Cell Biochem Biophys (2011) 61:355–366
DOI 10.1007/s12013-011-9218-0

ORIGINAL PAPER

Down-Regulation of Tumor-Associated NADH Oxidase, tNOX (ENOX2), Enhances Capsaicin-Induced Inhibition of Gastric Cancer Cell Growth

His-Ming Wang · Show-Mei Chuang · Yu-Ching Su · Yi-Hui Li · Pin Ju Chueh

Published online: 7 July 2011

Abstract Gastric cancer is a common human malignancy and a major contributor to cancer-related deaths worldwide. Unfortunately, the prognosis of most gastric cancer patients is poor because they are generally diagnosed at a late stage after the cancer has already metastasized. Most current research, therefore, emphasizes selective targeting of cancer cells by apoptosis-inducing agents. One such therapeutic agent is capsaicin, a component of chili peppers that has been shown to possess anti-growth activity against various cancer cell lines. Here, we examined the effect of capsaicin on SNU-1 and TMC-1 gastric cancer cells and found differing outcomes between the two cell lines. Our results show that capsaicin induced significant cytotoxicity with increases in oxidative stress, PARP cleavage, and apoptosis in sensitive SNU-1 cells. In contrast, TMC-1 cells were much less sensitive to capsaicin, exhibiting low cytotoxicity and very little apoptosis in response to capsaicin treatment. Capsaicin-induced apoptosis in SNU-1 cells was associated with down-regulation of tumor-associated NADH oxidase (tNOX) mRNA and protein. On the contrary, tNOX expression was scarcely affected by capsaicin in TMC-1 cells. We further showed that tNOX-knockdown sensitized TMC-1 cells to capsaicin-induced apoptosis and G1 phase accumulation, and led to decreased cell growth, demonstrating that tNOX is essential for cancer cell growth. Collectively, these results indicate that capsaicin induces divergent effects of the growth of gastric cancer cells that parallel its effects on tNOX expression, and demonstrate that forced tNOX down-regulation restored capsaicin-induced growth inhibition in TMC-1 cells.

Keywords Apoptosis · Capsaicin · Gastric cancer cells · Tumor-associated NADH oxidase (tNOX)

H.-M. Wang · S.-M. Chuang · Y.-C. Su · Y.-H. Li · P. J. Chueh (✉)
Graduate Institute of Biomedical Sciences, National Chung Hsing University, Taichung 40227, Taiwan, Republic of China
e-mail: pjchueh@dragon.nchu.edu.tw

Introduction

Gastric cancer is one of the most common human malignancies and is a major contributor to cancer-related mortality worldwide [1]. Unfortunately, most patients experience cancer metastasis and their prognosis is poor, reflecting the fact that gastric cancers are generally diagnosed at an advanced stage. Therefore, current research places an emphasis on chemoprevention, with the goal of impeding or reversing tumorigenesis. A growing body of evidence suggests that the active anticancer property of chemopreventative agents is their ability to selectively induce apoptosis in cancer cells [2–5]. Among the compounds suggested to possess chemopreventative properties is capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a pungent component of chili peppers, which have long been consumed by humans all over the world [6]. Capsaicin possesses anti-growth activity in various cancer cell systems including human leukemic [7–9], prostate [10–12], colon [13, 14], hepatoma [15, 16], breast [17, 18], and gastric cancer [19, 20]. Research into the cytotoxic action of capsaicin in cancer cells has revealed that capsaicin activates an array of signaling mechanism, including generation of reactive oxygen species (ROS) [7, 21]; up-regulation of p53, p21, and Bax [10]; suppression of STAT3 activation [22]; and degradation of Fas-associated factor 1 (FAF1) [23]. Some studies on capsaicin-treated animals, however, have yielded controversial outcomes [24, 25].

Previous studies have identified a tumor-associated NADH oxidase (tNOX; ENOX2) that belongs to a family of growth-related NADH (or hydroquinone) oxidases [26–28]. The NADH oxidase activity initially identified in rat liver plasma membranes is responsive to growth factors and hormones (CNOX; ENOX1), whereas that from rat hepatoma is constitutive active [28] and has since been identified in various cancer cell lines [18, 29–31] and in the sera of cancer patients (tNOX; ENOX2) [32, 33]. In fact, tNOX protein was originally purified from pooled cancer patient sera [34], suggesting its clinical relevance. tNOX has since been cloned from a HeLa cDNA library, and site-directed mutagenesis studies have identified potential functional motifs, including a quinone-binding site, an adenine nucleotide-binding site, and a CXXXC cysteine pair essential for its activity have been reported [35, 36]. Numerous anti-cancer drugs, including capsaicin [18, 30], phenoxodiol [37], and the major green tea catechin (−)-epigallocatechin-3-gallate (EGCg) [30, 31] have been shown to inhibit tNOX enzymatic activity in association with a reduction in cancer cell growth. Both gain-of-function and loss-of function assays support the idea that tNOX increases the ability of cancer cells to acquire aggressive characteristics [38]. Moreover, HeLa cells in which tNOX expression is depleted by RNA interference (RNAi) exhibit a cell growth-repressed and cell migration-inhibited phenotype [39], reinforcing the interpretation that tNOX protein is essential for the growth of transformed cells. Consistent with this, we have demonstrated that capsaicin induces apoptosis in human cancer cells in association with a concomitant down-regulation of tNOX protein [20, 30], confirming a close link between tNOX protein level and cancer cell survival. Here, we extend our study to two gastric cancer lines, SNU-1 and TMC-1, both of which express tNOX. Our results demonstrated that capsaicin affects tNOX expression differently in the two cell lines, leading to divergent outcomes. Importantly, RNAi-mediated tNOX knockdown restores capsaicin-induced growth reduction to capsaicin-resistant TMC-1 cells.

Materials and Methods

Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Anti-poly(ADP) ribose polymerase (PARP), anti-Bcl-2, anti-phospho-p53, anti-phospho-Rb, anti-cyclin D1, and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-Bax and anti-Bak antibodies were from Upstate (Lake Placid, NY, USA). The anti-β-actin antibody was from Millipore Corp. (Temecula, CA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes Inc. (Eugene, OR, USA). Di-hexyloxocarbocyanine iodide (DiOC6(3)) was purchased from Calbiochem (San Diego, CA, USA). Antisera to tNOX used in Western blot analyses were generated as described previously [29] and the tNOX protein band recognized by this antisera was also identified by the commercial available anti-tNOX polyclonal antibody (Protein Tech Group, Inc. Chicago, IL) [39]. The anti-p53 and anti-mouse IgG antibodies as well as other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA), unless otherwise specified.

Cell Culture and Transfection

The SNU-1 human gastric carcinoma (suspension) and TMC-1 human metastatic gastric carcinoma (adherent) cell lines, kindly provided by Dr. Chun-Ying Wu (Department of Gastroenterology, Taichung Veterans General Hospital, Taiwan), were grown in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was replaced every 2–3 days. In this study, cells were treated with different concentrations of capsaicin dissolved in ethanol and the controls were treated with the same volume of ethanol as in the capsaicin groups.

TMC-1 cells were transiently transfected with short hairpin RNA (shRNA) against tNOX, or scrambled shRNA (control) using the jetPEI transfection reagent according to the manufacturer's protocol (Polyplus-transfection SA, Illkirch Cedex, France), as described previously [39].

Trypan Blue Exclusion Assay

Cells were seeded at 10^4 cells per dish and, after culturing for the indicated time periods, were trypsinized (adherent TMC-1 cells only), collected by centrifugation and washed with phosphate-buffered saline (PBS). Cell pellets were then suspended in 50 μl PBS and 50 μl of a 0.4% (w/v) trypan blue stain solution, and cell numbers were counted and recorded. Each data point represents the mean of three separate experiments (mean ± SD).

Colony-Formation Assay

Two hundred cells were seeded onto a 6-cm dish and incubated in culture medium with different concentrations of capsaicin for 10 days to allow colony formation. After incubation, colonies were fixed in 1.25% glutaraldehyde at room temperature for 30 min, rinsed with distilled water

and stained with a 0.05% methylene blue solution. The number of colonies was counted and recorded.

Apoptosis Determination

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Jose, CA, USA). Cells cultured in 6-cm dishes were trypsinized and collected by centrifugation. The cell pellet was washed, resuspended in 1× binding buffer and stained with annexin V-FITC, as recommended by the manufacturer. Cells were also stained with propidium iodide (PI) to detect necrosis or late apoptosis. The distribution of viable (FITC/PI double-negative), early apoptotic (FITC-positive), late apoptotic (FITC/PI double-positive), and necrotic (PI-positive/FITC-negative) cells was analyzed using a Beckman Coulter FC500. The results are expressed as a percentage of total cells.

