NSAID-activated gene-1 as a molecular target for capsaicin-induced apoptosis through a novel molecular mechanism involving GSK3β, C/EBPβ and ATF3

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Capsaicin, a natural product of the Capsicum species of red peppers, is known to induce apoptosis and suppress growth. Non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) is a cytokine associated with pro-apoptotic and anti-tumorigenic property in colorectal and lung cancer. Our data demonstrate that capsaicin leads to induction of apoptosis and up-regulates NAG-1 gene expression at the transcriptional level. Overexpression of CCAAT/enhancer binding protein β (C/EBPβ) caused a significant increase of basal and capsaicin-induced NAG-1 promoter activity. We subsequently identified C/EBPβ binding sites in the NAG-1 promoter responsible for capsaicin-induced NAG-1 transactivation. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay confirmed binding of C/EBPβ and Protein Kinase C pathways. Knockdown of C/EBPβ, GSK3β or ATF3 ameliorates NAG-1 expression induced by capsaicin treatment. These data indicate that C/EBPβ phosphorylation through GSK3β may mediate capsaicin-induced expression of NAG-1 and apoptosis through cooperation with ATF3 in human colorectal cancer cells.

Introduction

Capsaicin is a homovanillic acid derivative (trans-8-methyl-N-vanillil-6-nonenamide) and a pungent ingredient found mostly in hot chili pepper. Recent reports have demonstrated that it has chemopreventive and chemotherapeutic activities in various cancer models (1–6). In an in vivo study, capsaicin treatment suppressed azoxymethane-induced aberrant cryptic foci formation in rats (7). Various mechanisms of capsaicin-induced apoptosis have also been proposed from different cancer cell models. Capsaicin induces apoptosis through activation of caspase-3 and the intracellular Ca²⁺ release pathway in esophagus carcinoma cells (3). In leukemia, capsaicin induces apoptosis through a p53-dependent mechanism (5), but in prostate cancer cells, capsaicin’s effect is p53 independent (2). Capsaicin-induced apoptosis is elevated by co-treatment of the adenine monophosphate-activated protein kinase activator in colorectal cancer cells (8). Thus, it is likely that various mechanisms are involved in capsaicin-induced apoptosis, and the efficacy of capsaicin is dependent on cell or tissue context.

Non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) is a member of the transforming growth factor β superfamily and is cloned by our group from cyclooxygenase deficient human colorectal cancer cells (9). Expression of the full-length NAG-1-protein results in increased apoptosis and decreased tumor size in the xenograft mouse model (9). Results from our previous study indicated that transgenic mice overexpressing NAG-1 (NAG-Tg) were resistant to azoxymethane-induced aberrant cryptic foci, and NAG-Tg/APE mice showed less tumor load in the small intestine compared with littermate APCMin control mice (10). Recently, we have also shown that NAG-1 expression causes less tumor formation in urethane-induced lung tumor model (11). Thus, NAG-1 expression results in a suppression of tumor formation in colon and lung cancer animal models. Along with these findings, we have also reported that NAG-1 expression is induced not only by NSAIDs (9,12) but also by several antitumorigenic compounds, including PPARγ ligands (13), and dietary chemopreventive compounds, such as conjugated linoleic acid (14), indole-3-carbinol (15), 6-gingerol (16), resveratrol (17), catechins (18), and genistein (19). NAG-1 expression is mediated by a tumor suppressor protein such as p53 (17,19), early growth response gene-1 (12,20), androgen expression in prostate cancer cells (9). Thus, NAG-1 expression is induced not only by NSAIDs (9,12) but also by several antitumorigenic compounds, including PPARγ ligands (13), and dietary chemopreventive compounds, such as conjugated linoleic acid (14), indole-3-carbinol (15), 6-gingerol (16), resveratrol (17), catechins (18), and genistein (19). NAG-1 expression is mediated by a tumor suppressor protein such as p53 (17,19), early growth response gene-1 (12,20), androgen expression in prostate cancer cells (9).

Here, we report for the first time that treating cells with capsaicin results in an increase of NAG-1 expression through GSK3β and ATF3 pathways. Knockdown of C/EBPβ, GSK3β or ATF3 ameliorates NAG-1 expression induced by capsaicin treatment. These data indicate that C/EBPβ phosphorylation through GSK3β may mediate capsaicin-induced expression of NAG-1 and apoptosis through cooperation with ATF3 in human colorectal cancer cells.

