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Report

The antioxidant caffeic acid phenethyl ester induces apoptosis associated with selective scavenging of hydrogen peroxide in human leukemic HL-60 cells

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Caffeic acid phenethyl ester (CAPE), an active component of propolis, has many biological and pharmacological activities including antioxidation and tumor cell cytotoxicity. We examined the type of cell death in human leukemic HL-60 cells after CAPE treatment in order to elucidate the relationship between CAPE-induced alterations of the redox state and apoptosis. CAPE treatment (6 μg/ml) resulted in marked growth inhibition up to 70.3$\pm$4.0% at day 2. This inhibition was partially blocked by pretreatment with *N*-acetyl-L-cycteine (NAC). Agarose gel electrophoresis showed evident DNA fragmentation after CAPE treatment. CAPE induced a significant decrease in mitochondrial transmembrane potential to about half of the untreated level after 6 h and a rapid depletion of intracellular glutathione (GSH) down to 41.7$\pm$6.0% after 1 h. Pretreatment of HL-60 cells with NAC reversed the GSH depletion and partially rescued cells from CAPE-induced apoptosis. With regard to intracellular reactive oxygen species, CAPE caused a fast and profound scavenging of H_2O_2 (19% of untreated cells after a 2-h treatment) but not of superoxide anion. These results suggest that apoptosis induced by CAPE is associated with mitochondrial dysfunction, GSH depletion and selective scavenging of H_2O_2 in human leukemic HL-60 cells. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, caffeic acid phenethyl ester (CAPE), glutathione, hydrogen peroxide, HL-60 cells, mitochondria.

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Introduction

Honeybee propolis has been widely used as a folk medicine. Caffeic acid phenethyl ester (CAPE), an active ingredient of propolis,[1] has a broad spectrum of biological activities including antioxidant,[2–4] anti-inflammatory,[5–7] anti-viral actions,[8] prevention of reperfusion injury[9] and anticancer effects.[1,10,11]

Apoptosis of tumor cells can be induced via different pathways by various treatments such as irradiation,[12] Fas/APO-1 ligand[13] and cytotoxic agents.[14,15] There is convincing evidence that disturbance of mitochondrial function is involved in the apoptotic processes.[16,17] Loss of mitochondrial transmembrane potential and a subsequent generation of reactive oxygen species (ROS) have been demonstrated as early events following induction of apoptosis by various stimuli.[18] However, several reports have indicated that ROS alone activate apoptosis, but are not required to execute the apoptotic processes.[19,20] Glutathione (GSH), a major intracellular thiol-containing antioxidant, maintains an optimal cellular redox potential to protect cells against oxidative stress.[21] One investigation has shown that thiol depletion can effectively activate caspase-3 and subsequently induce cancer cell apoptosis.[22] Taken together, these findings demonstrate a close relationship between the cellular redox state and execution of apoptosis.

CAPE has been observed to induce apoptosis in transformed cells and cytotoxicity in oral cancer cells,[23] but the mechanism remains unclear. Differential cytotoxicity toward tumor cells has been demonstrated through modulating the cellular redox state.[23] Hence, CAPE is a potent antioxidant as well as an apoptosis-inducing agent; however, conceptually,

these two activities seem to conflict. To elucidate the relationship between these two activities, we assessed changes in the redox state during CAPE-induced apoptosis. In particular, we investigated whether CAPE-induced apoptosis was accompanied with changes in mitochondrial transmembrane potential, intracellular GSH content, hydrogen peroxide (H_2O_2) level and superoxide anion ($O_2^{\cdot -}$) level inside human leukemic HL-60 cells.

Materials and methods

Cell culture and treatments

The human myeloid leukemic cell line, HL-60, was obtained from Dr MAS Moore (Memorial Sloan-Kettering Cancer Center, New York, NY). The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), passaged every 3 days and maintained in an exponential growth status. For induction of apoptosis, the cells were incubated in 35- or 100-mm Petri dishes at an initial concentration of 1×10^5/ml and grown at 37°C in a humidified 5% CO_2 incubator. CAPE was synthesized by Dr Ming-Shi Shiao according to the procedure of Grunberger *et al.*[1] with modification. Purified CAPE (⩾99%) was dissolved in absolute ethanol (stock solution). CAPE (6 μg/ml) was added to the cell cultures with a final ethanol concentration of 0.1% v/v. In our previous studies, this concentration of ethanol had no significant effect on the growth of HL-60 cells. *N*-Acetyl-L-cysteine (NAC) (Sigma, St Louis, MO) 15 mM was added 1 h before CAPE treatment to examine the role of GSH in CAPE-induced apoptosis. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) (Sigma), stocked in dimethyl sulfoxide, was used as a positive control for the generation of ROS.

