Counteracting effect of TRPC1-associated Ca\(^{2+}\) influx on TNF-\(\alpha\)-induced COX-2-dependent prostaglandin E\(_2\) production in human colonic myofibroblasts

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Hai L, Kawarabayashi Y, Imai Y, Honda A, Inoue R. Counteracting effect of TRPC1-associated Ca\(^{2+}\) influx on TNF-\(\alpha\)-induced COX-2-dependent prostaglandin E\(_2\) production in human colonic myofibroblasts. Am J Physiol Gastrointest Liver Physiol 301: G356–G367, 2011. First published May 5, 2011; doi:10.1152/ajpgi.00354.2010.—TNF-\(\alpha\)-NF-\(\kappa\)B signaling plays a central role in inflammation, apoptosis, and neoplasia. One major consequence of this signaling in the gut is increased production of prostaglandin E\(_2\) (PGE\(_2\)) via cyclooxygenase-2 (COX-2) induction in myofibroblasts, which has been reported to be dependent on Ca\(^{2+}\). In this study, we explored a potential role of canonical transient receptor potential (TRPC) proteins in this Ca\(^{2+}\)-mediated signaling using a human colonic myofibroblast cell line CCD-18Co. In CCD-18Co cell, treatment with TNF-\(\alpha\) greatly enhanced Ca\(^{2+}\) influx induced by store depletion along with increased cell-surface expression of TRPC1 protein (but not of the other TRPC isoforms) and induction of a Gd\(^{3+}\)-sensitive nonsselective cationic conductance. Selective inhibition of TRPC1 expression by small interfering RNA (siRNA) or functionally effective TRPC1 antibody targeting the near-pore region of TRPC1 (T1E3) antagonized the enhancement of store-dependent Ca\(^{2+}\) influx by TNF-\(\alpha\), whereas potentiated TNF-\(\alpha\)-induced PGE\(_2\) production. Overexpression of TRPC1 in CCD-18Co produced opposite consequences. Inhibitors of NF-\(\kappa\)B (curcumin, SN-50) attenuated TNF-\(\alpha\)-induced enhancement of TRPC1 expression, store-dependent Ca\(^{2+}\) influx, and COX-2-dependent PGE\(_2\) production. In contrast, inhibition of calcineurin-nuclear factor of activated T-cell proteins (NFAT) signaling by FK506 or NFAT Activation Inhibitor III enhanced the PGE\(_2\) production without affecting TRPC1 expression and the Ca\(^{2+}\) influx. Finally, the suppression of store-dependent Ca\(^{2+}\) influx by T1E3 antibody or siRNA knockdown significantly facilitated TNF-\(\alpha\)-induced NF-\(\kappa\)B nuclear translocation. In aggregate, these results strongly suggest that, in colonic myofibroblasts, NF-\(\kappa\)B and NFAT serve as important positive and negative transcriptional regulators of TNF-\(\alpha\)-induced COX-2-dependent PGE\(_2\) production, respectively, at the downstream of TRPC1-associated Ca\(^{2+}\) influx.

Ca\(^{2+}\) signaling; nuclear factor of activated T-cell proteins; NF-\(\kappa\)B; cytokine; inflammation; transient receptor potential; cyclooxygenase-2

SUBEPITHELIAL MYOFIBROBLASTS are located at the interface between the epithelium and lamina propria in most mucosal tissues. They have ultrastructural features reminiscent of both smooth muscle cells and fibroblasts (40). A growing body of evidence has been accumulated that myofibroblasts play crucial roles in intestinal homeostasis, inflammation, and neoplasia. The major mediator produced in myofibroblasts is prostaglandin E\(_2\) (PGE\(_2\)), which plays both protective and destructive roles in the gut. Although genetic deletion of the PGE\(_2\) receptor EP4 is detrimental to the gut, high concentrations of PGE\(_2\) analogs are also shown to exacerbate clinical colitis leading to tumorigenicity, presumably through the induction of proinflammatory responses (12, 33, 36). The formation of PGE\(_2\) in myofibroblasts is catalyzed mainly by cyclooxygenase-2 (COX-2), which is distinct from another isoform COX-1. COX-1 is a housekeeping enzyme and is constitutively expressed in most tissues, whereas COX-2 is expressed at low levels under unstimulated conditions but is rapidly induced upon inflammation and proliferation in response to cytokines, growth factors, and tumor promoters (35). The rate of PGE\(_2\) production is significantly enhanced via induction of COX-2 by tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), which is central to the inflammation and acts as an endogenous tumor promoter (15). Therapeutic antibodies against TNF-\(\alpha\) exert dramatic ameliorating effects on inflammatory bowel syndrome, in which myofibroblasts are found to play a vital role (4).