Materials and Methods

Materials

Human colorectal cancer cells (HCT-116, SW480, HT-29, and LoVo) were purchased from American Type Culture Collection (Manassas, VA), and culture media were purchase from Bio Whittaker (Rockland, ME). Capsaicin was purchased from Biomol (Plymouth Meeting, PA) and dissolved in ethanol. RO-31-8220, rotterlin, PD98059, SP600125, SB203585, AG490, MGI-32, wortmannin, AR-A014418, and rapamycin were purchased from Calbiochem (San Diego, CA). Antibody for Sp1 was purchased from Upstate (Lake Placid, NY) and antibodies for C/EBPz, C/EBPβ, CAMP response element binding (CREB), retinoic acid receptor (RARα), ATF3, p53, actin and ATF3 small interfering RNA (siRNA) were purchased from Santa Cruz (Santa Cruz, CA). Antibody for phosphor-SerThr was purchased from BD Bioscience (San Jose, CA) and antibody for GSK3β or GSK3β siRNA were purchased from Cell Signaling (Beverly, MA). The antibody for V5 was purchased from Invitrogen (Carlsbad, CA) and NAG-1 antibody was described previously (9). Control and C/EBPβ short hairpin RNA (shRNA) was kindly provided by Dr Jessica Schwartz (University of Michigan, Ann Arbor, MI). All chemicals were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

Cell culture

HCT-116 and HT-29 cells were maintained in McCoy’s 5A medium, and SW480 and LoVo cells were maintained in RPMI and Ham’s F-12 medium, respectively. All media contained 10% fetal bovine serum. The cells were treated with capsaicin under serum-free media for 6–24 h as indicated in the figure 1–6 legends.

Human NAG-1 promoters

The NAG-1 promoters were described previously (23). Internal deletion clones were created using QuickChange II mutagenesis kit (Stratagene, La Jolla, CA)

Abbreviations: ATF3, activating transcription factor 3; C/EBPβ, CCAAT enhancer binding protein; CREB, cAMP response element binding; GSK3β, glycogen synthase kinase 3β; NAG-1, non-steroidal anti-inflammatory drug-activated gene-1; RARα, retinoic acid receptorα

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with the following primers: pNAG-1-1A1-100-103, forward 5'-caccagagca-
cccggctattggaagctgatgcagaa-3', and reverse 5'-gtctgtggtttggttcattggagttggaagctgatgcagaa-3'. pNAG-1-87/-80, forward 5'-tgtgttgatctggatctggatctggatctggatctggatctggatctggagttggaagctgatgcagaa-3', and reverse 5'-gtctgtggtttggttcattggagttggaagctgatgcagaa-3'.

Expression vectors

Full-length C/EBPβ, C/EBPδ, and CHOP (C/EBPβ) cDNAs were amplified from human lung cDNA (Clontech, Mountain View, CA) using ReadyMix Taq polymerase (Sigma, St Louis, MO) with the following primers: C/EBPβ, forward 5'-tgctggtttggttcattggagttggaagctgatgcagaa-3', and reverse 5'-caccagagcacccggctattggaagctgatgcagaa-3'; pNAG-1-87/-80, forward 5'-tgtgttgatctggatctggatctggatctggatctggatctggatctggagttggaagctgatgcagaa-3', and reverse 5'-gtctgtggtttggttcattggagttggaagctgatgcagaa-3'.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed as described previously (12). Briefly, the cells were fixed with 1% formaldehyde for 10 min at 37°C and sonicated four times for 10 s. The cell lysates (0.2 ml) were diluted with immunoprecipitation buffer (1.8 ml) and immunoprecipitated with 5 μg of specific antibodies for immunoglobulin G, C/EBPβ, C/EBPδ, RARα, CREB, and AT3 at 4°C for overnight. The chromatin-associated DNA was eluted, reverse cross-linked by heating at 65°C for 4 h and treated with protease K at 45°C for 2 h. DNA was purified by phenol/chloroform extraction, and precipitated DNA was amplified using the following primer pairs: forward, 5'-caccagagcacccggctattggaagctgatgcagaa-3' and reverse, 5'-gtctgtggtttggttcattggagttggaagctgatgcagaa-3'. PCR products (268 bp) were separated using a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Statistical analysis was performed with the Student's unpaired t-test.