Cell growth kinetics

After treatment with CAPE, cells were harvested by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ). The numbers of viable cells were counted on day 2 using a Trypan blue dye exclusion test.

DNA extraction and gel electrophoresis

HL-60 cells (1×10^6/ml) were lysed with 0.5 ml lysis buffer (5 mM Tris–borate at pH 8.0, 0.25 ml Nonidet P-40 and 1 mM EDTA), followed by the addition of RNase (Sigma) at a final concentration of 20 μg/ml and incubated at 37°C for 1 h. Cells were further treated with proteinase K (300 μg/ml) for another 1 h and DNA was then isolated from the cultured cells as described.[24] Electrophoresis was carried out on 1.5% agarose gel in Tris–borate buffer (pH 8.0) containing 1 mM EDTA. The DNA on the gel was stained with ethidium bromide.

Quantitation of apoptosis

Hypodiploid DNA was analyzed using propidium iodide (PI) staining and flow cytometry as in our previous study.[24] Both treated and untreated leukemic cells were harvested, washed with PBS and resuspended (1×10^6/ml) in 1.5 ml hypotonic fluorochromic solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) (Sigma) for 60 min at 4°C in the dark. The PI fluorescence of individual nuclei was analyzed by a FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ). As an estimate of the proportion of apoptotic cells, the percentage of hypodiploid cells in a population of 10 000 cells was calculated using ModFIT cell cycle analysis software version 2.01.2 (Becton Dickinson, Lincoln Park, NJ).

Analysis of mitochondrial transmembrane potential

Mitochondrial transmembrane potential was assessed by a modified method of Rottenberg *et al.*[25] using a FACScalibur flow cytometer (Becton Dickinson). Briefly, PBS-washed HL-60 cells were incubated with 40 nM 3,3′-dihexyloxacarbocyanine [$DiOC_{6(3)}$] (Molecular Probes, Eugene, OR) for 15 min at 37°C. Green fluorescence (FL-1 channel) was measured to indicate the change of mitochondrial transmembrane potential with excitation and emission settings of 488 and 530 nm, respectively.

Fluorocytometric analysis of intracellular GSH, H_2O_2 and superoxide anion levels

Intracellular GSH, H_2O_2 and superoxide anion levels were assessed by staining with 5-chloromethylfluorescein diacetate (CMF-DA) (Molecular Probes), 2′-7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) and dihydroethidine (HE) (Sigma), respectively. Briefly, PBS-washed HL-60 cells were incubated with 1 μM CMF-DA, 10 μM DCFH-DA or 10 μM HE for 15 min at 37°C before treatment with CAPE. Cells were treated with CAPE 6 μg/ml and the fluorocytometric analysis was performed every 10 min thereafter. The GSH and H_2O_2 levels were measured with the FL-1 channel (green fluorescence), and the super-

oxide level was assessed with the FL-2 channel (red fluorescence with excitation and emission settings of 488 and 585 nm). The CMF-DA was used to measure the amount of intracellular non-protein thiol. Coates *et al.* have reported that, using this method, the GSH content is proportional to the amount of non-protein thiol.[26]

Statistics

Data are expressed as mean ± SEM. An unpaired Student's *t*-test was used to analyze the differences in growth inhibition and apoptosis between CAPE-treated cells with and without NAC pretreatment. The time-dependent changes in intracellular GSH, H_2O_2 and superoxide levels between untreated and CAPE-treated cells were evaluated by analysis of variance with repeated measurements followed by Bonferroni's test.

Results

CAPE-induced growth inhibition was partially blocked by pretreatment with NAC

Figure 1(a) demonstrates the growth inhibition of HL-60 cells by CAPE (6 μg/ml) in the presence or absence of NAC (15 mM). After 2 days' incubation, the growth inhibition of CAPE-treated cells was 70.3 ± 4.0% compared to the untreated cells. However, the growth inhibition induced by CAPE was partially prevented by pretreatment with NAC (37.3 ± 3.4%), a GSH precursor.

Effect of CAPE on DNA fragmentation in HL-60 cells

After treatment with 6 μg/ml CAPE for 2 h, a DNA ladder was not detectable but became obvious at 6 h (Figure 2).