The myofibroblast cell line CCD-18Co was established from the mucosal tissue of human colon. CCD-18Co expresses COXs and secretes PGE\(_2\), the rate of which is significantly enhanced by TNF-\(\alpha\) or interleukin-1\(\beta\) (IL-1\(\beta\)) (14). There is evidence that COX-2 expression and PGE\(_2\) production in myofibroblasts are controlled by intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (25, 47). However, what sources of Ca\(^{2+}\) contribute thereto remain entirely unclear. In general, there are two distinct sources for Ca\(^{2+}\) that can elevate [Ca\(^{2+}\)]\(_i\), i.e., Ca\(^{2+}\) influx across the plasma membrane and Ca\(^{2+}\) release from the endoplasmic reticulum (ER). Ca\(^{2+}\) influx can occur through voltage-gated (VGC), receptor-operated (ROC), and store-operated Ca\(^{2+}\) channels (SOC). Recent studies have shown that the canonical members of the transient receptor potential (TRPC) proteins may contribute to SOC and ROC. The TRPC family consists of seven distinct isoforms designated as TRPC1-TRPC7 (1, 8, 10, 30). Presently, TRPC1 is regarded as one of plausible candidate molecules for SOC in many cell types (1, 30) although early studies in T-lymphocytes and other hematopoietic cells demonstrated that the molecular identification of the bona fide SOC pore-forming subunit is Orai1 (9). Recent evidence suggests that the Ca\(^{2+}\)-transporting activity of these SOC molecules is regulated by an ER Ca\(^{2+}\) sensor protein, the stromal interacting molecule 1 (STIM1), which relays the store depletion signal to the plasma membrane to activate SOC (19, 32, 46). In some cell types, TRPC1 dynamically assembles with both STIM1 and Orai1 to generate more complexity in store-dependent Ca\(^{2+}\) influx mechanisms (27) although whether TRPC1 serves as a pore-forming subunit of SOC still remains unclear.

The nuclear factor of activated T-cell proteins (NFATs) and the NF-\(\kappa\)B are widely distributed, Ca\(^{2+}\)-dependent transcription factors that regulate a multitude of physiological and pathophysiological processes (3, 39, 44). NFAT is activated

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via dephosphorylation by calcineurin, which is activated upon binding of Ca\(^{2+}\)/calmodulin. NFAT has been reported to regulate COX-2 expression in colon carcinoma cells (5), and its activation can occur through Ca\(^{2+}\) influx associated with TRPC1-, TRPC3-, or TRPC6-associated SOC or ROC activities (26, 38). On the other hand, the activation of NF-κB is initiated by the phosphorylation of IkB (inhibitors of NF-κB) proteins, which under unstimulated conditions prevent the nuclear localization of the p65 protein, a subunit of inactive NF-κB dimer. When phosphorylated on serine residues in response to proinflammatory stimuli, IkBs are ubiquitinated and then degraded by the proteasome (2, 13). In human gingival fibroblasts, TNF-α-induced PGE\(_2\) release has been assigned to COX-2 induction via an NF-κB-dependent pathway (23). Furthermore, the transcriptional upregulation of TRPC1 via TNF-α-NF-κB signaling has been reported in endothelial cells (29). Conversely, in epithelial cells, increased TRPC1 expression inhibits NF-κB activity as a result of enhanced Ca\(^{2+}\) influx (20, 21).

In this study, on the basis of the above knowledge, we hypothesized that in gut myofibroblasts TNF-α may stimulate COX-2-dependent PGE\(_2\) production via enhanced [Ca\(^{2+}\)]\(_i\), dynamics through activation of TRPC channels. To test this hypothesis, we investigated the effects of TNF-α on the expression level of TRPC proteins and associated Ca\(^{2+}\)-transporting activities. We further examined their impacts on PGE\(_2\) production in CCD-18Co myofibroblasts, with particular interest in the regulation of Ca\(^{2+}\)- dependent transcription factors NFAT and NF-κB. Part of this study has been communicated to the 36th Congress of the International Union of Physiological Sciences in Kyoto (2009).