Results

Capsaicin suppresses cell growth, increases apoptosis and NAG-1 expression in human colorectal cancer cells

To observe whether capsaicin affects growth of human colorectal adenocarcinoma cells, we treated HCT-116 cells with different concentrations of capsaicin and measured cell growth. As shown in Fig. 1A, treatment of 50 and 100 μM capsaicin for 24 h showed changes of cell morphology as well as decreased cell number (upper panel) and led to a significant decrease of cell growth (lower panel). These results are from capsaicin-induced cell death because the number of apoptotic cells significantly increased in the groups treated with 50 and 100 μM of capsaicin for 24 h in a dose-dependent manner (Figure 1B).

To investigate whether capsaicin affects NAG-1 expression, HCT-116 cells were incubated with 0, 10, 50 and 100 μM of capsaicin for 24 h and 48 h. As shown in Figure 1C (upper panel), NAG-1 protein levels increased in the cells treated with 50 μM of capsaicin at 24 and 48 h. It has been known that capsaicin increases p53 expression (25)
and NAG-1 is a p53-target gene (17,19), but capsaicin treatment does not increase p53 expression in HCT-116 cells (p53 wild-type). The NAG-1 mRNA level also increased in capsaicin-treated cells in a dose-dependent manner (Figure 1C, lower panel), suggesting that capsaicin affects NAG-1 expression at the transcription level. In the presence of 50 μM of capsaicin, NAG-1 protein began to increase at 6 h and showed a time-dependent induction (Figure 1D). Capsaicin treatment increased NAG-1 in LoVo (p53 wild) and HT-29 (p53 mutant) cells, but not in SW480 (p53 mutant) cells (Figure 1E), indicating cell specificity of capsaicin effects on cell growth in a p53-independent manner. The validation of NAG-1 knockdown is indicated (upper panel). Apoptosis was analyzed using propidium iodide staining as described in Materials and Methods (lower panel).

**Fig. 1.** Capsaicin suppresses cell growth, increases apoptosis and NAG-1 expression in human colorectal cancer cells. (A) HCT-116 cells were treated with 0, 10, 50, and 100 μM of capsaicin for 24 h, and the shape and cell morphology was observed under an optical microscope (upper panel). At the same time, the cell number was counted and expressed as % of inhibition versus vehicle-treated cells (lower panel). Values are expressed as mean ± SD of three replicates. **P < 0.05, **P < 0.01 versus vehicle-treated cells. (B) HCT-116 cells were treated with 0, 10, 50, and 100 μM of capsaicin for 24 h and apoptosis measured as described in Materials and Methods. (C) HCT-116 cells were treated with indicated concentrations of capsaicin for 24 or 48 h and western blot was performed for NAG-1, p53 and actin as described in Materials and Methods (upper panel). HCT-116 cells were treated with indicated concentrations of capsaicin for 24 h, and Reverse transcription–polymerase chain reaction was performed as described in Materials and Methods (lower panel) (D) HCT-116 cells were treated with 50 μM of capsaicin for indicated time points, and western blot was performed for NAG-1 and actin. (E) Three different human colorectal cancer cells (LoVo, HT-29, and SW480) were treated with 0, 50, or 100 μM of capsaicin for 24 h, and western blot was performed for NAG-1 and actin. (F) HCT-116 cells were transfected with control vector (pSuper-retro-puro) or NAG-1 shRNA (pSuper-retro-puro-shNAG-1) as described previously (14) and then treated with 100 μM capsaicin for 24 h. The validation of NAG-1 knockdown is indicated (upper panel). Apoptosis was analyzed using propidium iodide staining as described in Materials and Methods (lower panel).
capsaicin-induced NAG-1 promoter activity (Figure 2B, right panel). To further identify potential regulatory cis-acting elements mediating capsaicin effects, we constructed internal deletion clones lacking potential binding sites for both C/EBPs. Since C/EBPβ binds to this region and its expression results in NAG-1 promoter activity (Figure 2B and C), the cells were transfected with wild-type or deletion constructs lacking C/EBPβ binding sites and treated with 50 μM of capsaicin for 24 h. As shown in Figure 2D, deletion of the distal C/EBP binding site (−110/−103) slightly decreased capsaicin’s effect, whereas deletion of the proximal C/EBP binding site (−87/−80) did not affect luciferase activity. Unexpectedly, capsaicin’s effect was completely abolished in cells transfected with a double deletion promoter construct and then treated with 50 μM of capsaicin for 24 h. The x-axis shows relative luciferase unit. *P < 0.001 versus vehicle-treated cells. RLU, relative luciferase unit.