Loss of mitochondrial transmembrane potential by CAPE treatment

As shown in Figure 3, the mean of $DiOC_{6(3)}$ fluorescence intensity of untreated cells was 106.1 ± 0.3. HL-60 cells exposed to 6 μg/ml CAPE for only 30 min showed a sharp decline to 75.1 ± 2.9 and further loss of $DiOC_{6(3)}$ fluorescence down to 54.4 ± 3.0 after 6 h. The decline of $DiOC_{6(3)}$ fluorescence intensity indicates the loss of mitochondrial transmembrane potential in CAPE-treated HL-60 cells.

Effect of CAPE on intracellular GSH level

After treatment with CAPE, the intracellular GSH level decreased very rapidly, decreasing to be 57.0 ± 8.2% of the level of untreated control cells at 30 min and eventually down to only 41.7 ± 6.0% after 60 min. This depletion of GSH was, however, reversed by pretreatment with 15 mM NAC (Figure 4).

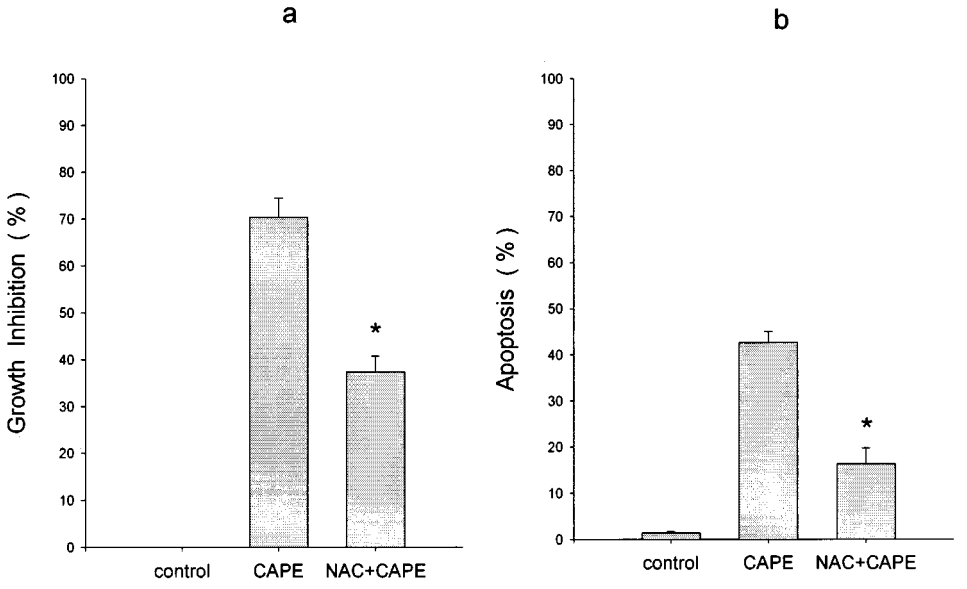

Figure 1. Effect of CAPE (6 μg/ml for 48 h) on growth inhibition (a) and apoptotic percentage (b) of HL-60 cells with or without NAC (15 mM for 1 h) pretreatment. Data from four separate experiments are expressed as mean ± SEM. *$p < 0.05$ when the extents of growth inhibition of NAC-pretreated cells were compared with CAPE-treated cells.

The role of GSH in CAPE-induced apoptosis

Since NAC prevented CAPE-induced GSH depletion and partially blocked CAPE-induced growth inhibition, we wondered whether GSH plays a role in CAPE-induced apoptosis. Our results show that NAC partially blocks the CAPE-induced apoptosis of HL-60 cells (Figure 1b).

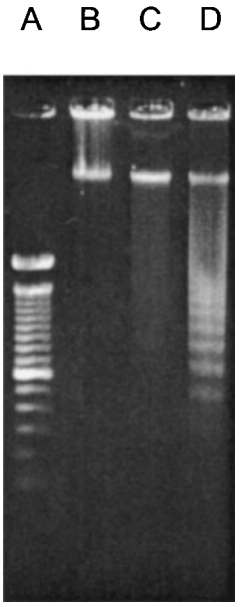

Figure 2. DNA fragmentation of HL-60 cells after CAPE (6 μg/ml) treatment. Lane A, molecular weight marker; lane B, untreated cells; lanes C and D, cells treated with CAPE (6 μg/ml) for 2 h and 6 h, respectively.

