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Involvement of NADPH Oxidase-mediated Generation of Reactive Oxygen Species in the Apototic Cell Death by Capsaicin in HepG2 Human Hepatoma Cells

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Although capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a pungent ingredient in a variety of red peppers of the genus *Capsicum*, has been shown to induce apoptotic cell death in many cancer cells, the exact mechanism of this action of capsaicin is not completely understood. In this study, we investigated the possible mediation of the NADPH oxidase-modulated production of reactive oxygen species (ROS) in the apoptotic mechanism of capsaicin in HepG2 human hepatoblastoma cells. Capsaicin induced apoptotic cell death in a time- and dose-dependent manner. Capsaicin at the concentration of inducing apoptosis also markedly increased the level of ROS. The capsaicin-induced generation of ROS and apoptosis was significantly suppressed by treatment with antioxidants, DPPD and tocopherol. In addition, inhibitors of NADPH oxidase, diphenylene iodonium, apocynin and neopterine, profoundly blocked the capsaicin-induced ROS generation and apoptosis. The expression of Rac1N17, a dominant negative mutant of Rac1, also significantly inhibited the capsaicin-induced apoptosis. Activation of nuclear factor-κB, a transcription factor essentially involved in ROS-induced apoptosis, was also observed by treatment with capsaicin. Collectively, these results suggest that the NADPH oxidase-mediated generation of ROS may be essentially involved in the mechanism of capsaicin-induced apoptosis in HepG2 cells. These results further suggest that capsaicin may be a valuable agent for the therapeutic intervention of human hepatomas.

Keywords: Capsaicin; Apoptosis; Reactive oxygen species; NADPH oxidase; HepG2 cells

Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DPI, diphenylene iodonium; DPPD, *N*,*N*′-diphenyl-*p*-phenylenediamine; FCCP, carbonylcyanide-*p*-(trifluoromethoxy) phenylhydrazone; NF-κB, nuclear factor-κB; NMMA, N^G-monomethyl-L-arginine; PI, propodium iodide; ROS, reactive oxygen species

INTRODUCTION

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), is a pungent ingredient in the genus *Capsicum* that has been widely used as food condiments for almost thousand years. Capsaicin has been shown to inhibit growth and induce apoptosis in various types of cancer cells, but not in normal counterpart cells.[1–4] Intracellular Ca^{2+} and reactive oxygen species (ROS) have been suggested as the signaling molecules associated with apoptotic effects of capsaicin in cancer cells.[3,5] However, the source of ROS generated and the exact mechanism of the apoptotic action of capsaicin were not completely unrevealed.

Excessively produced ROS may result in cellular damage through their interaction with cellular macromolecules and structures.[6] ROS are proposed as a common mechanism by which various agents induce apoptosis.[7] The apoptosis-inducing mechanisms of ROS include activation of proteases and nucleases,[6] altered gene expression[8] and changes in membrane permeability.[6]

Although the major biological process leading to oxygen-derived generation of ROS is electron transport associated with mitochondrial membranes,[9] the membrane-bound NADPH oxidase appeared to

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play a role in production of ROS in conjunction with apoptosis induced by various agents in HepG2 cells.[10,11] The NADPH oxidase is originally known to produce ROS during the respiratory burst in neutrophils.[12] It has also been found functionally active in many non-phagocytic cells.[13–16] Activation of this enzyme proceeds through a multistep assembly at the plasma membrane of several components including the two subunits of cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$), the small GTP-binding proteins (Rac and Rap1A), and the cytosolic factors ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$).[17] These components has also been detected and functionally active in HepG2 cells.[18,19]

Thus, the purpose of this study was to investigate (i) whether capsaicin induces apoptosis in the HepG2 cells, (ii) whether the production of ROS is involved in the mechanism of the capsaicin-induced apoptosis and (iii) whether NADPH oxidase mediates the generation of ROS and apoptosis induced by capsaicin.

MATERIALS AND METHODS

Materials

Powdered Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, capsaicin, tocopherol, DPPD, rotenone, malonate, antimycin A, metyrapone, allopurinol, NMMA, FCCP, hydroxyurea, DPI, apocynin, neopterine, sodium pyruvate, probenecid, PI, ribonuclease A and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). Capsaicin was dissolved in ethanol prior to use. DCFH-DA was from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). DCFH-DA was prepared as stock solutions in dimethyl sulfoxide (DMSO), then diluted with aqueous medium to the final desired concentrations. The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences, Ann Arbor, MI).

Cell Line and Cell Culture

HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). HepG2 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

DNA Isolation and Electrophoresis

DNA isolation was done according to Hockenbery *et al.*[20] HepG2 cells were collected by centrifugation (200*g*, 10 min), washed twice in phosphate buffered saline (PBS) (pH 7.4) and resuspended at a density of 4×10^6 cells/400 μl in hypotonic lysing buffer (5 mM Tris, 20 mM EDTA, pH 7.4) containing 0.5% Triton X-100 for 30 min at 4°C. The lysates were centrifuged at 13,000*g* for 15 min at 4°C. Fragmented DNA was extracted from the supernatant with phenol-chloroform-isoamylalcohol, precipitated by addition of 2 volumes of absolute ethanol and 0.1 volume of 3 mM sodium acetate, and treated with RNAse A (500 U/ml) at 37°C for 3 h. The pattern of DNA fragmentation was visualized by electrophoresis in 1.8% agarose gel containing ethidium bromide and photographed under UV light.