Cell Cycle Analysis

In brief, 10^6 cells were collected and washed in PBS, slowly fixed in 75% ethanol, and kept at −20°C for at least 1 h. The cell pellet was then washed again with PBS, and centrifuged at 500×*g* for 5 min. The pellet was resuspended in 200 μl cold PBS and stained in the dark with PI solution (20 mM Tris, pH 8.0, 1 mM NaCl, 0.1% NP-40, 1.4 mg/ml RNase A, 0.05 mg/ml PI) for 30 min on ice. Total cellular DNA content was analyzed with a Beckman Coulter FC500.

Cytofluorimetric Analysis of Mitochondrial Membrane Potential

Changes in mitochondrial membrane potential characteristic of apoptosis were studied using the cationic lipophilic dye DiOC6(3). Cells were treated with different concentrations of capsaicin for 18 h. At the end of treatment, cells were washed, incubated with 40 nM DiOC6(3) for 30 min at 37°C, and then washed with PBS, after which cell pellets were collected and resuspended in PBS. The fluorescence intensity of cells was analyzed using a Beckman Coulter FC500.

Measurement of Oxidative Stress

Oxidative stress was determined by measuring the level of hydrogen peroxide generated in the cells with the staining method of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA). The basis for this assay is that the nonpolar, nonionic H_2-DCFDA is cell permeable and is hydrolyzed to nonfluorescent H_2-DCF by intracellular esterases. In the presence of peroxide, H_2-DCF is rapidly oxidized to highly fluorescent DCF. At the end of capsaicin treatment, cells (2×10^5) were washed with PBS and incubated with 5 μM H_2DCFDA in DMSO for 30 min. Cells were collected by trypsinization and centrifugation, washed with PBS, centrifuged at 200×*g* for 5 min and analyzed immediately using a Beckman Coulter FC500 flow cytometer.

Western Blot Analysis

Cell extracts were prepared in lysis buffer (20 mM Tris–HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM PMSF, 10 ng/ml leupeptin, and 10 μg/ml aprotinin), and volumes of extract containing equal amounts of proteins (40 μg) were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked, washed, and probed with primary antibody. After washing to remove primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The blots were washed again, and developed using enhanced chemiluminescence (ECL) reagents, according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA from gastric cancer cells was isolated using the TRIzol reagent (GIBCO, Carlsbad, CA, USA). The first strand cDNA was synthesized from 1 μg of total RNA using Superscript II (Life Technologies, Rockville, MD, USA). The following primers sets were used for PCR amplifications: tNOX, 5′-TGG CTG TGG GTC TAC GAA A-3′ (sense) and 5′-GGA GTC TGC CTG TGT CCT TC-3′ (antisense); p21, 5′-CCT CTT CGG CCC AGT GGA C-3′ (sense) and 5′-CCG TTT TCG ACC CTG AGA G-3′ (antisense); β-actin, 5′-GAT GAT GAT ATC GCC GCG CT-3′ (sense) and 5′-TGG GTC ATC TTC TCG CGG TT-3′ (antisense). Reaction conditions consisted of 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by a final extension of 7 min at 72°C. PCR produces were resolved by electrophoresis was performed on 0.8% agarose gels and visualized by ethidium bromide staining.

Determination of tNOX Transcript Stability

Cells were pre-treated with 5 μg/ml of actinomycin D followed by the treatments of capsaicin. Cultures were

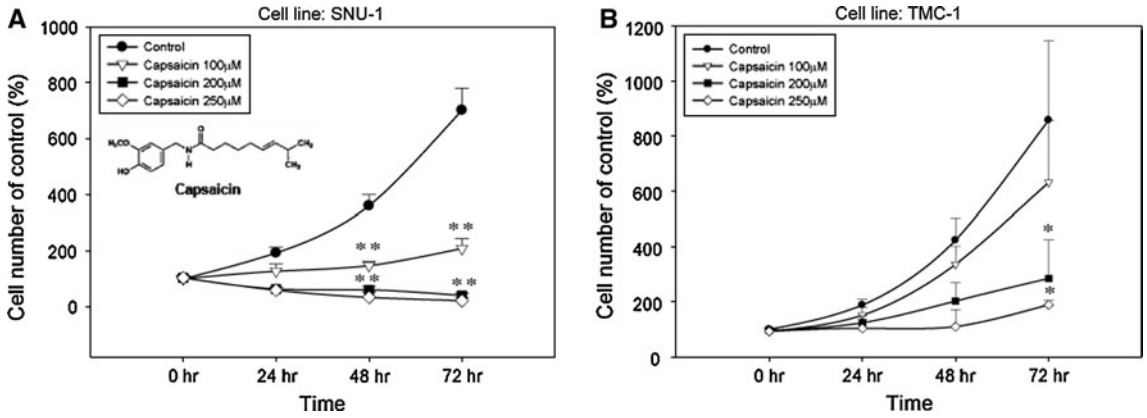

Fig. 1 Capsaicin differentially inhibits gastric cancer cell growth in SNU-1 (**a**) and TMC-1 (**b**) cells. Cells were treated with different concentrations of capsaicin, and the number of viable cells was determined by trypan blue exclusion assay after treatment. Values (mean ± SE) are from three independent experiments performed in at least triplicates. There was a significant difference in cells treated with capsaicin compared to the control group (*$p < 0.05$, **$p < 0.01$)

harvested at various times and total RNA was isolated and analyzed for tNOX transcript stability by RT-PCR.

Statistics

All data are expressed as the mean ± SD of three independent experiments. The differences between control and treatment groups were calculated using a one-way ANOVA followed by Dunnett's test to evaluate significance levels.

Results

Capsaicin Inhibits the Growth of Stomach Cancer Cells

Although the cytotoxic effects of capsaicin have been reported in a range of cancer cell lines, few studies have focused on gastric cancer lines [19, 20, 40]. To elucidate the molecular mechanism of capsaicin, we studied the effects of capsaicin on the growth of SNU-1 cells, derived from a poorly differentiated human gastric carcinoma, and TMC-1

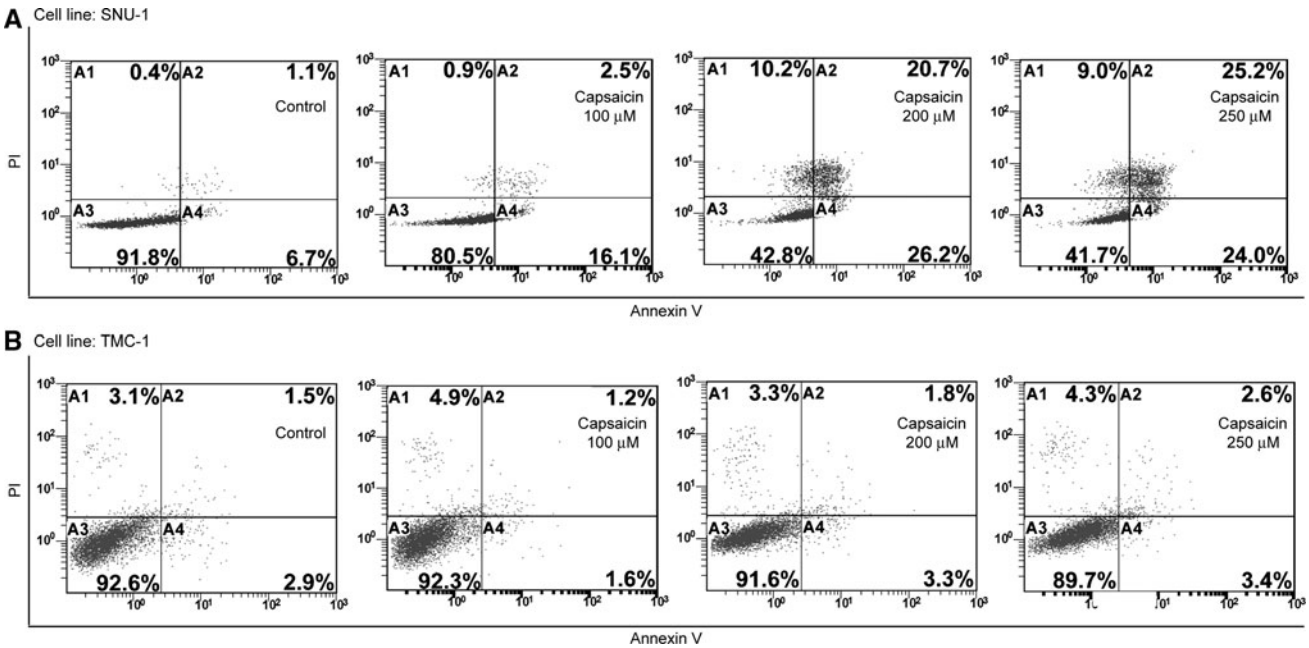

Fig. 2 Capsaicin induces apoptosis in SNU-1 cells (**a**), but not in TMC-1 cells (**b**). Cells were treated with different concentrations of capsaicin for 24 h, and the percentages of apoptotic cells were determined by flow-cytometry. The distribution of viable, early apoptotic, later apoptotic, and necrotic cells was analyzed. The graphs depict apoptotic and necrotic populations of cells double-stained with PI- and FITC-labeled annexin V. The results are expressed as a percentage of total cells