GSK3β and PKC-mediated phosphorylation of C/EBPβ induces NAG-1 expression

To gain further insight into signaling factors regulating NAG-1 expression by capsaicin, HCT-116 cells were pretreated with vehicle (dimethyl sulfoxide) or different kinase inhibitors for 30 min and incubated with 50 μM capsaicin. Pretreatment of cells with RO-31-8220 (PKC inhibitor) and AR-A014418 (selective GSK3 inhibitor) abolished capsaicin-induced NAG-1 expression, suggesting that these pathways mediate capsaicin-induced NAG-1 expression (Figure 3A). Since NAG-1 expression is mediated by PKCδ in prostate cancer cells (26), we tested the effect of rottlerin (a PKCδ-selective inhibitor) on NAG-1 expression. Pretreatment of rottlerin abolished capsaicin-induced NAG-1 activation (Figure 3B), indicating an involvement of PKCδ on capsaicin-induced NAG-1 expression. Next, to confirm the possible regulatory effect of GSK3β on capsaicin-induced NAG-1 expression, the cells were transfected with GSK3β siRNA, followed by capsaicin treatment. Knockdown of GSK3β attenuated NAG-1 induction by capsaicin (Figure 3C), indicating that GSK3β plays an important role in capsaicin-induced NAG-1 expression.

Furthermore, we measured the phosphorylated serine/threonine form of C/EBPβ using immunoprecipitation to see whether capsaicin affects phosphorylation of C/EBPβ protein. The cells were treated with capsaicin, and pulled down with C/EBPβ antibody, followed by immunoblot with antibody against phosphor serine/threonine or

Fig. 2. Identification of the responsible promoter region for capsaicin-induced NAG-1 transactivation. (A) HCT-116 cells were transfected with a reporter gene containing NAG-1 promoter and then treated with 50 μM capsaicin for 24 h. Luciferase activity was presented as relative luciferase unit. *P < 0.001 versus vehicle-treated cells. (B) The NAG-1 promoter (pNAG-1−133/+41) was co-transfected with indicated expression vector as described in Materials and Methods and then treated with 50 μM capsaicin for 24 h. The x-axis (right panel) shows relative luciferase unit. *P < 0.001 versus vehicle-treated cells. Left panel represents validation of expression vectors. Western blot analysis was performed after transfection with the indicated expressing vector. Empty indicates a pcDNA3.1 vector. (C) Chromatin immunoprecipitation assay was performed using a DNA–protein complex treated with 50 μM capsaicin for 24 h as described in Materials and Methods. The sequence of the NAG-1 promoter region (−131/+137) was amplified by PCR primer pairs as indicated by the arrows. The input represents PCR products obtained from 1% aliquots of chromatin pellets escaping immunoprecipitation. (D) HCT-116 cells were transfected with each internal deletion construct of the NAG-1 promoter and then treated with 50 μM of capsaicin for 24 h. The x-axis shows relative luciferase unit. *P < 0.001 versus vehicle-treated cells. RLU, relative luciferase unit.
C/EBPβ. As shown in Figure 3D, capsaicin treatment induced phosphorylation of serine/threonine residues in C/EBPβ by providing evidence that capsaicin enhances the phosphorylation of C/EBPβ. We further examined whether phosphorylation of C/EBPβ is mediated by PKC and GSK3β. As shown in Figure 3E, phosphorylation of C/EBPβ by capsaicin was inhibited in the presence of inhibitors of these pathways. These results suggest that capsaicin affects GSK3β activation, as compared to expression of GSK3β proteins using immunoblot analysis. Interestingly, capsaicin treatment increased total protein levels of GSK3β (Figure 5A). The mRNA level of GSK3β was also increased (data not shown). It is notable that GSK3β expression begins to increase at 6 h time point (Figure 5B), when NAG-1 expression begins to increase (Figure 1D). Next, we examined GSK3β cellular localization because it has been known that apoptotic signals increase nuclear accumulation of GSK3β and their target genes are activated (28,29). As shown in Figure 5C, capsaicin treatment increased accumulation of GSK3β in the nucleus. These results suggest that capsaicin enhances the expression of GSK3β, thereby accumulating nuclear GSK3β protein and provide the hypothesis that GSK3β may directly associate and phosphorylate C/EBPβ in the nucleus in the presence of capsaicin. To investigate the possibility of direct physical interaction between C/EBPβ and GSK3β, we used histidine tag pull-down assays, followed by western blot after transient transfection of C/EBPβ expression vector (pcDNA3.1/C/EBPβ/V5/His) in the presence of capsaicin. As shown in Figure 5D, capsaicin treatment increases protein–protein interaction between GSK3β and C/EBPβ. These interactions can be suppressed by the inhibition of PKC and GSK3β pathways (Figure 5E).