Flow Cytometric Analysis of Apoptosis

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml PI and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine percentage of nuclei with hypodiploid content indicative of apoptosis.[21]

The normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. Thus, annexin-V binding was also employed as an indicator of apoptosis,[22] to demonstrate the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane. It was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). Cells were washed in cold PBS, and resuspended in binding buffer. A portion of cell suspension (500 μl) was exposed to Annexin-V-FLUOS. The cells were gently vortexed, incubated at room temperature for 20 min in the dark, and then analyzed by FACScan within 1 h of staining.

Intracellular ROS Measurement

Relative changes in intracellular ROS in the HepG2 cells were monitored using a fluorescent probe, DCFH-DA.[23] DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed

intracellularly.[24] Cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 h at a final concentration of 5 μM at 37°C. Fluorescence (excitation wavelength set at 485 nm and the emission wavelength at 530 nm) was monitored in a well-stirred cuvette.

Western Blot Analysis

Cells were washed with PBS solution and centrifuged at 1000*g* for 5 min. Cell pellets was lysed for 15 min at 4°C in whole cell extraction buffer containing 50 mM Hepes (pH 7.4), 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 40 mM α-glycerophosphate, 0.1 mM DTT. Lysates were centrifuged at 20,000*g* for 10 min at 4°C, and supernatant proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England) at 30 V for overnight. The membrane was blocked with 5% skim milk in Tween-20 containing Tris buffered saline (TTBS) (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) and incubated with primary anti-human NF-κB (p65) antibody in TTBS containing 5% skim milk. After incubation with horseradish peroxidase-conjugated anti-IgG antibody, immuno-detected proteins were visualized by using enhanced chemiluminescence assay kit (Amersharm Life Science, Buckinghamshire, England). The immuno-blots were scanned and analyzed on an imaging densitometer (UVP, Cambridge, UK).

Transfection of Cells

Cells at 60% confluence were transiently transfected with pEXV-myc-N17rac1, pEXV-wtrac1 or empty pEXV vector with FuGENE6 transfection solution (Roche) in a serum free medium for 24 h. After the medium was changed to fresh full growth medium, cells were further incubated for 24 h, then, treated with capsaicin. The expression levels of transfected wtRac1 and Rac1 mutant proteins were detected by immunoblotting with anti-Rac1 antibody (Santa Cruz Biotechnology).

Data Analysis

All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *p*-Values less than 0.05 were considered statistically significant.

RESULTS

Induction of Apoptotic Cell Death by Capsaicin

The effect of capsaicin on apoptotic cell death of HepG2 cells was examined using three independent methods. Capsaicin induced DNA fragmentation in a concentration-dependent manner studied by agarose gel electrophoresis as depicted in Fig. 1A. Capsaicin also induced loss of phospholipid asymmetry, resulting in appearance of phosphatidylserine on the outer layer of the plasma membrane assessed by annexin-V binding, as depicted in Fig. 1B. Furthermore, capsaicin induced apoptosis in a time-related manner tested by flow cytometry by determining hypodiploid DNA content stained with PI[21] as shown in Fig. 1C. The apoptotic effect of capsaicin was prominent at the concentration of 200 μM. Taken together, these results indicate that capsaicin induced apoptotic cell death in the HepG2 cells.

Role of ROS in the Capsaicin-induced Apoptosis

To determine the role of ROS in the capsaicin-induced apoptosis of HepG2 cells, we first examined whether capsaicin generates ROS assessed by DCF fluorescence.[23] As shown in Fig. 2A and B, capsaicin (250 μM) induced a slow and sustained increase in intracellular ROS level, which was significantly inhibited by antioxidants, DPPD (10 μM) and tocopherol (l00 μM). To determine the role of ROS in the capsaicin-induced apoptosis, we investigated whether DPPD and tocopherol could suppress the apoptosis induced by capsaicin. As shown in Fig. 3C and D, DPPD (10 μM) and tocopherol (l00 μM) significantly lowered hypodiploid DNA content induced by capsaicin, indicating that ROS may be involved in the mechanism of the capsaicin-induced apoptosis in the HepG2 cells.

Role of NADPH Oxidase in the Capsaicin-induced ROS Generation and Apoptosis

To clarify the sites of the capsaicin-induced ROS production, we investigated the effects of various drugs that inhibit the mitochondrial respiratory chain enzymes, cytosolic non-mitochondrial ROS-generating enzymes or plasma membrane NADPH oxidase, on the capsaicin-induced ROS generation and apoptosis. The effects of capsaicin (250 μM) on the ROS level and apoptosis were not significantly altered either by inhibition of mitochondrial electron transport with inhibitors of Complex I,