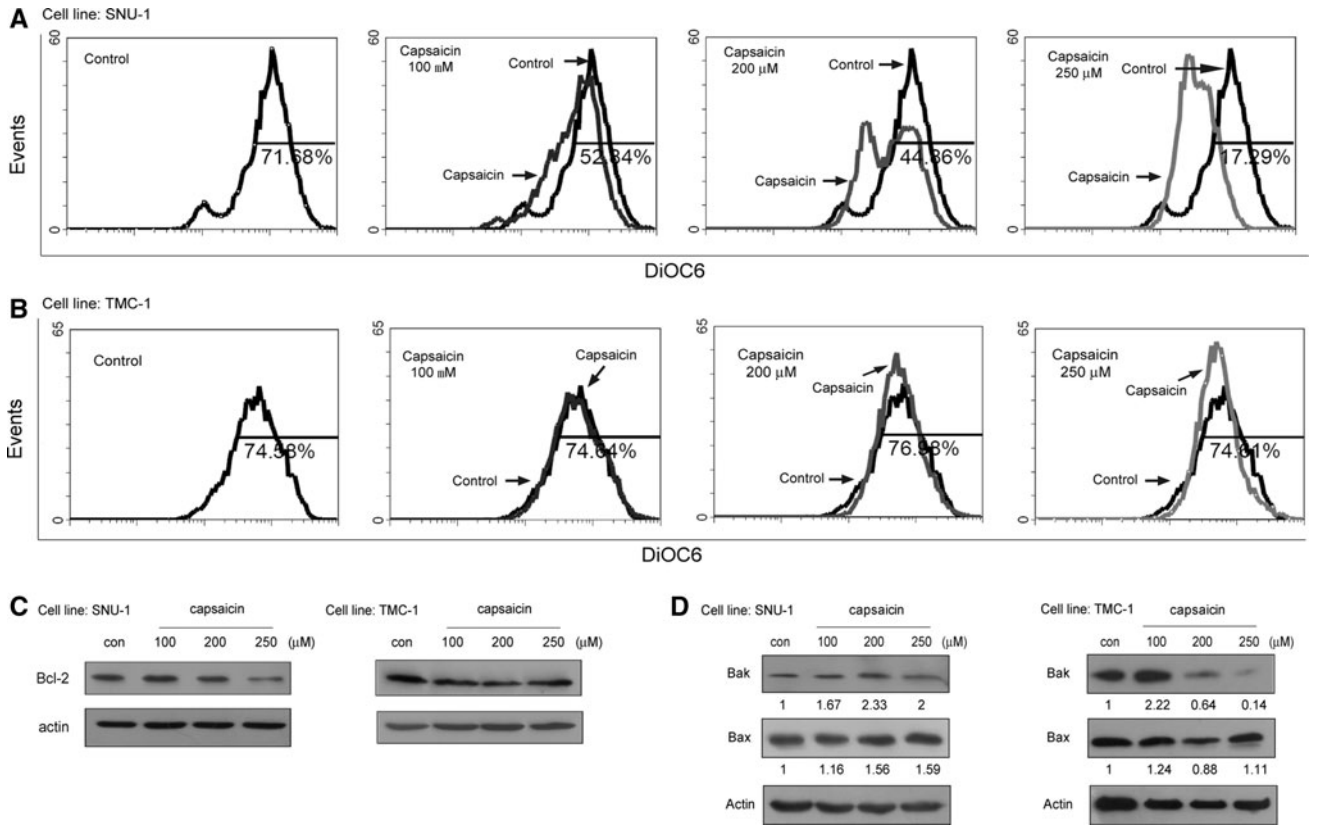

Fig. 3 Capsaicin induces mitochondria-dependent apoptosis in SNU-1 cells, but not in TMC-1 cells. **a**, **b** The mitochondrial function was assessed by flow cytometric analysis of $DiOC_6(3)$ retention in SNU-1 (**a**) or TMC-1 (**b**) cells treated with different concentrations of capsaicin for 18 h. **c**, **d** Cells were treated with different concentrations of capsaicin for 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for Bcl-2 expression (**c**) and for Bak and Bax expression (**d**) by Western blotting analysis. *β*-Actin was used as an internal control to monitor for equal loading. Bands in (**d**) were quantified by densitometry; *numbers below lanes* indicate the ratio of individual protein to actin. Representative images are shown

cells, a metastatic gastric carcinoma line. We first tested the dose-dependence of capsaicin cytotoxicity, measuring cell numbers by trypan blue exclusion assays. At 100 μM, capsaicin induced a significantly time-dependent decrease in the growth of SNU-1 cells, reducing cell numbers to less than 41 and 30% of control values at 48 and 72 h incubation, respectively (Fig. 1a). In contrast, capsaicin at 100 μM was not significantly cytotoxic toward TMC-1 cells at all the exposure-durations tested (Fig. 1b). However, at higher concentrations (≥200 μM), growth of TMC-1 was severely suppressed, but still had a twofold increase in cell number at 72 h. Meanwhile, SNU-1 cells were completely inhibited by 200 μM capsaicin, suggesting a substantial difference in capsaicin sensitivity between these two cell lines.

Capsaicin Induces Mitochondria-Dependent Apoptosis in SNU-1 But Not TMC-1 Cells

To further analyze whether the capsaicin-induced growth inhibition was due to apoptosis, we analyzed cells for apoptotic subpopulations by flow cytometry. At 24-h post-exposure, 16.1 and 26.2% of SNU-1 cells exposed to 100 and 200 μM capsaicin, respectively, belonged to an early apoptotic population, compared to 6.7% of control cells (Fig. 2a). In contrast, only 3.4% of TMC-1 cells treated with 250 μM capsaicin belonged to an early apoptotic population, compared with 2.9% in controls (Fig. 2b), indicating that TMC-1 cells were largely impervious to the apoptotic effect of capsaicin. Furthermore, an assessment of mitochondrial function, determined by measuring DiOC6(3) retention, suggested that capsaicin triggered a concentration-dependent loss of mitochondrial membrane potential in SNU-1 cells (Fig. 3a), but had little effect on mitochondrial membrane potential in TMC-1 cells (Fig. 3b). Consistent with our flow cytometric results, Western blot analyses revealed that high concentrations of capsaicin triggered considerable down-regulation of the anti-apoptotic protein Bcl-2 in SNU-1 cells but not in TMC-1 cells (Fig. 3c). Conversely, an increase in pro-apoptotic Bak protein up to 200 μM of capsaicin was observed in treated SNU-1 cells but not in TMC-1 cells while the changes in Bax protein were less substantial, confirming the operation of a capsaicin-induced, mitochondria-dependent pathway (Fig. 3d).

