Arch Pharm Res Vol 27, No 8, 845-849, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Capsaicin-Induced Apoptosis of H-Ras-Transformed Human Breast Epithelial Cells is Rac-Dependent *via* ROS Generation

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(Received July 6, 2004)

Many studies have focused on the anticarcinogenic, antimutagenic or chemopreventive activities of capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) which is a major pungent ingredient in red pepper. We have previously shown that capsaicin selectively induces apoptosis in H-ras-transformed MCF10A human breast epithelial cells but not in their normal cell counterparts (*Int. J. Cancer*, 103, 475-482, 2003). In this study, we investigated the possible roles of reactive oxygen species (ROS) and Rac1 in capsaicin-induced apoptosis of H-ras MCF10A cells. Selective induction of ROS generation by capsaicin treatment was observed only in H-ras MCF10A cells. Pretreatment of H-ras MCF10A cells with an antioxidant N-acetylcysteine (NAC) significantly reversed capsaicin-induced growth inhibition, suggesting that ROS may mediate the apoptosis of H-ras-transformed cells induced by capsaicin. Rac1 was prominently activated by H-ras in MCF10A cells. Based on the studies using a wild type Rac1 and a dominant negative Rac1 constructs, we propose that Rac1 activity is critical for inhibitory effect of capsaicin on growth of H-ras-transformed MCF10A cells possibly through ROS generation.

Key words: Capsaicin, Reactive oxygen species (ROS), Rac, H-ras, MCF10A cells

INTRODUCTION

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), the pungent ingredient found in red pepper, has long been used as spices, food additives and drugs (Iwai *et al.*, 1979; Suzuki *et al.*, 1984; Cordell and Araujo, 1993). Although capsaicin has been subjected to extensive investigations with regard to its possible tumorigenicity and genotoxicity, the compound has attracted considerable attention because of its chemoprotective properties against certain carcinogens and mutagens (reviewed in Surh *et al.*, 1998).

Capsaicin is an inhibitory quinone analog that can inhibit the plasma membrane NADH oxidase of transformed cells (Morre *et al.*, 1995). Previous studies including ours have demonstrated that capsaicin preferentially represses the growth of some transformed cells of human origin (Morre *et al.*, 1995; Takahata *et al.*, 1999; Jung *et al.*, 2001) possibly *via* induction of apoptosis (Wolvetang *et al.*, 1996; Macho *et al.*, 1999; Lee *et al.*, 2000) but normal cells were largely unaffected by capsaicin. We have previously shown that capsaicin selectively induces apoptosis in H-ras-transformed MCF10A cells, while it has little effect on viability of the parental MCF10A human breast epithelial cells (Kang *et al.*, 2003).

Regulation of ROS generation by the small GTP-binding proteins, Rac1 and Rac2, has been reported (Bokoch, 2000; Bokoch and Diebold, 2002). In neutrophils, the activity of the NADPH oxidase system is regulated by Rac2 (Knaus *et al.*, 1991), whereas in macrophages, the NADPH oxidase appears to be regulated by Rac1 (Abo *et al.*, 1991). Rac1 leads to an increase in ROS in fibroblasts (Sundaresan *et al.*, 1996).

Ras expression enhances the sensitivity of fibroblasts for apoptosis-inducing stimuli in which ras-mediated superoxide anion production is involved (Schwieger *et al.*, 2001). Since Ras-transformed fibroblasts were shown to produce ROS through a mechanism which is dependent of Rac1 (Irani *et al.*, 1997), the capsaicin modulation of H-ras pathway and ROS generation merits additional investigation. In the present study, we investigated the possible roles of Rac1 and ROS in capsaicin-induced apoptosis of H-ras MCF10A cells. Our data suggest that ROS may mediate the apoptosis of H-ras-transformed cells induced by capsaicin. We also provide evidence that Rac1 activity is critical for inhibitory effect of capsaicin on growth of H-

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ras-activated MCF10A cells possibly through ROS generation.

MATERIALS AND METHODS

Cell lines

MCF10A is a spontaneously immortalized "normal" breast epithelial cell line. (Soule *et al.*, 1990). H-ras MCF10A cells were established as previously described (Moon *et al.*, 2000). Briefly, a retroviral vector containing a mutant H-ras (pBW1423) with a mutation of Gly to Asp in amino acid codon 12 was introduced in MCF10A cells. MCF10A and H-ras MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 2 ng/mL EGF, 0.1 μg/mL cholera enterotoxin, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin and 0.5 μg/mL fungizone.