**MATERIALS AND METHODS**

**Cell culture.** Human colonic subepithelial myofibroblast cell line, CCD-18Co, was purchased from the American Type Culture Collection (CRL-1459) and grown in MEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM nonessential amino acids. CCD-18Co myofibroblasts were passaged 12 to 17 times and, when necessary, transfected with the stealth siRNA of TRPC1 or full-length human TRPC1 cDNA inserted into the pCIneo vector by the aid of a transfectant Lipofectamine 2000 (Invitrogen).

**RT-PCR.** Total RNA extracted from CCD-18Co cells was subjected to semiquantitative RT-PCR using the following protocol: preheating at 94°C for 1 min followed by 20, 30, or 35 cycles of denaturation at 94°C for 10 s, annealing at 58–65°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 10 min. RNA extract from whole rat brain was used as a positive control. Primer pairs used for each TRPC isoform are listed in Table 1. PCR amplicons were electrophoresed and visualized by SYBR Green (Qiagen).

**PGE\(_2\) immunoassay.** CCD-18Co myofibroblasts were seeded at the density of 5 × 10\(^5\) cells/cm\(^2\) and grown to confluence in a 24-well culture plate filled with MEM containing 10% FBS, 2 mM L-glutamine, and 0.1 mM nonessential amino acids. After being cultured for 24 h at 37°C in the presence or absence of drugs listed below, cells were washed with Dulbecco’s PBS (Sigma). After being incubated in fresh prewarmed PBS at 37°C, the resulting supernatant was collected and immediately frozen at −80°C. The remaining cells were collected to measure protein concentration. PGE\(_2\) levels were determined for appropriately diluted supernatants by using a PGE\(_2\) enzyme immunoassay kit (R&D Systems) according to the manufacturer’s instructions. The obtained values were normalized and expressed per unit of protein weight to minimize variations among different experimental conditions. According to the manufacturer’s information, the antibody used in the PGE\(_2\) immunoassay crossreacts 17.5% with PGE\(_3\), 11.9% with PGE\(_1\), 7.0% with PGI\(_2\), 6.0% with PGF\(_2α\), 2.5% with 6-keto-PGF\(_1α\), but negligibly with the other eicosanoid pathway products.

**Immunoblot analysis.** Immunoblotting experiments were performed to examine the protein level of TRPC1, STIM1, Orai1, COX-2, NFAT3, IkB-α, and NF-κB/p65 in CCD-18Co myofibroblasts. Total cell lysates were prepared in sample buffer. Cytoplasmic and nuclear proteins were extracted from CCD-18Co cells by using the ProteoJET, a cytoplasmic and nuclear protein extraction kit (Fermentas), according to the manufacturer’s instructions. Protein concentrations of samples were determined using a BCA protein assay kit (Pierce). Just before electrophoresis, 5% (vol/vol) 2-mercaptoethanol and 1% (wt/vol) bromophenol blue were added to the sample, and proteins were separated by 10% (wt/vol) SDS-PAGE and electrothermically transferred to a PVDF membrane. The membrane was blocked with 5% (wt/vol) skim milk dissolved in Tween-PBS and then incubated overnight with anti-TRPC1 antibody (Alomone) (1: 200), anti-Na\(^+/K^+\) ATPase-α1 (NKA) antibody (Upstate) (1:500), anti-COX-2 antibody (BD Transduction Laboratories) (1:200), anti-β-actin antibody (Abcam) (1:2,000), anti-GOK/STIM1 antibody (BD Transduction Laboratories) (1:500), anti-Orai1 antibody (Alomone) (1:200), anti-NFAT3 antibody (Cell Signaling) (1:1,000), anti-IκB-α antibody (Cell Signaling) (1:1,000), anti-NF-κB p65 antibody (Cell Signaling) (1:1,000), or anti-Lamin B antibody (Santa Cruz Biotechnology) (1:1,000), respectively. Protein expressions were detected by immunoblotting experiments were performed to examine the protein level of TRPC1, STIM1, Orai1, COX-2, NFAT3, IkB-α, and NF-κB/p65 in CCD-18Co myofibroblasts. Total cell lysates were prepared in sample buffer. Cytoplasmic and nuclear proteins were extracted from CCD-18Co cells by using the ProteoJET, a cytoplasmic and nuclear protein extraction kit (Fermentas), according to the manufacturer’s instructions. Protein concentrations of samples were determined using a BCA protein assay kit (Pierce). Just before electrophoresis, 5% (vol/vol) 2-mercaptoethanol and 1% (wt/vol) bromophenol blue were added to the sample, and proteins were separated by 10% (wt/vol) SDS-PAGE and electrothermically transferred to a PVDF membrane. The membrane was blocked with 5% (wt/vol) skim milk dissolved in Tween-PBS and then incubated overnight with anti-TRPC1 antibody (Alomone) (1: 200), anti-Na\(^+/K^+\) ATPase-α1 (NKA) antibody (Upstate) (1:500), anti-COX-2 antibody (BD Transduction Laboratories) (1:200), anti-β-actin antibody (Abcam) (1:2,000), anti-GOK/STIM1 antibody (BD Transduction Laboratories) (1:500), anti-Orai1 antibody (Alomone) (1:200), anti-NFAT3 antibody (Cell Signaling) (1:1,000), anti-IκB-α antibody (Cell Signaling) (1:1,000), anti-NF-κB p65 antibody (Cell Signaling) (1:1,000), or anti-Lamin B antibody (Santa Cruz Biotechnology) (1:1,000), respectively. Protein expressions were detected by