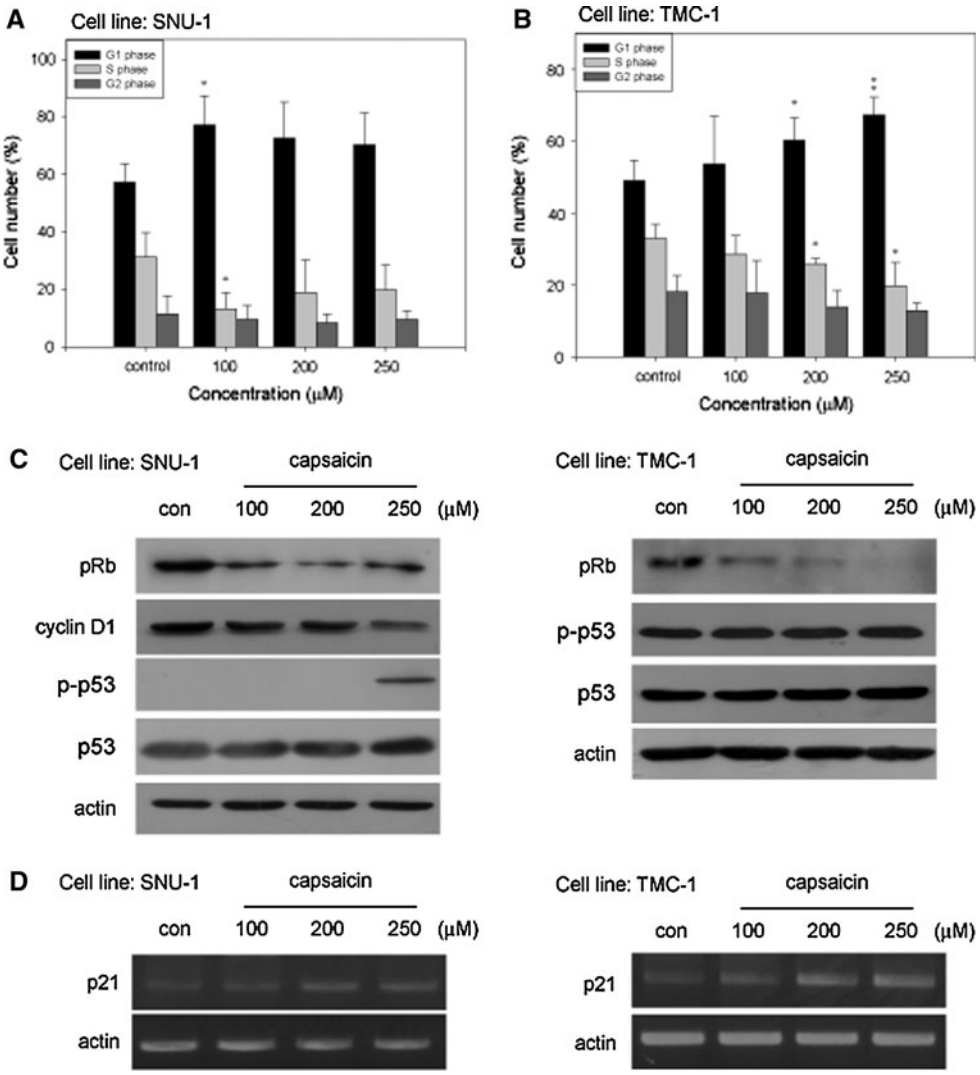

Fig. 4 Capsaicin induces G1 accumulation in SNU-1 and TMC-1 cells. **a**, **b** Cells were incubated with or without capsaicin for 24 h and assayed for cell cycle phase in SNU-1 (**a**) and TMC-1 (**b**) cells. The graphs are representative of three independent experiments. There was a significant difference in cells treated with capsaicin compared to the control group ($*p < 0.05$). **c** Cells were incubated with or without capsaicin for 24 h and aliquots of cell lysates were separated by SDS-PAGE and analyzed for phospho-Rb, cyclin D1, phospho-p53, and total p53 by Western blotting. β-Actin was used as an internal control to monitor for equal loading. **d** p21 mRNA levels were determined by RT-PCR using β-actin as an internal control

Capsaicin Induces Cell Cycle Arrest in Both Gastric Cancer Cell Lines

To address other possible mechanisms that might account for capsaicin-induced growth inhibition, we assessed the cell cycle phase distribution in SNU-1 and TMC-1 cells treated with capsaicin by examining nuclear DNA staining with PI using flow cytometry. The percentage of G0/G1 cells was increased from 57 to 77% by treatment with 100 μM capsaicin in the SNU-1 line (Fig. 4a). At a concentration of 250 μM, capsaicin had a similar effect on TMC-1 cells, gradually increasing the percentage cells in G0/G1 phase from 49% in control to 67% (Fig. 4b). Thus, capsaicin induced G1 accumulation in both cell lines, albeit with different potency. Additional supporting evidence for the cell cycle effects of capsaicin is provided by Western blot analyses, which showed a decrease in Rb phosphorylation and cyclin D1 in capsaicin-treated SNU-1 cells, as well as augmented p53 phosphorylation (Fig. 4c). In TMC-1 cells, the phosphorylated form of Rb was similarly reduced by capsaicin, and cyclin D1 levels were undetectable, even in controls (data not shown). In contrast, phosphorylated p53 levels remained high regardless of capsaicin exposure (Fig. 4c). Our RT-PCR data also demonstrated that capsaicin increased p21 mRNA levels in both cell lines (Fig. 4d), suggesting that accumulation of cells in the G0/G1 phase contributes, at least in part, to the antiproliferative effect of capsaicin on gastric cancer cells.

Oxidative Stress and tNOX Level Influence Capsaicin-Inhibited Gastric Cancer Survival

Given previous reports that capsaicin-induced apoptosis is associated with ROS signaling [7, 21], we next examined the effect of capsaicin on oxidative stress utilizing the fluorescent dye H_2DCFDA which exhibits enhanced fluorescence in the presence of intracellularly generated oxidative stress. Treatment with capsaicin for 1 h induced a concentration-dependent increase in oxidative stress in SNU-1 cells; this increase was inhibited by pretreatment

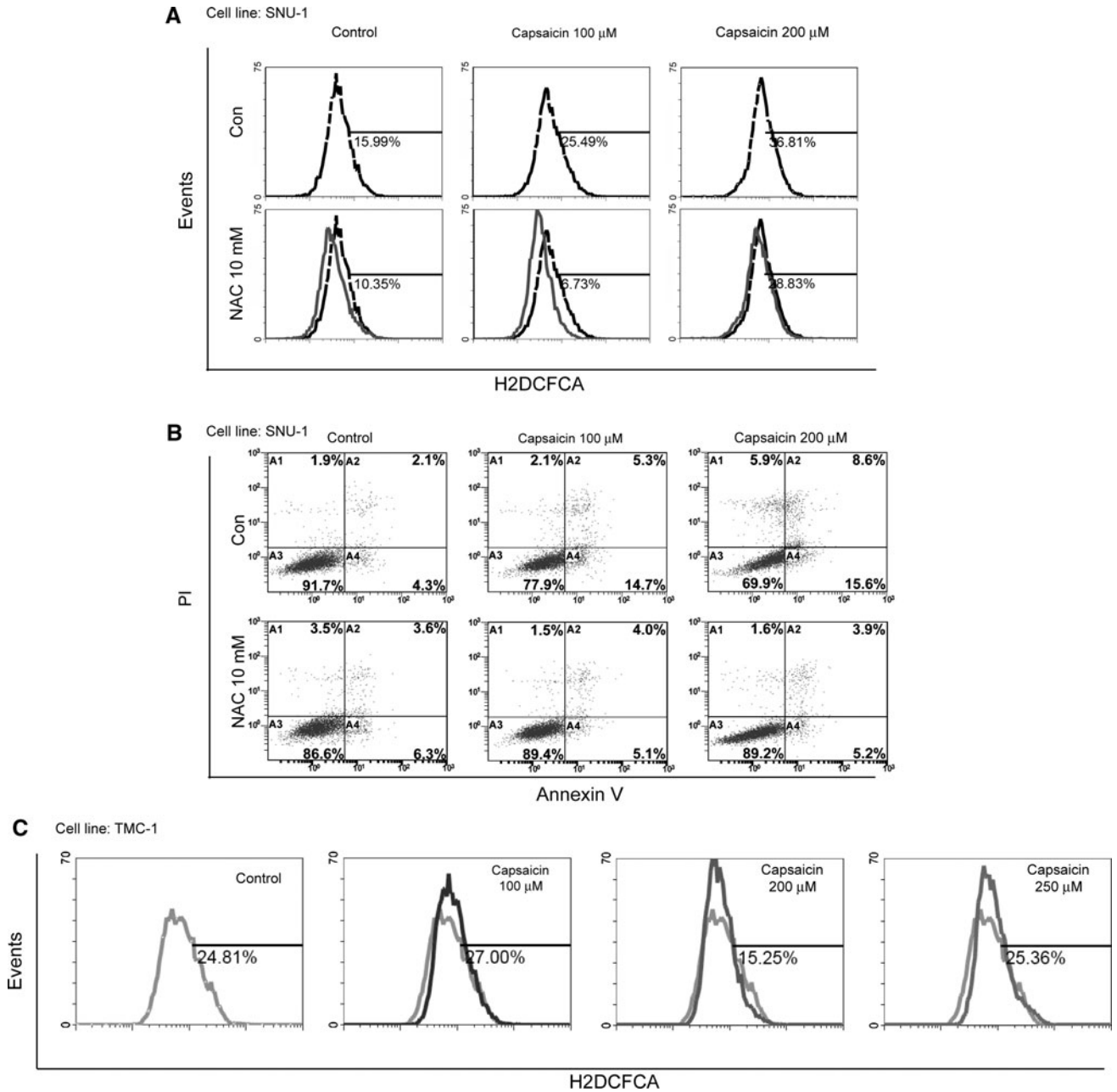

Fig. 5 Capsaicin induces intracellular ROS generation, resulting in apoptosis in SNU-1 cells, but not in TMC-1 cells. **a**, **b** SNU-1 cells were pre-incubated with or without 10 mM NAC at 37°C for 2 h before exposure to capsaicin treatment. The percent change in intracellular ROS generation was measured after capsaicin exposure for 1 h (**a**). The distribution of viable, early apoptotic, later apoptotic, and necrotic cells was analyzed after capsaicin treatment for 24 h (**b**). The graphs depict apoptotic and necrotic populations of cells double-stained with PI- and FITC-labeled annexin V. The results are expressed as a percentage of total cells. **c** TMC-1 cells were pre-incubated with or without 10 mM NAC for 2 h before exposure to capsaicin for 7 h. The percent change in intracellular ROS generation was measured