Detection of intracellular ROS generation

Cells were incubated with 5 μg/mL 2',7'-dicholorofluorescein (DCF)-DA (Molecular Probe, Eugene, OR) for 20 min. After incubation, cells were harvested and resuspended in phenol-free DMEM/F12. Fluorescence was measured with a FACS Calibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and emission at 530 nm.

Confluent cells were treated with 100 μM capsaicin for 1 h, washed with serum-free DMEM/F12 media containing DCF-DA and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 5 min. The cells were observed under confocal microscope.

Growth inhibition

Cells (1.5×10^5) in a 48-well plate were cultured in the presence of various concentrations of capsaicin for 72 h. Control cells were treated with DMSO alone. Treated cells were counted and cell viability was determined by a trypan blue exclusion assay.

Transfection

Transfection was performed using Lipofectamine reagent (Life Technologies, Inc., Rockville, MD) following the manufacturer's instruction. DNA constructs of wild type Rac1 and a dominant negative mutant of Rac1 (DN Rac1), N17Rac1, were provided by Dr. H. Kim (Seoul National University, Seoul, Korea).

Rac activity assay

The level of Rac-GTP was measured by affinity precipitation using GST-PBD of PAK1 as previously described (Benard *et al.*, 1999). Briefly, activated Rac-GTP was precipitated from whole cell lysates, prepared in lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1mM EDTA, 2% glycerol, 2 μg/mL leupeptin and 2 μg/mL aprotinin with glutathione Sepharose 4B beads at 4°C. Proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted for Rac1 antibodies purchased from Upstate Biotech. Inc. (Lake Placid, NY). ECL system was used for detection.

RESULTS

Capsaicin increases ROS generation in H-ras MCF10A cells

It has been reported that ras mediates ROS generation in various cell systems (Irani *et al.*, 1997). We detected ROS levels in MCF10A and H-ras-transformed MCF10A cells. As shown in Fig. 1A, the level of intracellular ROS was higher in H-ras MCF10A cells compared to the parental MCF10A cells. The increased ROS generation in H-ras MCF10A cells was confirmed by confocal microscopy (Fig. 1B). Treatment with 100 μM capsaicin for 1 h markedly enhanced ROS production in H-ras MCF10A cells while the ROS level of MCF10A cells was not affected by the same treatment. The data demonstrate that capsaicin increases H-ras-induced ROS generation

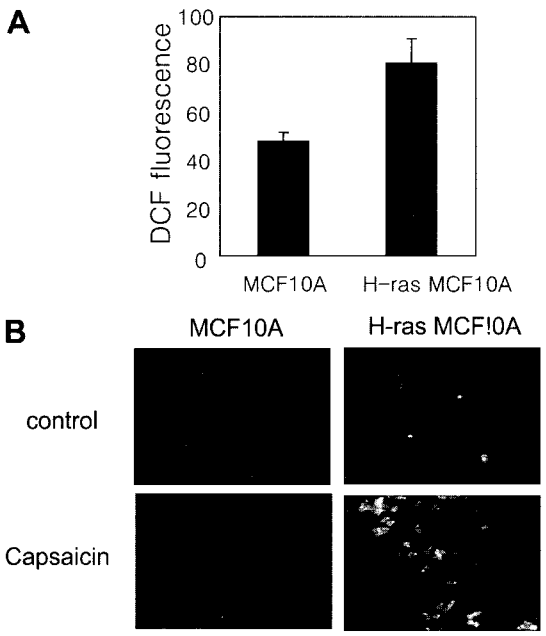

Fig. 1. Capsaicin increases ROS generation in H-ras MCF10A cells. A, MCF10A and H-ras MCF10A cells were incubated with DCF-DA for 20 min. DCF fluorescence, reflecting the relative levels of ROS (arbitrary units), was determined by FACS analysis with excitation and emission settings of 488 and 530 nm, respectively. Values represent means ± SE of duplicate from two independent experiments. B, Confluent cells were treated with 100 μM capsaicin for 1 h, washed with serum-free DMEM/F12 media containing DCF-DA and incubated for 5 min. Cells were then observed under confocal microscope (×400).

in MCF10A cells.