Effect of CAPE on intracellular ROS level

As demonstrated in Figure 5(a), the intracellular H_2O_2 level of CAPE-treated cells decreased rapidly, finally down to 19% of the level in untreated cells. However, we found that CAPE did not significantly affect the intracellular superoxide anion level (Figure 5b). In the same experiment, we used TPA 10^{-7}M as a positive control, and it produced large amounts of both H_2O_2 and superoxide anion (data not shown).

Discussion

Our findings indicate that CAPE is a potent apoptosis-inducing agent whose action is accompanied by mitochondrial dysfunction, GSH depletion and selective scavenging of H_2O_2.

Mitochondrial dysfunction, as indicated by loss of mitochondrial transmembrane potential, has been shown to be involved in many types of tumor cell apoptosis.[27] We found that CAPE treatment results in a progressive loss of mitochondrial transmembrane potential at the early stage of apoptosis in HL-60 cells. Moreover, CAPE induced rapid and profound depletion of intracellular GSH earlier than the loss of mitochondrial transmembrane potential. Biswal *et al.* showed that pretreatment with NAC completely blocked the nordihydroguaiaretic acid-

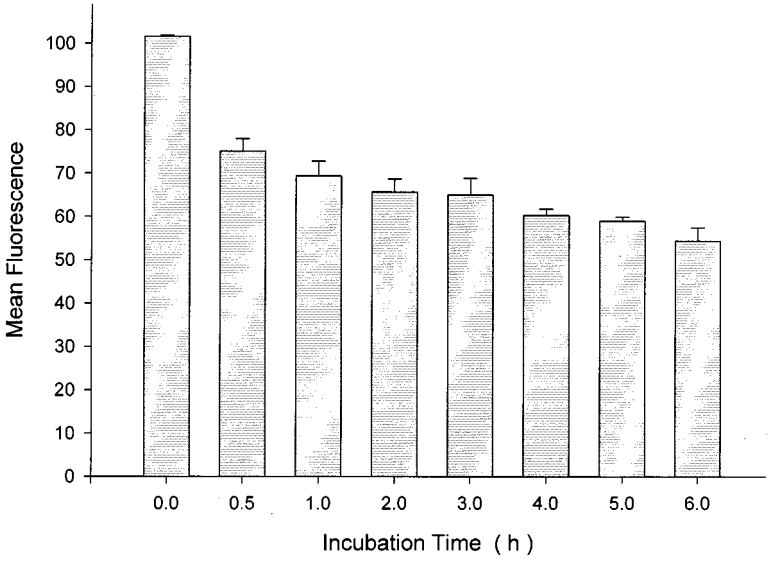

Figure 3. Time-dependent effect of CAPE (6 μg/ml) on mitochondrial transmembrane potential of HL-60 cells. Data from three separate experiments are expressed as mean $\pm$ SEM.

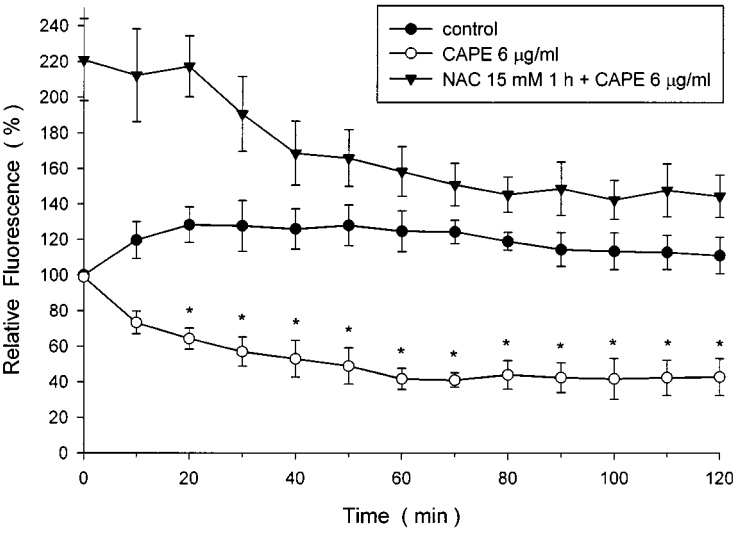

Figure 4. Time-dependent effect of CAPE (6 μg/ml) on intracellular GSH content of HL-60 cells with or without NAC pretreatment. Data from three separate experiments are expressed as mean $\pm$ SEM. *$p < 0.05$ when the GSH contents of CAPE-treated cells were compared with untreated cells.