with the ROS scavenger, *N*-acetyl cysteine (NAC), which reduced the capsaicin-induced increase in fluorescence (Fig. 5a). NAC pretreatment effectively abrogated capsaicin-induced apoptosis, reducing the percentage of cells in the early apoptotic population from 19.6% to 6.2% with a 24-h treatment (Fig. 5b). On the other hand, 250 μM capsaicin had no significant effect on oxidative stress in TMC-1 cells even after a 7-h treatment (Fig. 5c) or apoptosis induction (Fig. 2b). These results indicate that oxidative stress is essential for capsaicin-induced, mitochondria-dependent apoptosis in SNU-1 cells. Since there was neither H_2DCFDA-detected oxidative stress nor

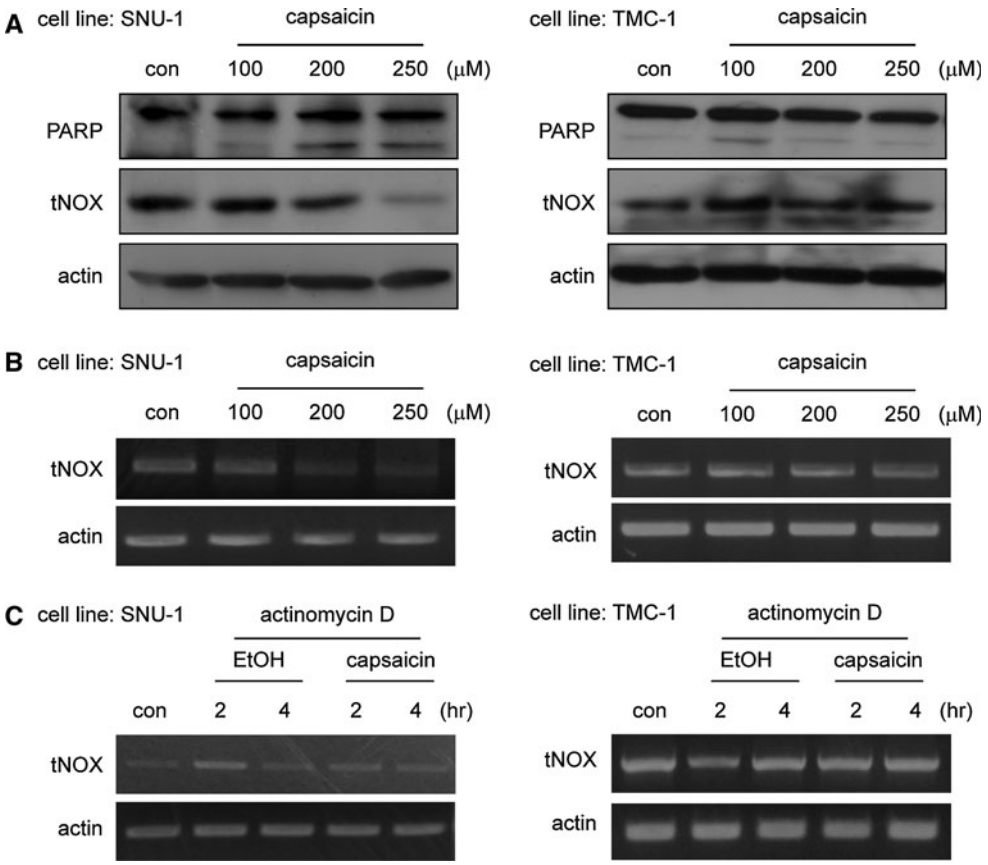

Fig. 6 Capsaicin induces PARP cleavage and tNOX downregulation. Cells were incubated with or without capsaicin for 24 h. **a** Aliquots of cell lysates were separated by SDS-PAGE and analyzed for PARP and tNOX by Western blotting. β-actin was used as an internal control to monitor for equal loading. **b** tNOX mRNA levels were determined by RT-PCR using β-actin as an internal control. **c** Cells were pre-treated with actinomycin D followed by capsaicin treatment and assayed for tNOX transcript stability

apoptosis in capsaicin-treated TMC-1 cells, it remains undecided whether the oxidative stress is important in apoptosis induction in TMC-1 cells.

Previously, we have shown that the anti-growth effect of capsaicin is associated with decreased tNOX activity and tNOX protein levels [18, 20, 30]. In addition, a clear correlation between cytotoxic benzo[*b*]thiophenilsulfonamide 1,1-dioxide (BTS) derivatives induced oxidative stress and tNOX activity inhibition has been reported [41]. To examine whether tNOX is involved in capsaicin-induced oxidative stress and inhibition of gastric cancer growth, we further explored its association with cellular changes caused by capsaicin treatment. We found that capsaicin induced a concentration-dependent increase in PARP cleavage, indicative of caspase-3 activation, in SNU-1 cells; this increase was accompanied by a decrease in tNOX expression (Fig. 6a). In contrast, for 200 and 250 μM capsaicin had little effect on PARP cleavage or tNOX expression in TMC-1 cells (Fig. 6a). This differential effect of capsaicin on tNOX protein expression in the two lines was confirmed by RT-PCR analysis, which showed that capsaicin induced a marked concentration-dependent reduction in tNOX mRNA in SNU-1 cells, but had relatively little effect on those in TMC-1 cells (Fig. 6b). Furthermore, we found that the RNA level of tNOX diminished more slowly in the TMC-1 cells compared with that in SNU-1 cells (Fig. 6c).

To study whether tNOX protein level is important for gastric cancer cell survival, we employed a loss-of-function approach, using shRNA to knock down tNOX expression in capsaicin-resistant TMC-1 cells. Preliminary experiments showed that tNOX-shRNA efficiently inhibited tNOX protein expression in TMC-1 cells (Fig. 7a). Subsequent experiments showed that shRNA-mediated tNOX knockdown in TMC-1 cells resulted in a more SNU-1-like response to capsaicin: the apoptotic cell population was increased (Fig. 7b); mitochondrial membrane potential changes were enhanced (Fig. 7c); and intracellular oxidative stress level was slightly increased compared with wild-type TMC-1 cells (Fig. 7d). Moreover, PARP cleavage was observed in tNOX-shRNA-expressing cells in response to capsaicin treatment, confirming that tNOX deficiency rendered TMC-1 cells sensitive to the pro-apoptotic effect of capsaicin (Fig. 7e). In addition, capsaicin-induced G1 accumulation (Fig. 8a) and inhibition of cell proliferation (Fig. 8b) were enhanced by tNOX knockdown in TMC-1 cells, supporting a central role for tNOX in TMC-1 cell survival.

