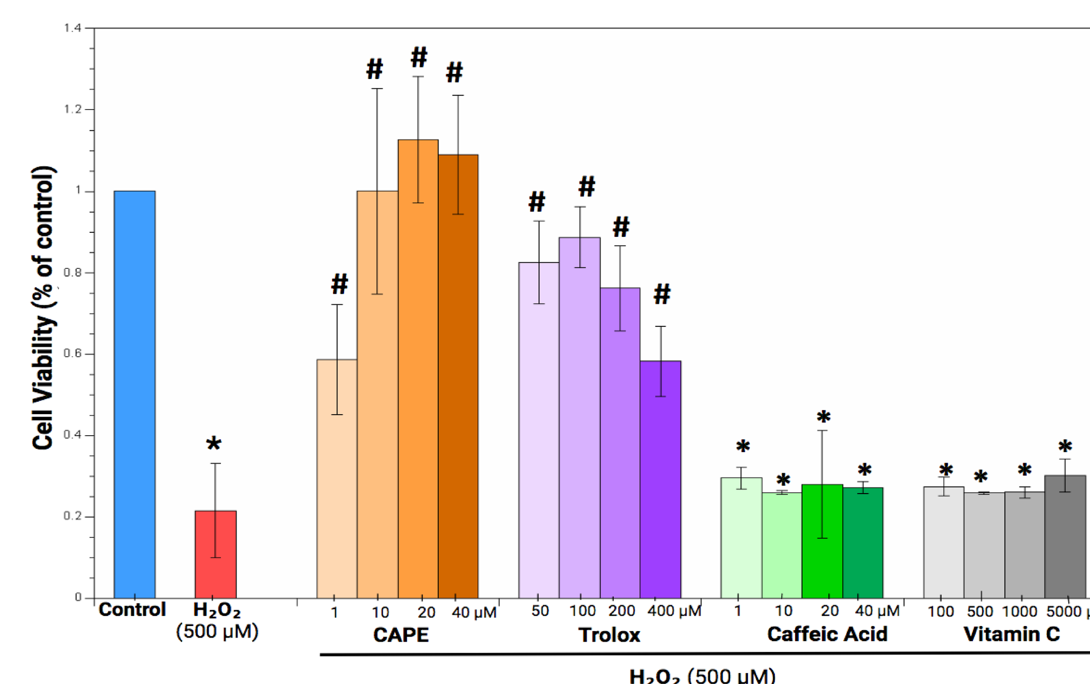

Figure 4. Dose-dependent effects of CAPE (1-40 μM, n=5), Trolox (50-400 μM, n=4), Caffeic Acid (1-40 μM, n=5), and Vitamin C (100-5000 μM, n=3) 24 hour pretreatment on H_2O_2 (500 μM)-induced cell death (*: $p < 0.05$ vs. control, #: $p < 0.05$ vs H_2O_2).