On the other hand, our previous studies showed that ATF3 plays an important role in the NAG-1 induction (14,18), and recent other studies demonstrated that ATF3 and C/EBPβ may act in concert to regulate their target gene (30,31). Interestingly, nuclear ATF3 level was increased after capsaicin treatment (Figure 5C). Thus, we examined ATF3 protein as an interacting protein with C/EBPβ in capsaicin-induced NAG-1 expression. As shown in Figure 5D, C/EBPβ is able to associate with ATF3 at the basal level and capsaicin treatment increases interaction between C/EBPβ and ATF3. Interestingly, this interaction was also inhibited in the presence of GSK3β and PKC inhibitors (Figure 5E), indicating that interaction of C/EBPβ with ATF3 depends on its phosphorylation. However, incubation with capsaicin for 2 h did not affect formation of transcription factor complex (Figure 5E).

Association of C/EBPβ with GSK3β and ATF3
GSK3β activity is mainly regulated by phosphorylation, transcriptional level, or cellular localization (27). To gain further information of how capsicain affects GSK3β activation, we compared expression of GSK3β proteins using immunoblot analysis. Interestingly, capsaicin treatment increased total protein levels of GSK3β (Figure 5A). The mRNA level of GSK3β was also increased (data not shown). It is notable that GSK3β expression begins to increase at 6 h time point (Figure 5B), when NAG-1 expression begins to increase (Figure 1D). Next, we examined GSK3β cellular localization because it has been known that apoptotic signals increase nuclear accumulation of GSK3β and their target genes are activated (28,29). As shown in Figure 5C, capsicain treatment increased accumulation of GSK3β in the nucleus. These results suggest that capsicain enhances the expression of GSK3β, thereby accumulating nuclear GSK3β protein and provide the hypothesis that GSK3β may directly associate and phosphorylate C/EBPβ in the nucleus in the presence of capsicain. To investigate the possibility of direct physical interaction between C/EBPβ and GSK3β, we used histidine tag pull-down assays, followed by western blot after transient transfection of C/EBPβ expression vector (pcDNA3.1/C/EBPβ/V5/His) in the presence of capsicain. As shown in Figure 5D, capsicain treatment increases protein–protein interaction between GSK3β and C/EBPβ. These interactions can be suppressed by the inhibition of PKC and GSK3β pathways (Figure 5E).

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To confirm the interaction of GSK3β with C/EBPβ and ATF3, the reverse immunoprecipitation using V5 antibody for GSK3β pull down and C/EBPβ or ATF3 antibodies for immunoblotting was performed in the same way. The association of GSK3β with C/EBPβ and ATF3 could be detected in basal level and this association was induced by capsaicin treatment (Figure 5F).

Chromatin immunoprecipitation assay was performed to see whether ATF3 is associated with formation of complex, which binds to the NAG-1 promoter. As shown in Figure 5G, ATF3 associated with NAG-1 promoter. Since electrophoretic mobility shift assay indicated that ATF3 did not bind directly to the NAG-1 promoter (data not shown), it is likely that ATF3 is involved in formation of complex with C/EBPβ, thereafter enhancing capsaicin-induced NAG-1 expression.

Knockdown of C/EBPβ blocks capsaicin-induced NAG-1 expression and apoptosis

Finally, we tested whether C/EBPβ and ATF3 are required for the induction of NAG-1 in response to capsaicin. The cells were transfected with control or C/EBPβ shRNA and then treated with 50 µM capsaicin. Western blot confirmed complete knockdown of C/EBPβ expression (Figure 6A). NAG-1 expression was increased in capsaicin-treated cells; however, C/EBPβ shRNA-transfected cells completely abolished capsaicin-induced NAG-1 expression (Figure 6A).