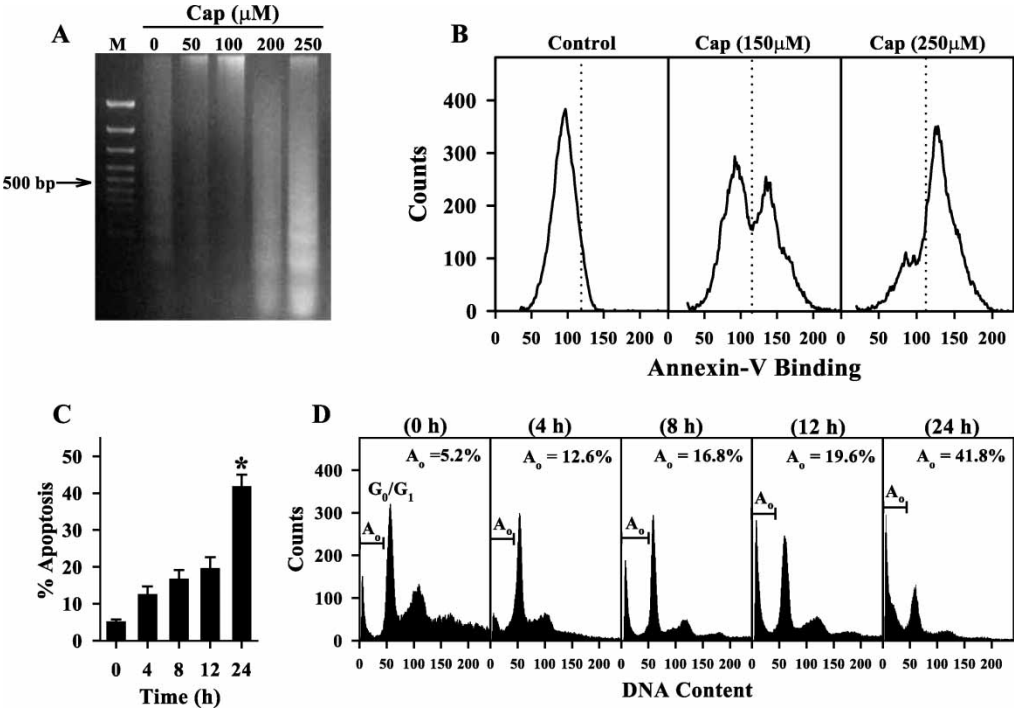

FIGURE 1 Capsaicin (Cap) induces apoptosis in a concentration- and time-dependent manner in HepG2 human hepatoblastoma cells. In the experiments of (A) cells were treated for 24 h with or without each concentration of capsaicin. DNA was isolated from the cells and analyzed by 1.8% agarose gel electrophoresis. Lane M represent DNA marker. In the experiments of (B) the cells were incubated in the absence (control) or in the presence of capsaicin for 24 h. Cells were stained with Annexin-V-FLUOS and analyzed by flow cytometry. Note that in the presence of capsaicin there is a shift in Annexin-V-FLUOUS fluorescence. This is due to the binding of annexin-V to membrane phospholipids of cells undergoing apoptosis. Results are representative of four experiments. In the experiments of (C and D) the cells were incubated with capsaicin (250 μM) for each designated time. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (C) the data represent the mean values of four replications with bars indicating SEM. *$p < 0.05$ compared to control.

succinate dehydrogenase or Complex III (10 μM rotenone + 5 mM malonate and 5 μM antimycin A, respectively) or by the mitochondrial uncoupler, FCCP (0.1 μM) as summarized in Table I. These mitochondrial enzyme inhibitors did not have a significant influence on the basal level of ROS and apoptosis, except that antimycin A and FCCP significantly increased only the basal level of ROS. These results indicate that mitochondria may not be the site of ROS production induced by capsaicin. In addition, the capsaicin-induced ROS production and apoptosis was not significantly influenced by treatment with specific inhibitors of the enzymes which can potentially generate ROS, cytochrome P-450, nitric oxide synthase, xanthine oxidase and ribonucleotide reductase (500 μM metyrapone, 100 μM NMMA, 100 μM allopurinol and 1.5 mM hydroxyurea, respectively). However, inhibitors of the membrane-bound NADPH oxidase, 5 μM DPI,[25] 500 μM apocynin[26] and100 μM neopterine,[27] significantly suppressed both the ROS generation (Fig. 3A and B) and apoptosis (Fig. 3C and D) induced by capsaicin (250 μM). As a matter of fact, it has been detected the activity of NADPH oxidase similar to that found in neutrophils and the presence of Rac1 which is an important component for activation of NADPH oxidase in the HepG2 cells.[18] Thus, we further investigated the involvement of Rac1 in NADPH oxidase-dependent apoptosis by capsaicin by measuring the apoptosis of the cells transfected with wild-type Rac1 (wtRac1) or dominant negative mutant of Rac1 (Rac1N17) in the presence of capsaicin (250 μM). Transfection of the cells with empty vector itself did not affect the capsaicin-induced apoptosis as shown in Fig. 4. HepG2 cells expressing wtRac1 showed enhanced apoptosis, whereas the capsaicin-induced apoptosis was significantly decreased in the cells with Rac1N17. These results strongly suggest that non-phagocytic NADPH oxidase may play an important role in apoptotic cell death induced by capsaicin in the HepG2 cells.