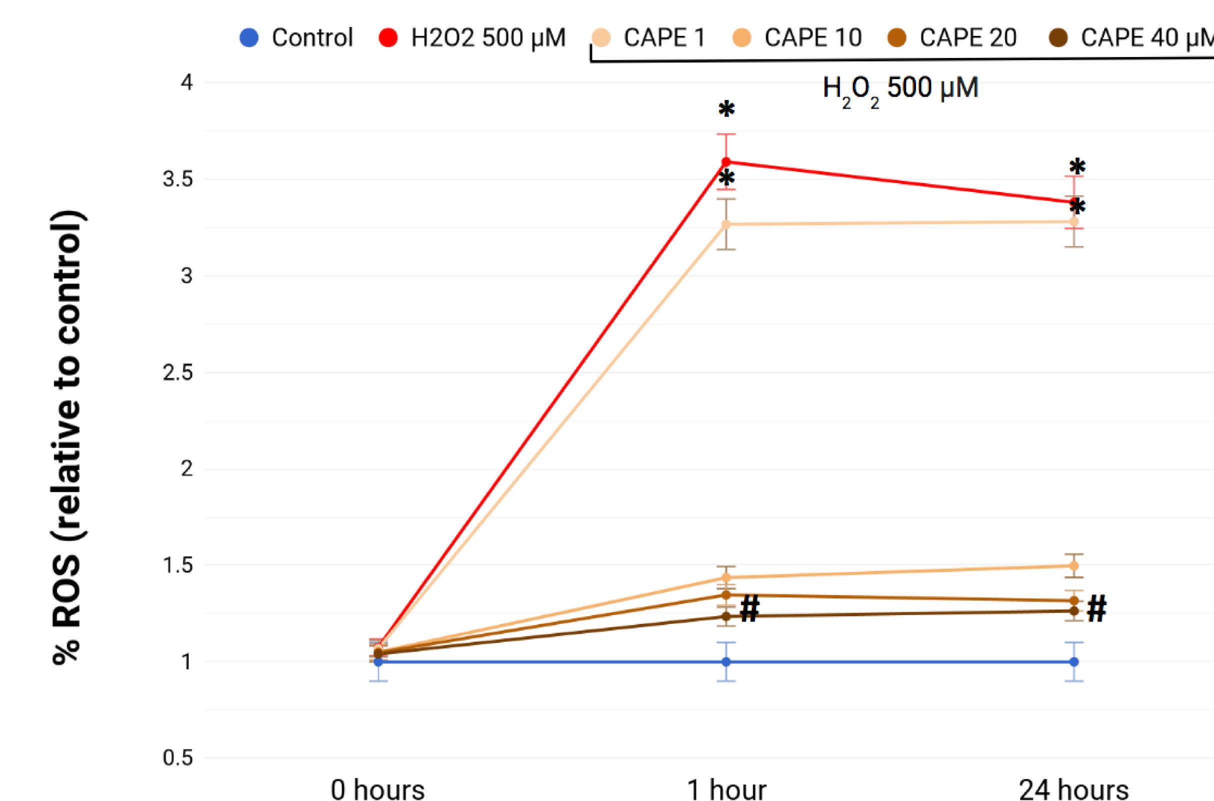

Figure 5. Dose-dependent effects of CAPE (1-40 μM, n=3) on H_2O_2-induced H9C2 intracellular ROS compared to control (n=3, *: $p < 0.05$ vs. control, #: $p < 0.05$ vs H_2O_2).

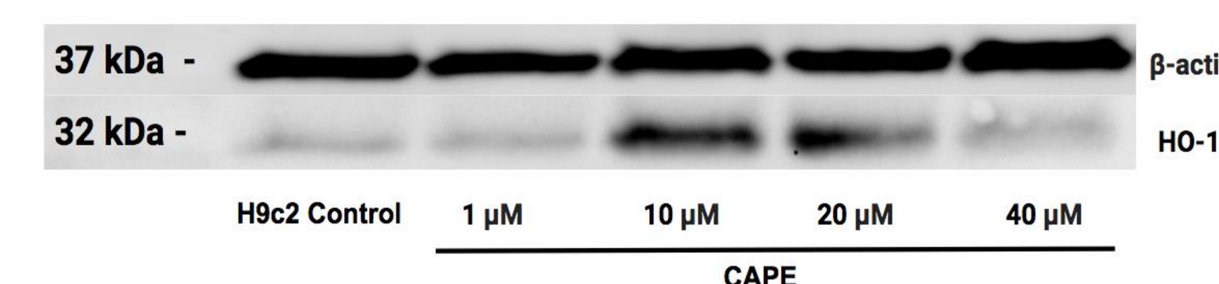

Figure 6. Dose-dependent effects of CAPE pretreatment (1-40 μM) on HO-1 induction in H9c2 cells. β-Actin was probed as internal control.

Conclusions

We found that H_2O_2 dose-dependently reduces H9c2 cell viability. CAPE and Trolox dose-dependently rescue cell viability, while caffeic acid and vitamin C do not protect against H_2O_2-induced cell damage. We demonstrated that CAPE dose-dependently reduces intracellular ROS and induces HO-1 expression. In summary, CAPE cardioprotective and antioxidant effects in H9c2 myoblasts be mediated by HO-1 induction. Future studies will test if CAPE's effects are reduced by using a HO-1 inhibitor in H9c2 myoblasts under oxidative stress.

References

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Acknowledgements

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