To examine whether C/EBPβ has any relevance to the induction of apoptosis by capsaicin, we blocked C/EBPβ expression, and apoptosis analysis was performed by flow cytometry. As shown in Figure 6B, fold increase of apoptosis by capsaicin was lower in C/EBPβ shRNA-transfected cells compared with control vector-transfected cells (1.8-fold versus 3.5-fold; P < 0.0001), suggesting a pivotal role of C/EBPβ expression in capsaicin-induced apoptosis in HCT-116 cells. We also examined the role of ATF3 in capsaicin-induced NAG-1 expression using ATF3 siRNA. As shown in Figure 6C, knockdown of the ATF3 gene suppressed capsaicin-induced NAG-1 expression, demonstrating a significant role of ATF3 in NAG-1 expression in response to capsaicin treatment.

Discussion

In this study, we report that capsaicin increases apoptosis and activates proapoptotic gene NAG-1 through C/EBPβ activation in human colorectal cancer cells. We also demonstrate a novel pathway that phosphorylation of C/EBPβ by GSK3β and PKC in the presence of capsaicin is associated with a bZIP protein, ATF3.

The relevance of the NAG-1 gene in apoptosis and cell proliferation by several phytochemicals and chemopreventive drugs has been reported in various cancer models (9,13,14,26). We also observed that NAG-1 induction is implicated in apoptotic activity by capsaicin in human colorectal cancer cells (Figure 1F). Capsaicin-induced cell growth arrest and induction of NAG-1 expression was observed at 50 µM in HCT-116 cells. A low dose (10 µM) of capsaicin is effective to inhibit cell proliferation in endothelial cancer cells (32), whereas a much higher concentration of capsaicin is required to induce apoptosis in colorectal cancer cells (8), prostate cancer cells (2), and gastric carcinoma cells (33).

In terms of physiological concentration of capsaicin, blood concentration of capsaicin was estimated to be 581 ng/ml (equivalent to 2 µM) after intravenous administration of 2 mg capsaicin per kg body wt to rats (34), and topical application of capsaicin resulted in blood capsaicin concentrations up to 10–20 µM (35). According to a recent human study, blood concentration of capsaicin is estimated at 2.47 ng/ml (equivalent to 8.1 nM) in male adults, after administrating 0.4 mg capsaicin per kg body wt (36). However, actual daily intake of capsaicin in the countries that use more chili peppers is much higher (estimated at 2.8 mg/kg body wt) (37,38). Although the concentration of capsaicin (50 µM) we used is higher than plasma levels, we believe that a much higher amount of capsaicin could reach the lumen of the
Capsaicin and NAG-1 in human colorectal cancer

Capsaicin enhances GSK3β expression and protein interactions of GSK3β, C/EBPβ, and ATF3. (A) HCT-116 cells were treated with indicated concentrations of capsaicin for 24 or 48 h and western blot was performed for GSK3β and actin. (B) HCT-116 cells were treated with 50 μM of capsaicin for indicated time points, and western blot was performed for GSK3β and actin. (C) The cells were treated with 50 μM of capsaicin for 6 h and nuclear and cytosol fractions were isolated, and then western blot was performed for GSK3β, C/EBPβ, and actin. (D) The cells were transfected with C/EBPβ expression vector-tagged V5 (pcDNA3.1/C/EBPβ2/V5/His) and treated with 50 μM capsaicin for 6 h. The cell lysates were immunoprecipitated with ProBond nickel-chelating resin (Invitrogen), separated by sodium dodecyl sulfate–polyacrylamide gel, transferred to membranes, and then immunoblotted with V5, C/EBPβ, or ATF3 antibody. (E) HCT-116 cells were transfected with pcDNA3.1/C/EBPβ2/V5/His, pretreated with RO-31-8220 (2.5 μM) or AR-A014418 (30 μM) for 30 min then treated with 50 μM capsaicin for 2 or 6 h. Immunoprecipitation was performed as described in (D). (F) The cells were transfected with GSK3β expression vector (pcDNA3.1/GSK3β/V5/His) and treated with 50 μM capsaicin for 6 h. The cell lysates were immunoprecipitated with ProBond nickel-chelating resin (Invitrogen), separated by sodium dodecyl sulfate–polyacrylamide gel, transferred to membranes, and then immunoblotted with V5, C/EBPβ or ATF3 antibody. (G) Chromatin immunoprecipitation assay for ATF3 binding was performed using a DNA–protein complex treated with 50 μM capsaicin for 24 h as described in Materials and Methods. The sequence of the NAG-1 promoter region (−131/−137) was amplified by PCR primer pairs as indicated by the arrows. The input represents PCR products obtained from 1% aliquots of chromatin pellets escaping immunoprecipitation.