Further Evidence of ROS Involvement in Capsaicin-induced Effects: Activation of NF-κB

Since activation of NF-κB appears to be commonly involved in the ROS-induced apoptotic processes,[28] we further examined whether capsaicin activates NF-κB assessed by Western blot analysis. As depicted in Fig. 5, capsaicin (250 μM) reduced cytosolic NF-κB and I-κB that inhibits NF-κB activation, but increased

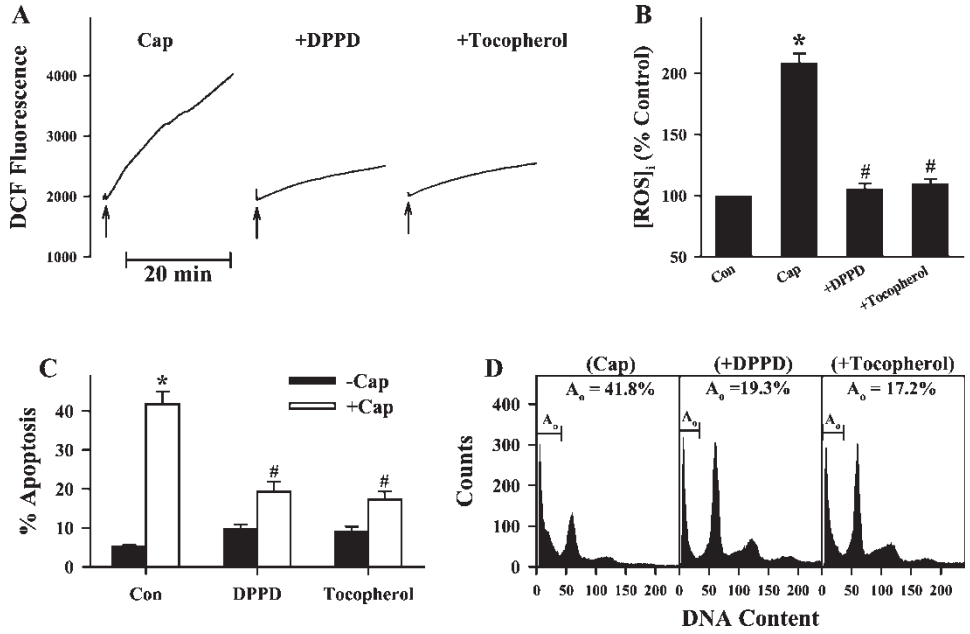

FIGURE 2 Effects of antioxidants on the generation of ROS (A and B) and apoptosis (C and D) induced by capsaicin (Cap) in HepG2 human hepatoblastoma cells. The data (A) show changes in levels of ROS as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of capsaicin (250 μM). DPPD (10 μM) and tocopherol (100 μM) were used. These drugs were incubated for 5 h before the detachment of the cells from the bottom of culture flask, and added again 10 min before capsaicin treatment. Quantitative changes (B) were expressed as percent changes of the maximum increase in DCF fluorescence induced by the drug compared to control condition in which the cells were treated with a drug-free vehicle. In the experiments of (C and D) the cells were incubated with or without capsaicin (250 μM) for 24 h. DPPD (10 μM) and tocopherol (100 μM) were given 30 min before capsaicin application. The measurement of apoptotic cells was the same as in Fig. 1. In bar graphs (B and D) the data represent the mean values of four replicates with bars indicating SEM. $^{*}p < 0.05$ compared to control condition in which the cells were incubated with capsaicin-free medium. $^{\#}p < 0.05$ compared to capsaicin alone.

nuclear level of NF-κB in a time-dependent manner. These results demonstrate that ROS act as an essential component in the mechanism of the capsaicin-induced apoptosis in the HepG2 cells.

DISCUSSION

Capsaicin has been reported to induce apoptosis in various cancer cells.[14,16–19] Consistently, the results