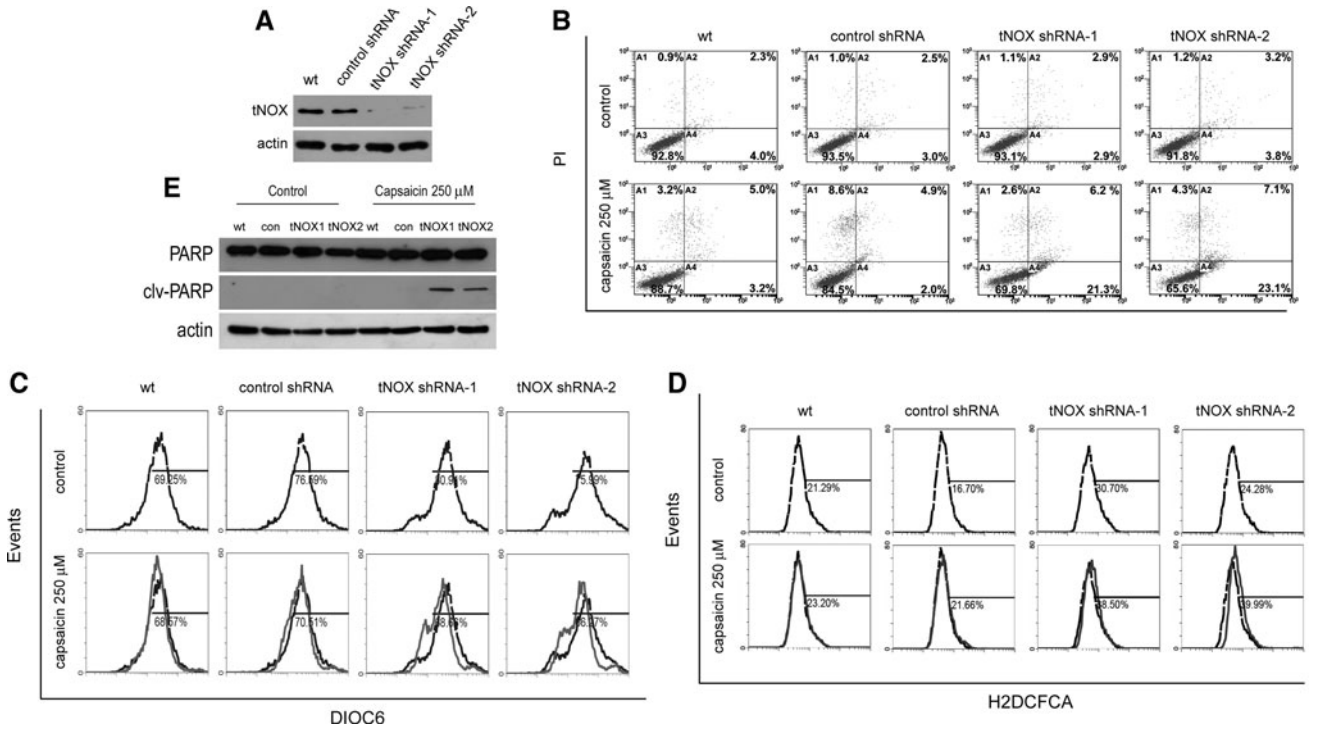

Fig. 7 tNOX knockdown enhances capsaicin-induced apoptosis, mitochondria membrane potential changes, and ROS generation. TMC-1 cells were transfected with control shRNA or tNOX shRNA as described in "Materials and Methods" section. a Aliquots of cell lysates were separated by SDS-PAGE and analyzed for tNOX level by Western blotting. β-Actin was used as an internal control to monitor for equal loading. **b–e** Cells were incubated with or without 250 μM capsaicin. The distribution of viable, early apoptotic, later apoptotic, and necrotic cells was analyzed after capsaicin exposure for 24 h (**b**). The loss of mitochondrial membrane potential was assessed by flow cytometric analysis of $DiOC_6(3)$ retention after 18 h (**c**). The percent change in intracellular ROS generation was measured after 1 h (**d**). **e** Aliquots of cell lysates were separated by SDS-PAGE and analyzed for PARP expression by Western blotting after 24-h treatment. β-Actin was used as an internal control to monitor for equal loading. Representative images are shown

Discussion

In this study, we investigated the anti-proliferative effect of capsaicin on two lines of gastric cancer cells, SNU-1 and TMC-1. Our results suggested that capsaicin enhances oxidative stress and tNOX down-regulation in association with mitochondria-dependent apoptosis, leading to SNU-1 cell growth inhibition. In contrast, capsaicin is largely ineffective in inducing oxidative stress as well as tNOX protein repression, which thus results in almost no apoptosis induction and augmented cell survival in TMC-1 cells. Importantly, tNOX-knockdown sensitizes TMC-1 cells to capsaicin-mediated oxidative stress and mitochondria-dependent apoptosis, leading to cell growth suppression.

A diverse array of singling pathways is involved in capsaicin-induced apoptosis. However, many have highlighted the primary role played by oxidative stress, leading to loss of cell function and ultimately apoptosis [7, 12, 16, 21]. Cellular oxidative stress is mainly contributed by the generation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical ($^{\bullet}OH$) and the superoxide anion (O_2^-). ROS are highly reactive molecules that are produced primarily throughout the electron transport chain in the mitochondria within the cell [42, 43]. In addition to mitochondria, various compartments and numerous enzymes also produce ROS including peroxisomes [44], cyclooxygenases [45], and the NADPH oxidase family [46]. The short-lived free radicals do not penetrate the plasma membrane easily and react fast with other molecules, consequently, leaving little possibility for specific identification. On the other hand, hydrogen peroxide readily diffuses across the membrane and can function as a second messenger in redox signaling mediating diverse cellular responses including cell proliferation, differentiation, and migration [46]. In this study, we determined oxidative stress by measuring the level of hydrogen peroxide generated in the cells and validated that oxidative stress is an upstream event in capsaicin-induced apoptosis—specifically, mitochondria-dependent apoptosis—in SNU-1 cells, but not in TMC-1 cells. Supported by others, generation of hydrogen peroxide is shown to involve in the decreased viability and increased apoptosis in both in vitro and in vivo studies of pancreatic as well as colon cancer models [21, 47].

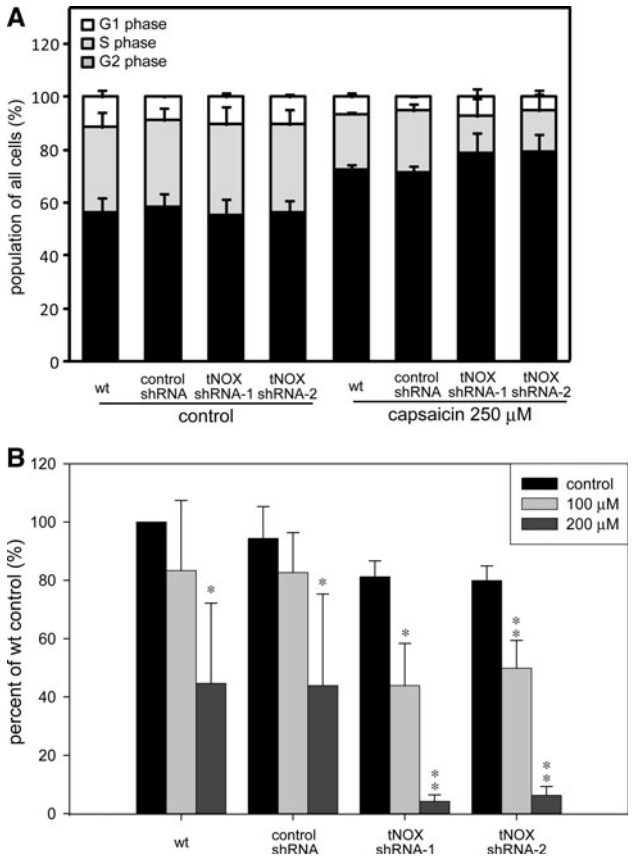

Fig. 8 tNOX knockdown enhances capsaicin-induced G1 accumulation and inhibition of colony-forming ability. TMC-1 cells were transfected with control shRNA or tNOX shRNA as described in "Materials and Methods" section. **a** Cells were then incubated with or without 250 μM capsaicin for 24 h and assayed for cell cycle phase. The graphs are representative of three independent experiments. **b** Cells seeded at 200 cells/dish were incubated with or without capsaicin for 10 days. Cells were allowed to form colonies and colony numbers were counted and recorded. Values (mean ± SE) are from three independent experiments. There was a significant decrease in foci number in cells treated with capsaicin compared with controls (*$p < 0.05$, **$p < 0.01$)

The transient receptor potential vanilloid subtype 1 (TRPV1) is identified as the capsaicin receptor as well as a non-selective cation channel belonging to the TRP family of ion channels [48]. TRPV1 is mainly expressed in the spinal cord and in trigeminal ganglia for the sensation of pain [49] and is activated by several noxious stimuli such as heat, voltage, and vanilloids. Capsaicin functions as an agonist to desensitize TRPV1, thus, commonly used as pain relief. However, TRPV1 also is expressed in diverse tissues suggesting its function in a broader context than pain perception. Specifically, several studies have supported that the anticancer action of capsaicin is through the interaction with the TRPV1 [50, 51] while TRPV1-independent mechanism has been documented as well [10, 18], indicating multiple molecular targets involved in capsaicin-induced apoptosis. Furthermore, cyclooxygenase, a ROS-generating enzyme is shown to involve in the capsaicin-induced apoptosis in human neuroblastoma cells [52]. Also, the plasma membrane oxidoreductase system is proposed to be a redox sensor that in combination with growth factors regulates cell proliferation and apoptosis can be inhibited by capsaicin to trigger Bcl-2-mediated apoptosis [9].