Gastrointestinal tract. This can be observed in other phytochemicals such as epigallocatechin gallate, which has a concentration 28–165 times higher in the gastrointestinal tract than in plasma (39).

The transcriptional regulation of NAG-1 gene is mediated by several mechanisms, including p53 tumor suppressor gene (17,19), and early growth response gene-1 (12,20) pathways. It has been reported that capsaicin induces apoptosis through a p53-dependent pathway in stomach cancer and leukemia cells (5,25). However, in colorectal cancer cells, it is unlikely that capsaicin-induced NAG-1 expression is p53 dependent, although NAG-1 is a target of p53 tumor suppressor gene.

Our data showed that knockdown of C/EBPβ suppressed capsaicin-induced NAG-1 expression (Figure 6A). These results, together with the observations that overexpression of C/EBPβ is associated with the activation of the NAG-1 promoter (Figure 2B) implicate a crucial role of C/EBPβ in NAG-1 induction by capsaicin. Thus, NAG-1 appears to be one of the target genes for C/EBPβ transcription factor. C/EBPβ seems to play a role in promoting tumorigenesis in some cancer models, including breast (40) and prostate (41). For colorectal cancer, overexpression of C/EBPβ protein results in cellular growth arrest and apoptosis (42), although C/EBPβ expression is increased in human colorectal cancer (43). It remains unclear how C/EBPβ is involved in tumor suppression through its interaction with specific downstream genes in cancer cells. Our data demonstrate that C/EBPβ phosphorylation modulates recruitment of proapoptotic protein such as ATF3, enhancing its activity in NAG-1 expression.

The current data show that phosphorylation of C/EBPβ by capsaicin is positively regulated by GSK3β pathway. Although we did not examine the changes of all phosphor form of C/EBPβ in this study, it has been shown that GSK3β phosphorylates C/EBPβ on Ser177, Ser183, and Thr189 residues (44). Although phosphorylation of C/EBPβ is regulated by GSK3β (44–46) in many types of cells, the detailed mechanisms are not fully elucidated. In this study, capsaicin treatment not only caused an increased expression of GSK3β, but also increased accumulation of GSK3β in nuclear fraction (Figure 5A–C). In addition, the nuclear GSK3β directly associates with C/EBPβ (Figure 5D–F). Thus, our data suggest that capsaicin-induced overexpression of GSK3β results in the direct interaction with C/EBPβ and subsequent phosphorylation of C/EBPβ. Indeed, GSK3β increases rapidly early in the process of apoptosis and is able to modulate gene expression through its regulation of transcription factor (28). We observed that capsaicin induced GSK3β mRNA (data not shown), implying transcriptional regulation of GSK3β by capsaicin. Further study is required to obtain more detailed information on how capsaicin regulates GSK3β gene expression.

Like other C/EBP members, C/EBPβ phosphorylation at several serine and threonine residues allows recruitment of the transcriptional coactivator (47). C/EBPβ is able to form heterodimers, and such interactions alter DNA binding affinity and gene transcription. In fact,
C/EBPβ has been shown to interact with non-bZIP proteins such as p50 subunit of nuclear factor-kappaB, glucocorticoid receptor, and retinoblastoma protein (48–51). Such heterodimerization indicates different transactivation potential and DNA binding affinity/specificity. Another interesting finding of this study is that ATF3 may cause NAG-1 expression, at least in part, through the recruitment of C/EBPβ. Although ATF3 does not bind to the NAG-1 promoter directly, the C/EBPβ–ATF3 complex would be an important factor for capsaicin-induced NAG-1 expression. These observations interpret direct evidence to support the importance of ATF3 in increased DNA-binding potential of C/EBPβ, which consequently mediates enhancement of C/EBPβ-driven transactivation of the NAG-1 gene. One speculation would be that ATF3 may play a role as a bridge protein that facilitates recruitment of C/EBPβ to the NAG-1 promoter during its activation. Despite the predominant cooperative regulation of C/EBPβ, until now the question of whether ATF3 interacts with other transcription factors has not been addressed in the literature. Indeed, the fact that double deletion of C/EBP binding sites are required to abolish luciferase activity of capsaicin-induced NAG-1 expression supports this hypothesis. Secondly, capsaicin may lead to the formation of a supracomplex, including C/EBPβ, ATF3 and other transcription factors because C/EBP dimers are able to be cross-coupled to other transcription factors and show DNA-binding characteristics distinct from those of the individual transcription factor partners (52).