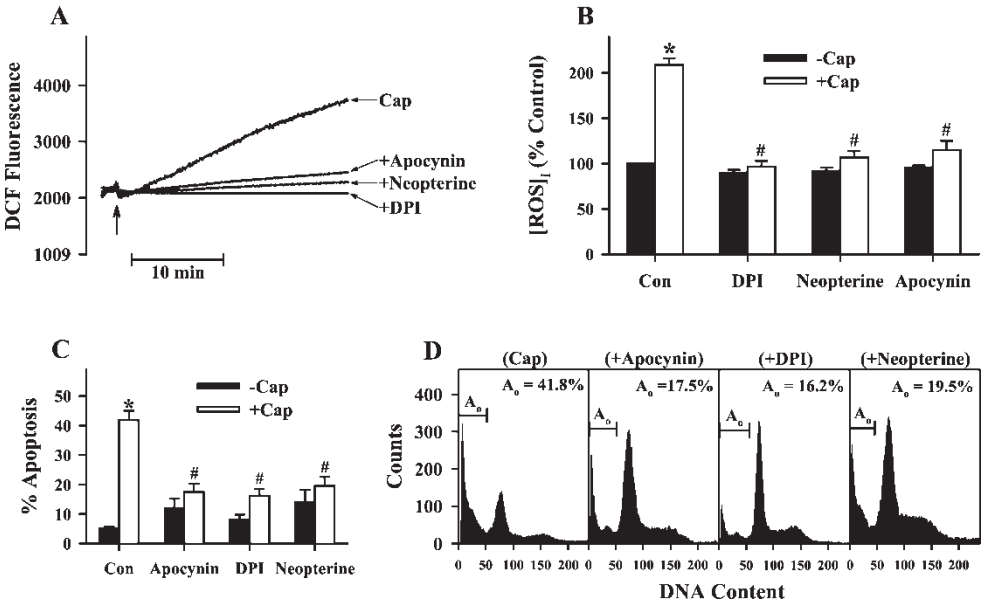

FIGURE 3 NADPH oxidase mediates the capsaicin (Cap)-induced generation of ROS (A and B) and apoptosis (C and D) in HepG2 human hepatoblastoma cells. Experimental procedures and data presentations are the same as in Fig. 2. In these experiments DPI (5 μM), apocynin (500 μM) and neopterine (100 μM) were used as an inhibitor of NADPH oxidase. These drugs were given 10 and 30 min before capsaicin (250 μM) application in the experiments of (A and B) and (C and D), respectively. $^{*}p < 0.05$ compared to control. $^{\#}p < 0.05$ compared to capsaicin alone.

TABLE I Effects of various inhibitors of ROS-generating enzymes on the capsaicin-induced ROS generation and apoptosis in HepG2 human hepatoblastoma cells

	$[ROS]_i$ (% increase)†		% Apoptosis‡	
Compound	− capsaicin	+capsaicin	− capsaicin	+capsaicin
None	120.4 ± 10.2	442.6 ± 53.5*	4.8 ± 0.5	41.8 ± 3.4*
Rotenone (10 μM)+				
Malonate (5 mM)	113.6 ± 18.9	434.2 ± 69.5*	9.8 ± 2.3	49.7 ± 6.3*
Antimycin A (5 μM)	262.9 ± 30.1*	494.1 ± 48.9*	5.9 ± 1.6	54.5 ± 7.8*
FCCP (0.1 μM)	280.7 ± 32.8*	524.1 ± 61.3*	7.5 ± 2.8	59.3 ± 8.6*
Metyrapone (500 μM)	113.4 ± 13.6	392.5 ± 42.5*	8.1 ± 1.4	48.9 ± 4.3*
NMMA (100 μM)	132.3 ± 14.5	443.5 ± 61.3*	7.8 ± 2.2	44.8 ± 4.2*
Allopurinol (100 μM)	149.2 ± 12.8	397.2 ± 76.1*	9.5 ± 1.0	47.8 ± 4.6*
Hydroxyurea (1.5 mM)	134.6 ± 17.4	453.7 ± 85.4*	8.6 ± 0.7	49.7 ± 5.8*

All data represent the mean values ± SEM. *$p < 0.05$ compared to control. Note that no drug tested did significantly alter both increased $[ROS]_i$ and apoptosis induced by capsaicin. † $[ROS]_i$ was measured by DCF fluorescence method, and the values are expressed as percent increase of DCF fluorescence intensity induced by treatment with or without capsaicin (250 μM) for 1 h compared to that of initial time. Each drug was added 10 min before capsaicin treatment. ‡ Apoptosis was measured by flow cytometry. In the experiments the cells were incubated with capsaicin (250 μM) for 48 h. Each drug was added 30 min before capsaicin treatment.

of the present study also demonstrated that capsaicin induced apoptotic cell death in HepG2 human hepatoblastoma cells, evaluated by three independent methods, detection of DNA fragmentation through agarose gel electrophoresis (Fig. 1A), detection of phosphatidylserine translocation through annexin-V binding assay (Fig. 1B), and measurement of hypodiploid DNA contents through flow cytometry (Fig. 1C).

The results of this study strongly suggest that NADPH oxidase is the upstream signaling molecule of the capsaicin-induced generation of ROS, and in turn, induction of apoptosis in HepG2 cells. These conclusions are based on (i) antioxidants which completely blunted the generation of ROS by capsaicin (Fig. 2A and B), significantly prevented induction of apoptosis (Fig. 2C and D), (ii) capsaicin induced activation of NF-κB, a common downstream mediator of ROS[28] (Fig. 5), (iii) specific inhibitors of NADPH oxidase significantly suppressed the capsaicin-induced generation of ROS (Fig. 3A and B) and apoptosis (Fig. 3C and D) and (iv) transfection of the cells with a dominant negative mutant of Rac1 (Rac1N17), an essential component for the activation of NADPH oxidase,[17,18] significant reduced the capsaicin-induced apoptosis (Fig. 4).