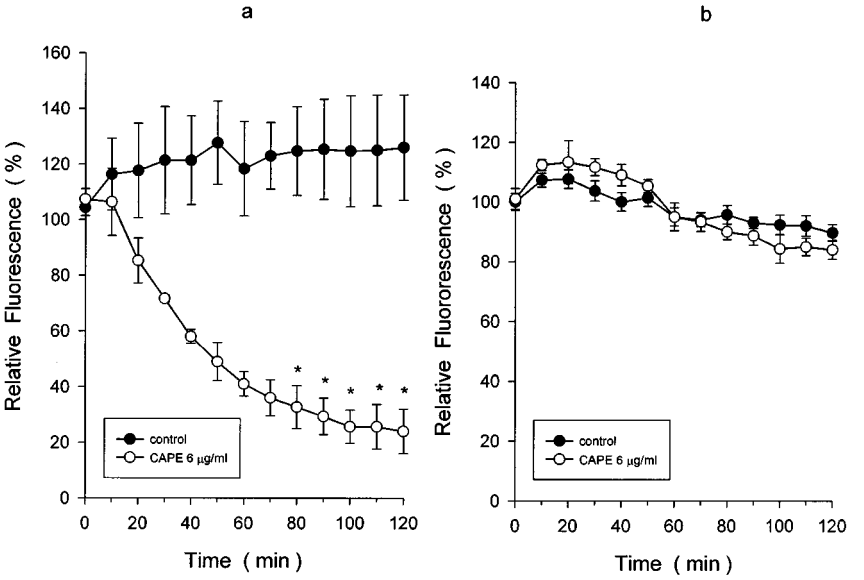

Figure 5. Time-dependent effect of CAPE (6 μg/ml) on intracellular ROS levels of HL-60 cells. (a) H_2O_2. (b) superoxide anion level. Data from three separate experiments are expressed as mean $\pm$ SEM. *$p < 0.05$ when the ROS levels of CAPE-treated cells were compared with untreated cells.

induced loss of mitochondrial transmembrane potential and apoptosis in hematopoietic FL5.12 cells.[28] The observation that pretreatment with NAC partially blocked the CAPE-induced growth inhibition and apoptosis in our study provides evidence for the possible role of GSH. Oda *et al.* reported that GSH depletion is one factor, but not a sufficient one, for the progress of apoptosis in leukemic U937 cells induced by ricin.[29] Thus, CAPE-induced apoptosis of leukemic HL-60 cells may involve multiple mechanisms including GSH depletion and subsequent loss of mitochondrial transmembrane potential.

In addition to its apoptosis-inducing activity, CAPE inhibits TPA-mediated H_2O_2 generation by HeLa cells with ED_{50} about 5 μM.[3] As we found, CAPE can inhibit

even background intracellular H_2O_2 production by HL-60 cells very rapidly and profoundly. Interestingly, there was no significant change in superoxide anion level after CAPE treatment. The mechanism by which CAPE selectively suppresses H_2O_2 formation is not yet known. It could be any of the enzymatic processes involved in the balance of the redox state inside cells, such as alteration in activities of nicotinamide-adenine dinucleotide phosphate oxidase, superoxide dismutase or catalase. Decreased H_2O_2 production has also been shown as an early event during dexamethasone-induced apoptosis of rat thymocytes.[30]

Unlike most chemotherapeutic agents, CAPE possesses both apoptosis-inducing and antioxidant activities, but these two may be independent events. CAPE apparently induces apoptosis by targeting intracellular GSH and mitochondria, whereas the antioxidant activity probably relates to ROS scavenging of H_2O_2.

A high concentration of intracellular thiol is an important cause of resistance to cytotoxic and irradiation in cancer cells.[31,32] Since CAPE depletes GSH, the most important of the thiol reserves, it may be this action that causes it to overcome resistance to chemotherapeutic drugs and radiotherapy in tumor cells. Research on naturally occurring compounds such as CAPE as new adjuncts to cancer treatment is promising, because these compounds have negligible toxicity and they are usually effective at low doses. We suggest that CAPE may resensitize malignant cells that are resistant to radiation and chemotherapy.

Conclusion

CAPE, a known antioxidant, induces apoptosis associated with mitochondrial dysfunction, GSH depletion and selective scavenging of H_2O_2 in human leukemic HL-60 cells.

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