ROS may mediate capsaicin-induced apoptosis of H-ras MCF10A cells

In order to investigate the role of ROS in apoptosis induced by capsaicin in H-ras MCF10A cells, we examined the effect of capsaicin on the growth of H-ras MCF10A cells treated with an antioxidant *N*-acetylcysteine (NAC). As shown in Fig. 2, pretreatment with NAC reversed capsaicin-induced growth inhibition. Treatment of H-ras MCF10A cells with 50 μM capsaicin for 28 h inhibited the growth of H-ras MCF10A cells to 56% of control. The growth inhibitory effect of capsaicin was significantly alleviated (to 84% of control) by pretreatment with 2 mM NAC for 30min, indicating the involvement of ROS in capsaicin-induced growth inhibition. The data suggest that ROS may mediate the apoptosis of H-ras MCF10A cells induced by capsaicin.

Rac is activated by H-ras

Since mounting evidence indicates the regulation of ROS generation by Rac1 (Sundaresan *et al.*, 1996; Bokoch, 2000; Bokoch and Diebold, 2002), we sought to determine whether Rac pathway is activated in H-ras MCF10A cells. Enhanced ROS generation by H-ras (Fig. 1) suggests that H-ras might be an effective activator of the Rac pathway in MCF10A cells. To directly assess the abilities of H-ras and N-ras to activate Rac, we performed a Rac activity assay which specifically recognizes the activated GTP-bound form of Rac (Sander *et al.*, 1998). As shown in Fig. 3, a prominent activation of Rac was induced by H-ras, demonstrating that Rac pathway is activated in H-ras MCF10A cells.

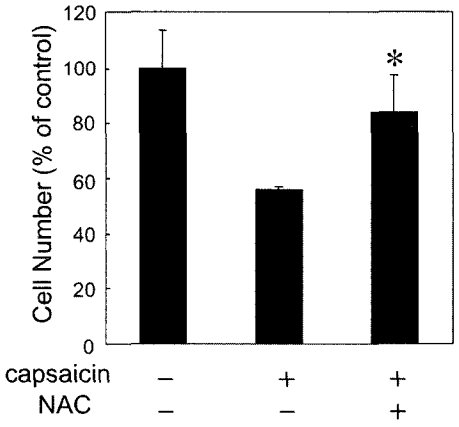

Fig. 2. ROS is required for capsaicin-induced growth inhibition of H-ras MCF10A cells. Cells (1.5×10^5) in 48-well plates were incubated with 50 μM capsaicin for 28 h with or without pretreatment with 2 mM NAC for 30 min. The percentage of cell survival was normalized to the control cells. The results presented are means±S.E. of triplicates. *, Statistically different from capsaicin-treated cells without pretreatment with NAC at $p<0.05$ by the independent two sample Student's *t*-test.

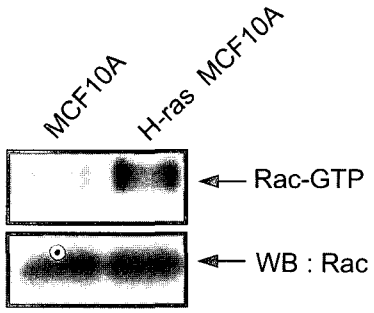

Fig. 3. Rac is activated by H-ras in MCF10A cells. Equal amounts of cell lysates were incubated with GST-PBD fusion protein, and the bound active Rac-GTP molecules were analyzed by immunoblotting using anti-Rac antibody.

Capsaicin-iuduced growth inhibition was Rac-dependent

We then examined the role of Rac pathway in capsaicin-induced growth inhibition in H-ras MCF10A cells. Growth of parental MCF10A cells was inhibited by capsaicin in a dose-dependent manner when the cells were transfected with the wild type Rac1 (Fig. 4A). The data demonstrate