Oxidative stress is proposed as a common mechanism of apoptosis.[7] In the HepG2 cells used in this study, ROS appear to play an essential role in apoptosis induced by various agents including tamoxifen,[10] *N*-ethylmaleimide,[11] serum,[29] fenofibrate,[30] and arsenic trioxide.[31] The results of this study further support this notion that ROS act as a common mediator of apoptosis. Although the major

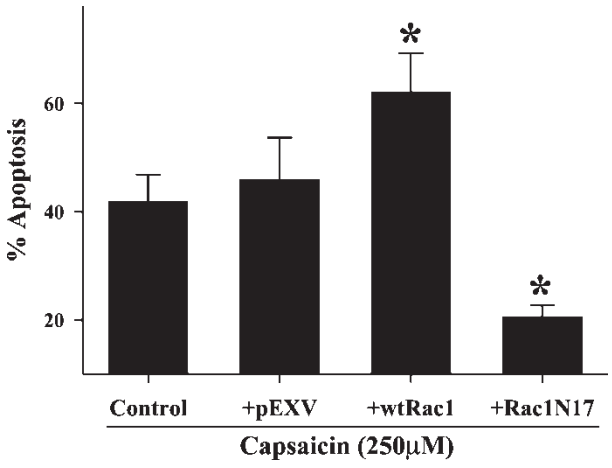

FIGURE 4 Transfection with a dominant-negative mutant of Rac1 (N17Rac1) reverses the apoptotic effect of capsaicin in HepG2 human hepatoblastoma cells. Cells were transfected with wt Rac1, myc-tagged Rac1N17, and empty pEXV vector. The number of apoptotic cells was measured by flow cytometry, as described in Fig. 1. In these experiments 250 μM of capsaicin was used. *$p < 0.05$ compared to control.

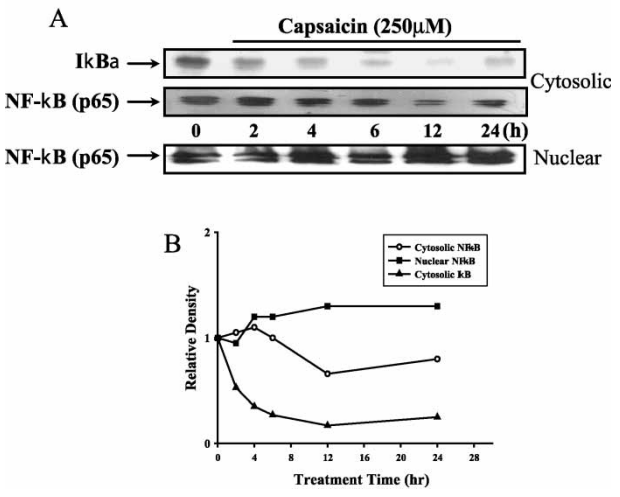

FIGURE 5 Capsaicin induces activation of NF-κB in HepG2 human hepatoblastoma cells. Cells were treated with capsaicin (250 μM) for 24 h. Cell lysates containing equal amount of cytosolic and nuclear proteins were analyzed by Western blot (A) using anti-I-κB or anti-NF-κB (p65) antibodies. In (B), the immunoblots were scanned and analyzed on an imaging densitometer (UVP, Cambridge, UK).

biological process leading to oxygen-derived generation of ROS is electron transport associated with mitochondrial membranes,[9] recent reports have demonstrated that the membrane-bound NADPH oxidase acts as a major source of ROS production particularly associated with apoptosis in many cellular systems, including various cancer cells,[10,11,32] lung epithelium[33] and mesangial cells.[34] The results of the present study showing that the capsaicin-induced generation of ROS and induction of apoptosis is due to the activation of NADPH oxidase, further support the active involvement of the enzyme in the mechanism of ROS-associated apoptosis.

The actions of capsaicin as an inhibitor of plasma membrane NADH oxidase (PMNO) have been closely linked to its induction of apoptosis in variety of transformed cells.[35–37] PMNO transfers electrons from cytoplasmic NADH via coenzyme Q to extracellular electron acceptors such as oxygen.[38] The enzyme appears to play an important role in the regulation of internal redox equilibrium in response to external stimuli.[39] Apoptosis induction of cancer cells by capsaicin has been suggested to result from generation of ROS through inhibition of PMNO.[37,40] Thus, although the NADPH oxidase seems to play a major role in the capsaicin-induced generation of ROS and induction of apoptosis in HepG2 cells, the involvement of the PMNO system in these actions of capsaicin is not completely excluded, and remains to be determined.

In this study, we did not determine how capsaicin activates the NADPH oxidase, and it remains to be studied in the future. Although speculated, Ca^{2+}/calmodulin-dependent protein kinase-II may be possibly involved in the mechanism of action of capsaicin. Because it appears to activate Tiam1,[41] a Rac1-specific exchange factor, and thus activate Rac1, which is essentially required for the generation of ROS by the NADPH oxidase in HepG2 cells.[18] Indeed, in the HepG2 cells we observed that capsaicin did increase intracellular Ca^{2+} concentration (data not shown), which is required for the activation of Ca^{2+}/calmodulin-dependent protein kinase-II.[42] Another possibility may include the involvement of arachidonic acid which seemed to be implicated in the mechanism of activation of the NADPH oxidase in phagocytic cells.[43,44]

Interestingly, in addition to generation of ROS, capsaicin has been also reported to act as an antioxidant.[45,46] In human glioblastoma cells, capsaicin appeared to induce apoptosis through reduction of the basal generation of ROS.[47] Although we do not know the exact reason of this discrepancy, capsaicin may act as either a prooxidant or antioxidant, depending on the cellular type. The intriguing possibility that existence of the NADPH oxidase system in the cells may determine this opposite actions of capsaicin, should be tested in the future study.