Previously, we showed that capsaicin preferentially inhibits the enzymatic activity of tNOX and the inhibition of activity is well correlated with the reduction of growth of transformed cells [18]. In addition, the cytotoxic benzo[*b*]thiophenilsulfonamide 1,1-dioxide (BTS) derivatives-mediated ROS overproduction is shown to be parallel to inhibition of tNOX activity, implying that inhibition of tNOX might contribute to the cytotoxicity of BTS derivatives [41], or even to a greater extent, tNOX might function as a modulator for ROS generation induced by BTS derivatives. A similar concept is applied to the superoxide-generating NADPH oxidase complex comprising a membrane-bound catalytic subunit NOX and a number of cytosolic regulatory subunits in response to growth factors, subsequently, affecting numerous intracellular signaling pathways [46]. Although not addressed directly, the possibility that tNOX is an upstream regulator of the intracellular ROS generation, an essential signaling intermediate in capsaicin-induced apoptosis and cell-growth inhibition, is suggested by our results.

Multiple aforementioned capsaicin targets may involve in ROS generation and excessive intracellular ROS production with insufficient antioxidant defense by capsaicin cause high cytotoxicity and apoptosis in SNU-1 cells while little effects are observed in TMC-1 cells. This interpretation is supported by a recent proteomic and genomic analyses of TMC-1 cells and SC-M1 gastric cancer cells that reveal differences in the protein expression profile [53]. Another comparative proteomic analysis study also concludes that different capsaicin-mediated oxidative stress levels in two different cancer cells presumably resulted from complicated expression patterns of many oxidative stress and antioxidant genes [15]. At this point, we are still uncertain whether hydrogen peroxide is the only form of ROS or the source of ROS in capsaicin-mediated apoptosis in our system. However, we suggest that tNOX may be involved in the complicated balance system of cellular redox environment and the tNOX/ROS subsequently turn on signal transductions important in participation of a variety of cellular processes. This concept is supported by data from this study demonstrating that tNOX deficiency in TMC-1 cells indeed results in increased intracellular oxidative stress, enhanced changes in mitochondrial membrane potential and increased apoptosis (Fig. 7).

ROS signaling also acts as the mediator of growth-regulatory molecules and modulate gene expression through key transcription factors, such as NF-κB, AP-1, STAT3, FOXO, and others [54–56]. Based on the analysis of the tNOX promoter sequence, some transcription factor candidates, such as NF-κB and AP-1, might regulate tNOX at transcriptional level, implying that tNOX transcription is regulated by ROS signaling. It is possible that those transcription factors regulating tNOX have different expression profile as supported by a proteomic and genomic study in gastric cancer cells [53], resulting in the dissimilarities in tNOX transcription and translation in SNU-1 and TMC-1 cells. An additional perspective to explain the discrepancy is that tNOX transcript is more stable in capsaicin-treated TMC-1 cells compared to that of SNU-1 cells that is resulted from the different expression profile in these two cell lines, as demonstrated by our results in this study (Fig. 6c), bringing about a higher level of tNOX protein expression in capsaicin-treated TMC-1 cells.

Taken together, our results show that capsaicin induces diverse inhibitory effects on the growth of gastric cancer cells, and demonstrate that tNOX down-regulation restores capsaicin-induced growth inhibition in initially resistant TMC-1 gastric cancer cells. These findings establish an essential function of tNOX in the capsaicin-induced oxidative stress, cell-cycle regulation, and survival of gastric cancer cells and may provide a rational framework for the further development of tNOX inhibitors as a novel class of anti-tumor agents.

Acknowledgments The authors thank Dr. Chun-Ying Wu for providing the SNU-1 and TMC-1 cells. Financial support was provided by the grants from the Department of Health, Executive Yuan [DOH-9712, to KNS], and the Ministry of Education, Taiwan, Republic of China under the ATU plan.

References

1. Parkin, D. M., Bray, F., Ferlay, J., & Pisa, P. (2005). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians, 55*, 74–108.
2. Aggarwal, B. B., & Shishodia, S. (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology, 71*, 1397–1421.
3. Hail, N, Jr. (2005). Mitochondria: A novel target for the chemoprevention of cancer. *Apoptosis, 10*, 687–705.
4. Hail, N, Jr., Cortes, M., Drake, E. N., & Spallholz, J. E. (2008). Cancer chemoprevention: A radical perspective. *Free Radical Biology and Medicine, 45*, 97–110. Review.
5. Sun, S. Y., Hail, N, Jr., & Lotan, R. (2004). Apoptosis as a novel target for cancer chemoprevention. *Journal of National Cancer Institute, 96*, 662–672.
6. Dorai, T., & Aggarwal, B. B. (2004). Role of chemopreventive agents in cancer therapy. *Cancer Letters, 215*, 129–140.
7. Ito, K., Nakazato, T., Yamato, K., Miyakawa, Y., Yamada, T., Hozumi, N., et al. (2004). Induction of apoptosis in leukemic cells by homovanillic acid derivative, capsaicin, through oxidative stress: Implication of phosphorylation of p53 at Ser-15 residue by reactive oxygen species. *Cancer Research, 64*, 1071–1078.
8. Lawen, L., Martinus, R. D., McMullen, G. L., Nagley, P., Vaillant, F., Wolvetang, E. J., et al. (1994). The universality of bioenergetic disease: The role of mitochondrial mutation and the putative inter-relationship between mitochondria and plasma membrane NADH oxidoreductase. *Molecular Aspects of Medicine, 15*, s13–s27.
9. Wolvetang, E. J., Larm, J. A., Moutsoulas, P., & Lawen, L. (1996). Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin. *Cell Growth Differentiation, 7*, 1315–1325.
10. Mori, A., Lehmann, S., O'Kelly, J., Kumagai, T., Desmond, J. C., Pervan, M., et al. (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Research, 66*, 3222–3229.
11. Sánchez, A. M., Sánchez, M. G., Malagarie-Cazenave, S., Olea, N., & Díaz-Laviada, I. (2006). Induction of apoptosis in prostate tumor PC-3 cells and inhibition of xenograft prostate tumor growth by the vanilloid capsaicin. *Apoptosis, 11*, 89–99.
12. Sánchez, A. M., Malagarie-Cazenave, S., Olea, N., Vara, D., Chiloeches, A., & Díaz-Laviada, I. (2007). Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. *Apoptosis, 12*, 2013–2024.
13. Kim, C. S., Park, W. H., Park, J. Y., Kang, J. H., Kim, M. O., Kawada, T., et al. (2004). Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferators-activated receptor gamma in HT-29 human colon cancer cells. *Journal of Medicinal Food, 7*, 267–273.
14. Kim, Y. M., Hwang, J. T., Kwak, D. W., Lee, Y. K., & Park, O. J. (2007). Involvement of AMPK signaling cascade in capsaicin-induced apoptosis of HT-29 colon cancer cells. *Annals of the New York Academy Sciences, 1095*, 496–503.
15. Baek, Y. M., Hwang, H. J., Kim, S. W., Hwang, H. S., Lee, S. H., Kim, J. A., et al. (2008). A comparative proteomic analysis for capsaicin-induced apoptosis between human hepatocarcinoma (HepG2) and human neuroblastoma (SK-N-SH) cells. *Proteomics, 8*, 4748–4767.
16. Lee, Y. S., Kang, Y. S., Lee, J. S., Nicolova, S., & Kim, J. A. (2004). Involvement of NADPH oxidase-mediated generation of reactive oxygen species in the apototic cell death by capsaicin in HepG2 human hepatoma cells. *Free Radical Research, 38*, 405–412.
17. Kang, H. J., Soh, Y., Kim, M. S., Lee, E. J., Surh, Y. J., Kim, H. R., et al. (2003). Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in ras-transformed human breast epithelial cells. *International Journal of Cancer, 103*, 475–482.
18. Morré, D. J., Chueh, P. J., & Morré, D. M. (1995). Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 1831–1835.
19. Kim, J. D., Kim, J. M., & Pyo, J. O. (1997). Capsaicin can alter the expression of tumor forming-related genes which might be followed by induction of apoptosis of a Korean stomach cancer cell line, SNU-1. *Cancer Letters, 120*, 235–241.
20. Wang, H. M., Chueh, P. J., Chang, S. P., Yang, C. L., & Shao, K. N. (2009). Effect of capsaicin on tNOX (ENOX2) protein expression in stomach cancer cells. *BioFactors, 34*, 209–217.
21. Zhang, R., Humphreys, I., Sahu, R. P., Shi, Y., & Srivastava, S. K. (2008). In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. *Apoptosis, 13*, 1465–7148.
22. Bhutani, M., Pathak, A. K., Nair, A. S., Kunnumakkara, A. B., Guha, S., Sethi, G., et al. (2007). Capsaicin is a novel blocker of