Our additional finding is that protein–protein interactions among GSK3β, C/EBPβ, and ATF3 were inhibited by pretreatment of GSK3β selective inhibitor (Figure 5E). These data suggest that phosphorylation of C/EBPβ by capsaicin increases recruitment of ATF3 for binding to C/EBPβ, which subsequently increases NAG-1 gene transcription. With respect to other kinase pathways, we observed that PKCδ is another mediator of C/EBPβ phosphorylation and NAG-1 induction by capsaicin (Figure 3). This is consistent with a previous study, showing that PKCδ targets NAG-1 gene transcription (26) and other proapoptotic signaling pathways in many cancer models (53,54).

We evaluated the effect of capsaicin on C/EBPβ phosphorylation and formation of transcription complex after shorter incubation (2 h) with capsaicin (Figures 3E and 5E). The results show that capsaicin did not affect C/EBPβ phosphorylation or complex formation at 2 h. It is notable that C/EBPβ phosphorylation usually occurs at a late time point (4–6 h) after stimulation (45,55). However, we do not exclude the possibility that C/EBPβ phosphorylation occurs via different signaling (GSK3β, PKC, or other kinase). It is still unclear how complex alterations including C/EBPβ and ATF3 are able to participate in NAG-1 induction, but upon cellular activation by capsaicin, it is speculated that phosphorylation of C/EBPβ enhances the formation of the transcriptional complex with ATF3 and DNA binding affinity to the promoter regions because C/EBPβ at the resting state has weak DNA binding activity due to an intramolecular inhibitory element, whereas the phosphorylation of C/EBPβ releases the intramolecular inhibition and exposes the DNA binding site (56). In conclusion, capsaicin activates the transcription of the NAG-1 gene by activation of the C/EBPβ-dependent pathway. GSK3β modulates C/EBPβ phosphorylation by

**Fig. 6.** Knockdown of C/EBPβ alters capsaicin-induced NAG-1 expression and apoptosis. (A) HCT-116 cells were transfected with control or C/EBPβ shRNA using Lipofectamine 2000 for 48 h and then treated with vehicle or 50 μM capsaicin for 24 h. Western analysis was performed for C/EBPβ, NAG-1, and actin. (B) HCT-116 cells were transfected with control or C/EBPβ shRNA and treated with 100 μM of capsaicin for 24 h. Apoptosis was analyzed as described in Materials and Methods. Values are expressed as mean ± SD of three replicates. (C) HCT-116 cells were transfected with control or ATF3 siRNA (200 nM) using TransIT-TKO Mirus transfection reagent for 48 h and then treated with ethanol or 50 μM of capsaicin for 24 h. Western analysis was performed for ATF3, NAG-1, and actin. (D) Proposed mechanism by which capsaicin induces NAG-1 transcription in human colorectal cancer. Capsaicin activates C/EBPβ through GSK3β- and PKCδ-dependent phosphorylation. Capsaicin increase nuclear accumulation of GSK3β and phosphorylates C/EBPβ through direct interaction with C/EBPβ. Activation of C/EBPβ increases the binding affinity of C/EBPβ onto NAG-1 promoter and activates transcription of NAG-1 genes. ATF3 may play a role as a bridge protein or formation of supracomplex, including ATF3 and other transcription factors, thereby enhancing the recruitment of C/EBPβ to the NAG-1 promoter. Upregulation of the NAG-1 gene results in an increase of apoptosis in colorectal cancer cells.
direct protein–protein interaction, and activation of C/EBPβ increases the binding affinity of C/EBPβ onto the NAG-1 promoter and increases transcription of NAG-1 genes. In this serial sequence, ATF3 may facilitate the recruitment of C/EBPβ to the NAG-1 promoter, and PKCδ also affects phosphorylation of C/EBPβ (Figure 6D).

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