In conclusion, capsaicin induced apoptosis in HepG2 human hepatoblastoma cells. The NADPH oxidase-mediated generation of ROS may be importantly involved in the mechanism of apoptosis induced by capsaicin. These results further suggest that capsaicin may be useful for the treatment of human hepatomas.

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References

[1] Morre, D.J., Chueh, P.J. and Morre, D.M. (1995) "Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture", *Proc. Natl Acad. Sci. USA* **92**, 1831–1835.
[2] Kang, H.J., Soh, Y., Kim, M.S., Lee, E.J., Surh, Y.J., Kim, H.R., Kim, S.H. and Moon, A. (2003) "Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells", *Int. J. Cancer* **103**, 475–482.
[3] Wolvetang, E.J., Larm, J.A., Moutsoulas, P. and Lawen, A. (1996) "Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin", *Cell Growth Differ.* **7**, 1315–1325.
[4] Zhang, J., Nagasaki, M., Tanaka, Y. and Morikawa, S. (2003) "Capsaicin inhibits growth of adult T-cell leukemia cells", *Leuk. Res.* **27**, 275–283.
[5] Macho, A., Calzado, M.A., Munoz-Blanco, J., Gomez-Diaz, C., Gajate, C., Mollinedo, F., Navas, P. and Munoz, E. (1999) "Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium", *Cell Death Differ.* **6**, 155–165.
[6] Yu, B.P. (1994) "Cellular defenses against damage from reactive oxygen species", *Physiol. Rev.* **74**, 139–162.
[7] Kamata, H. and Hirata, H. (1999) "Redox regulation of cellular signaling", *Cell. Signal* **11**, 1–14.
[8] Schiaffonati, L. and Tiberio, L. (1997) "Gene expression in liver after toxic injury: analysis of heat shock response and oxidative stress-inducible genes", *Liver* **17**, 183–191.
[9] Halliwell, B. (1989) "Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis", *Br. J. Exp. Pathol.* **70**, 737–757.
[10] Lee, Y.S., Kang, Y.S., Lee, S.H. and Kim, J.A. (2000) "Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells", *Cell Death Differ.* **7**, 925–932.
[11] Kim, J.A., Kang, Y.S., Park, S.H., Kim, H.W., Cho, S.Y. and Lee, Y.S. (2001) "Role of reactive oxygen species in apoptosis induced by *N*-ethylmaleimide in HepG2 human hepatoblastoma cells", *Eur. J. Pharmacol.* **7**, 925–932.
[12] Babior, B.M. (1995) "The respiratory burst oxidase", *Curr. Opin. Hematol.* **2**, 55–60.
[13] Jones, S.A., O'Donnell, V.B., Wood, J.D., Broughton, J.P., Hughes, E.J. and Jones, O.T. (1996) "Expression of phagocyte NADPH oxidase components in human endothelial cells", *Am. J. Physiol.* **271**, H1626–H1634.
[14] Kummer, W. and Acker, H. (1995) "Immunohistochemical demonstration of four subunits of neutrophil NAD(P)H oxidase in type I cells of carotid body", *J. Appl. Physiol.* **78**, 1904–1909.
[15] Marshall, C., Mamary, A.J., Verhoeven, A.J. and Marshall, B.E. (1996) "Pulmonary artery NADPH oxidase is activated in