constitutive and interleukin-6-inducible STAT3 activation. *Clinical Cancer Research, 13*, 3024–3032.
23. Ghosh, A. K., & Basu, S. (2010). Fas-associated factor 1 is a negative regulator in capsaicin induced cancer cell apoptosis. *Cancer Letters, 287*, 142–149.
24. Agrawal, R. C., Wiessler, M., Hecker, E., & Bhide, S. V. (1986). Tumor-promoting effect of chili extract in BALB/c mice. *International Journal of Cancer, 38*, 689–695.
25. Toth, B., & Gannett, P. (1992). Carcinogenicity of lifelong administration of capsaicin of hot pepper in mice. *In Vivo, 6*, 59–63.
26. Bruno, M., Brightman, A. O., Lawrence, J., Werderitsh, D., Morré, D. M., & Morré, D. J. (1992). Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. *Biochemistry Journal, 284*, 625–628.
27. Chueh, P. J. (2000). Cell membrane redox systems and transformation. *Antioxidants & Redox Signaling, 2*, 177–187.
28. Morré, D. J. (1998). NADH oxidase: A multifunctional ectoprotein of the eukaryotic cell surface. In H. Asard, A. Bérczi, & R. Caubergs (Eds.), *Plasma membrane redox systems and their role in biological stress and disease* (pp. 121–1561). Dordrecht: Kluwer Academic Publishers.
29. Chen, C. F., Huang, S., Liu, S. C., & Chueh, P. J. (2006). Effect of polyclonal antisera to recombinant tNOX protein on the growth of transformed cells. *BioFactors, 28*, 119–133.
30. Mao, L. C., Wang, H. M., Lin, Y. Y., Chang, T. K., Hsin, Y. H., & Chueh, P. J. (2008). Stress-induced downregulation of tumor-associated NADH oxidase during apoptosis in transformed cells. *FEBS Letters, 582*, 3445–3450.
31. Morré, D. J., Bridge, A., Wu, L. Y., & Morré, D. M. (2000). Preferential inhibition by (-)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. *Biochemical Pharmacology, 60*, 937–946.
32. Morré, D. J., & Reust, T. (1997). A circulating form of NADH oxidase activity responsive to the antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl)urea (LY181984) specific to sera of cancer patients. *Journal of Bioenergetics and Biomembranes, 29*, 281–289.
33. Morré, D. J., Caldwell, S., Mayorga, A., Wu, L. Y., & Morré, D. M. (1997). NADH oxidase activity from sera altered by capsaicin is widely distributed among cancer patients. *Archives of Biochemistry and Biophysics, 342*, 224–230.
34. Chueh, P. J., Morré, D. J., Wilkinson, F. E., Gibson, J., & Morré, D. M. (1997). A 33.5 kD heat- and protease-resistant NADH oxidase inhibited by capsaicin from sera of cancer patients. *Archives of Biochemistry and Biophysics, 342*, 38–47.
35. Chueh, P. J., Morré, D. M., & Morré, D. J. (2002). A site-directed mutagenesis analysis of tNOX functional domains. *Biochimica et Biophysica Acta, 1594*, 74–83.
36. Chueh, P. J., Kim, C., Cho, N., Morré, D. M., & Morré, D. J. (2002). Molecular cloning and characterization of a tumor-associated, growth-related, and time-keeping hydroquinone (NADH) oxidase (tNOX) of the HeLa cell surface. *Biochemistry, 41*, 3732–3742.
37. Morré, D. J., Chueh, P. J., Yagiz, K., Balicki, A., Kim, C., & Morré, D. M. (2007). ECTO-NOX target for the anticancer isoflavene phenoxodiol. *Oncology Research, 16*, 299–312.
38. Chueh, P. J., Wu, L. Y., Morré, D. M., & Morré, D. J. (2004). tNOX is both necessary and sufficient as a cellular target for the anticancer actions of capsaicin and the green tea catechin (-)-epigallocatechin-3-gallate. *BioFactors, 20*, 249–263.
39. Liu, S. C., Yang, J. J., Shao, K. N., & Chueh, P. J. (2008). RNA interference targeting tNOX attenuates cell migration via a mechanism that involved membrane association of Rac. *Biochemical and Biophysical Research Communication, 365*, 672–677.
40. Lo, Y. C., Yang, Y. C., Wu, I. C., Kuo, F. C., Liu, C. M., Wang, H. W., et al. (2005). Capsaicin-induced cell death in a human gastric adenocarcinoma cell line. *World Journal of Gastroenterology, 11*, 6254–6257.
41. Alonso, M. M., Enćio, I., Martínez-Merino, V., Gil, M. J., & Migliaccio, M. (2001). New cytotoxic benzo(b)thiophenilsulfonamide 1,1-dioxide derivatives inhibit a NADH oxidase located in plasma membranes of tumor cells. *British Journal of Cancer, 85*, 1400–1402.
42. Finkel, T. (2003). Oxidant signals and oxidative stress. *Curentr Opinion Cell Biology, 15*, 247–254.
43. Balaban, R. S., Nemoto, S., & Finkel, T. (2005). Mitochondria, oxidants and aging. *Cell, 120*, 483–495.
44. Schrader, M., & Fahimi, H. D. (2006). Peroxisomes and oxidative stress. *Biochimica et Biophysica Acta, 1763*, 1755–1766.
45. Pathak, S. K., Sharma, R. A., Steward, W. P., Mellon, J. K., Griffiths, T. R. L., & Gescher, A. J. (2005). Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: Targets for chemopreventive strategies. *European Journal Cancer, 41*, 61–70.
46. Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. *Nature Reviews Immunology, 4*, 181–189.
47. Lu, H. F., Chen, Y. L., Yang, J. S., Yang, Y. Y., Liu, J. Y., Hsu, S. C., et al. (2010). Antitumor activity of capsaicin on human colon cancer cells in vitro and colo 205 tumor xenografts in vivo. *Journal of Agricultural and Food Chemistry, 58*, 12999–13005.
48. Nagy, I., Sántha, P., Jancsó, G., & Urbán, L. (2004). The role of the vanilloid (capsaicin) receptor (TRPV1) in physiology and pathology. *European Journal of Pharmacology, 500*, 351–369. Review.
49. Julius, D., & Basbaum, A. I. (2001). Molecular mechanisms of nociception. *Nature, 413*, 203–210. Review.
50. Kim, S. R., Kim, S. U., Oh, U., & Jin, B. K. (2006). Transient receptor potential vanilloid subtype I mediates microglial cell death in vivo and in votro via Ca^{2+}-mediated mitochondrial damage and cytochrome c release. *Journal of Immunology, 177*, 4322–4329.
51. Amantini, C., Ballarini, P., Caprodossi, S., Nabissi, M., Morelli, M. B., Lucciarini, R., et al. (2009). Triggering of transient receptor potential vanilloid type I (TRPV1) by capsaicin induces Fas/CD95-mediated apoptosis of urothelial cancer cells in an ATM-dependent manner. *Carcinogenesis, 30*, 1320–1329.
52. Lee, Y. S., Kwon, E. J., Jin, D. Q., Park, S. H., Kang, Y. S., Huh, K., et al. (2002). redox status-dependent regulation of cycloxygenases mediates the capsaicin-induced apoptosis in human neuroblastoma cells. *Journal Environment Pathology Toxicology Oncology, 21*, 113–120.
53. Chen, Y. R., Juan, H. F., Huang, H. C., Huang, H. H., Lee, Y. J., Liao, M. Y., et al. (2006). Quantitative proteomic and genomic profiling reveals metastasis-related protein expression patterns in gastric cancer cells. *Journal of Proteome Research, 5*, 2727–2742.
54. Sen, C. K., & Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB Journal, 10*, 709–720.
55. Li, L., Cheung, S. H., Evans, E. L., & Shaw, P. E. (2010). Modulation of gene expression and tumor cell growth by redox modification of STAT3. *Cancer Research, 70*, 8222–8232.
56. Dansen, T. B. (2010). FOXO transcription factors: Key players in redox signaling. *Antioxidants & Redox Signaling, 14*, 559–561.