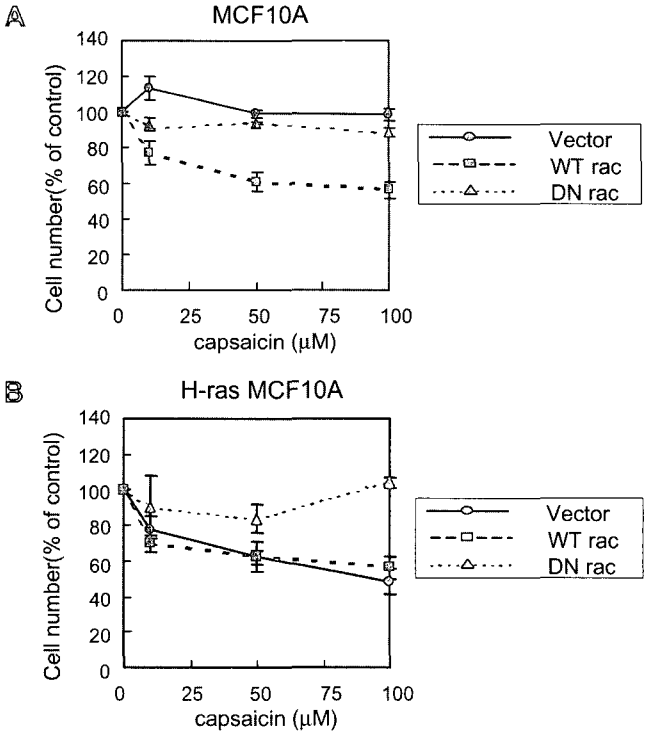

Fig. 4. Capsaicin-iuduced growth inhibition was Rac-dependent. Cells were transfected with control vector pcDNA3 (Vector), wild type Rac1 (WT rac) and a dominant negative Rac1 (DN rac) constructs. The transfectancts were treated with various concentrations of capsaicin for 72 h. The percentage of cell survival was normalized to the control cells. The results presented are means±S.E. of triplicates.

that overexpression of Rac1 rendered the MCF10A cells, which were originally not responsive to capsaicin-induced growth inhibition, sensitive to capsaicin. As shown in Fig. 4B, capsaicin-induced growth inhibition of H-ras MCF10A cells was markedly abolished by transfection with a DN Rac1, suggesting that the growth-inhibitory effect of capsaicin was dependent on Rac pathway in MCF10A cells.

DISCUSSION

Efforts have been made to develop a chemoprevention strategy that selectively triggers apoptosis in malignant cells but not in the normal cells (Kong *et al.*, 2000). In an attempt to evaluate the chemopreventive activity of capsaicin, we have previously investigated the effect of capsaicin in apoptosis of the parental and H-ras-transformed MCF10A human breast epithelial cells. We reported that capsaicin selectively induced apoptosis in H-ras MCF10A cells but not in their normal cell counterparts (Kang *et al.*, 2003). Consistently with our findings, Ras has been reported to enhance the sensitivity of fibroblasts for various apoptosis-inducing stimuli (Schwieger *et al.*, 2001).

ROS have been implicated as a main mediator of apoptosis in many different cellular systems (Jacobson, 1996). Apoptosis induction of cancer cells by capsaicin has been suggested to result from generation of ROS through inhibition of plasma membrane NADPH oxidase (Macho *et al.*, 1998; Lee *et al.*, 2004). This study was performed to examine the possible involvement of ROS and Rac in capsaicin-induced apoptosis in H-ras-transformed MCF10A cells. Our data show that ROS generation was enhanced by capsaicin treatment only in H-ras-activated cells, suggesting that capsaicin-induced ROS generation was mediated by H-ras signaling pathway. Given that our previous report proposed the roles of JNK-1 and p38 MAPK in selective induction of apoptosis by capsaicin in H-ras MCF10A cells (Kang *et al.*, 2003), further studies will be needed to determine whether capsaicin-induced ROS generation involves these ras-downstream effector molecules.

Uncontrolled activation of the ras signaling pathway is one of the most frequent defects in transformed cells (Barbacid, 1987) and ras mutations are most prevalent in epithelial-derived human carcinoma cells (Bos, 1989; Denhardt, 1996). We previously showed that H-ras induced malignant cell phenotypes such as cell invasion and migration while N-ras had no significant effect on the invasion and migration of MCF10A breast epithelial cells (Moon *et al.*, 2000; Kim *et al.*, 2003). Our findings which revealed the molecular mechanisms for the selective induction of apoptosis by capsaicin in H-ras-transformed malignant breast epithelial cells may suggest a potential use of capsaicin for breast cancer chemoprevention.

ACKNOWLEDGEMENTS

This work was supported by a 2003 Research Grant from Duksung Women's University, Seoul, Korea. We are grateful to Dr. Jae Hong Kim (School of Life Sciences and Technology, Korea University, Seoul, Korea) for the use of FACS. We also thank Ms. Hye-Jung Kang (R&D Center of Pharmaceuticals Institute of Science & Technology, CJ Corporation, Gyeonggi-Do, Korea) and Dr. Mi-Sung Kim (College of Pharmacy, Seoul National University, Seoul, Korea) for technical assistance.

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