hypoxic pulmonary vasoconstriction", *Am. J. Respir. Cell Mol. Biol.* **15**, 633–644.
[16] Youngson, C., Nurse, C., Yeger, H., Curnutte, J.T., Vollmer, C., Wong, V. and Cutz, E. (1997) "Immunocytochemical localization on O_2-sensing protein (NADPH oxidase) in chemoreceptor cells", *Microsc. Res. Tech.* **37**, 101–106.
[17] Babior, B.M. (1999) "NADPH oxidase: an update", *Blood* **93**, 1464–1476.
[18] Cool, R.H., Merten, E., Theiss, C. and Acker, H. (1998) "Rac1, and not Rac2, is involved in the regulation of the intracellular hydrogen peroxide level in HepG2 cells", *Biochem. J.* **332**, 5–8.
[19] Ehleben, W., Porwol, T., Fandrey, J., Kummer, W. and Acker, H. (1997) "Cobalt and desferrioxamine reveal crucial members of the oxygen sensing pathway in HepG2 cells", *Kidney Int.* **51**, 483–491.
[20] Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) "Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death", *Nature* **348**, 334–336.
[21] Bombeli, T., Karsan, A., Tait, J.F. and Harlan, J.M. (1997) "Apoptotic vascular endothelial cells become procoagulant", *Blood* **89**, 2429–2442.
[22] Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) "A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V", *J. Immunol. Methods* **184**, 39–51.
[23] LaBel, C.P., Ischiopoulos, H. and Bondy, S.C. (1992) "Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress", *Chem. Res. Toxicol.* **5**, 227–231.
[24] Shen, H.M., Shi, C.Y. and Ong, C.N. (1996) "Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1", *Free Radic. Biol. Med.* **21**, 139–146.
[25] O'Donnell, B.V., Tew, D.G., Jones, O.T. and England, P.J. (1993) "Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase", *Biochem. J.* **290**, 41–49.
[26] Stolk, J., Hiltermann, T.J., Dijkman, J.H. and Verhoeven, A.J. (1994) "Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol", *Am. J. Respir. Cell Mol. Biol.* **11**, 95–102.
[27] Kojima, S., Nomura, T., Icho, T., Kajiwara, Y., Kitabatake, K. and Kubota, K. (1993) "Inhibitory effect of neopterin on NADPH-dependent superoxide-generating oxidase of rat peritoneal macrophages", *FEBS Lett.* **329**, 125–128.
[28] Agostini, M., Di Marco, B., Nocentini, G. and Delfino, D.V. (2002) "Oxidative stress and apoptosis in immune diseases", *Int. J. Immunopathol. Pharmacol.* **15**, 157–164.
[29] Kang, S., Song, J., Kang, H., Kim, S., Lee, Y. and Park, D. (2003) "Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells", *Eur. J. Endocrinol.* **148**, 147–155.
[30] Jiao, H.L. and Zhao, B.L. (2002) "Cytotoxic effect of peroxisome proliferator fenofibrate on human HepG2 hepatoma cell line and relevant mechanisms", *Toxicol. Appl. Pharmacol.* **185**, 172–179.
[31] Kito, M., Akao, Y., Ohishi, N., Yagi, K. and Nozawa, Y. (2002) "Arsenic trioxide-induced apoptosis and its enhancement by buthionine sulfoximine in hepatocellular carcinoma cell lines", *Biochem. Biophys. Res. Commun.* **291**, 861–867.
[32] Chung, Y.M., Bae, Y.S. and Lee, S.Y. (2003) "Molecular ordering of ROS production, mitochondrial changes, and caspase activation during sodium salicylate-induced apoptosis", *Free Radic. Biol. Med.* **34**, 434–442.
[33] Zhang, X., Shan, P., Sasidhar, M., Chupp, G.L., Flavell, R.A., Choi, A.M. and Lee, P.J. (2003) "Reactive oxygen species and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase mediate hyperoxia-induced cell death in lung epithelium", *Am. J. Respir. Cell. Mol. Biol.* **28**, 305–315.
[34] Lodha, S., Dani, D., Mehta, R., Bhaskaran, M., Reddy, K., Ding, G. and Singhal, P.C. (2002) "Angiotensin II-induced mesangial cell apoptosis: role of oxidative stress", *Mol. Med.* **8**, 830–840.
[35] Morre, D.J., Chueh, P.J. and Morre, D.M. (1995) "Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture", *Proc. Natl Acad. Sci. USA* **92**, 1831–1835.
[36] Morre, D.J., Sun, E., Geilen, C., Wu, L.Y., de Cabo, R., Krasagakis, K., Orfanos, C.E. and Morre, D.M. (1996) "Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines", *Eur. J. Cancer* **32**, 1995–2003.
[37] Macho, A., Sancho, R., Minassi, A., Appendino, G., Lawen, A. and Munoz, E. (2003) "Involvement of reactive oxygen species in capsaicinoid-induced apoptosis in transformed cells", *Free Radic. Res.* **37**, 611–619.
[38] Crane, F.L., Sun, I.L., Clark, M.G., Grebing, C. and Low, H. (1985) "Transplasma-membrane redox systems in growth and development", *Biochim. Biophys. Acta* **811**, 233–264.
[39] Morre, D.J. and Brightman, A.O. (1991) "NADH oxidase of plasma membranes", *J. Bioenerg. Biomembr.* **23**, 469–489.
[40] Macho, A., Blazquez, M.V., Navas, P. and Munoz, E. (1998) "Induction of apoptosis by vanilloid compounds does not require *de novo* gene transcription and activator protein 1 activity", *Cell Growth Differ.* **9**, 277–286.
[41] Fleming, I.N., Elliott, C.M., Buchanan, F.G., Downes, C.P. and Exton, J.H. (1999) "Ca^{2+}/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation", *J. Biol. Chem.* **274**, 12753–12758.
[42] Fujisawa, H. (2001) "Regulation of the activities of multifunctional Ca^{2+}/calmodulin-dependent protein kinases", *J. Biochem. (Tokyo)* **129**, 193–199.
[43] Daniels, I., Lindsay, M.A., Keany, C.I., Burden, R.P., Fletcher, J. and Haynes, A.P. (1998) "Role of arachidonic acid and its metabolites in the priming of NADPH oxidase in human polymorphonuclear leukocytes by peritoneal dialysis effluent", *Clin. Diagn. Lab. Immunol.* **5**, 683–689.
[44] Shiose, A. and Sumimoto, H. (2000) "Arachidonic acid and phosphorylation synergistically induce a conformational change of $p47^{phox}$ to activate the phagocyte NADPH oxidase", *J. Biol. Chem.* **275**, 13793–13801.
[45] Joe, B. and Lokesh, B.R. (1994) "Role of capsaicin, curcumin and dietary *n*-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages", *Biochim. Biophys. Acta* **1224**, 255–263.
[46] Reddy, A.C. and Lokesh, B.R. (1992) "Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes", *Mol. Cell. Biochem.* **111**, 117–124.
[47] Lee, Y.S., Nam, D.H. and Kim, J.A. (2000) "Induction of apoptosis by capsaicin in A172 human glioblastoma cells", *Cancer Lett.* **161**, 121–130.