### Table 1. Primers for semi-quantitative RT-PCR

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<th>Reverse Primer (5′→3′)</th>
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### Primers for real-time RT-PCR

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<td>β-Actin</td>
<td>TACACCTGATCATTTAACCT</td>
<td>GATACCTTTCGAGCTTGT</td>
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Prostaglandin E\(_2\) (PGE\(_2\)) immunoassay. TRPC, transient receptor potential; COX, cyclooxygenase.
incubating the PVDF membrane with respective secondary antibodies linked to horseradish peroxidase.

**Cell surface biotinylation assay.** CCD-18Co cells were washed once with ice-cold PBS and added to 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) for 30 min on ice. Thereafter, biotin was quenched with 50 mM glycine on ice for 10–15 min. The cells were lysed using 500 μl of lysis buffer containing the following: 500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 10% sucrose, and 1 μg/ml aprotinin leupeptin (pH 7.5). The cell extracts were sonicated for 10 s six times, and insoluble material was spun down at 30,000 g for 20 min. Immobilized avidin beads (20 μl) (Sigma) in PBS were added to a 100-μl aliquot of cell extract and incubated on a rotator overnight at 4°C. The bound fraction was washed and released in SDS-PAGE sample buffer and analyzed by Western blotting with anti-TRPC1 or anti-NKA antibody as described above. NKA was used as an internal control of biotin-labeled membrane fraction.

**Measurement of [Ca^{2+}]i.** The intracellular Ca^{2+} concentration ([Ca^{2+}]i) of myofibroblasts was monitored by the fura-2 digital fluorescence imaging technique. Briefly, CCD-18Co myofibroblasts were plated on a poly-L-lysine-coated glass chamber placed on the stage of an inverted fluorescent microscope (DMI600B, Leica Microsystems). The cells were then loaded with fura-2 AM (5 μM with 0.01% pluronic acid) in the dark at room temperature for 30 min. The intensity of fura-2 fluorescence emitted at 510 nm (±10 nm) by 340- or 380-nm excitation was measured by means of a digital photo-imaging system composed of a fluorescent microscope (DMI600B, Leica) and a low-noise, high-intensifying EMCCD camera (Cascade, Nippon Roper). The data acquisition and analysis were made by the software SlideBook 4.2 (Intelligent Imaging Innovation). The obtained fluorescence was corrected for background fluorescence, and changes in [Ca^{2+}]i were defined as the ratio of corrected fluorescence intensities at 340 and 380 nm, respectively (F_{340}/F_{380}). To evaluate the magnitude of Ca^{2+} entry in CCD-18Co cells, they were first perfused with Ca^{2+}-free external solution containing cyclopiazonic acid (CPA; 10 μM) and then exposed to 1 mM Ca^{2+} re-introduced into the bath.

**Electrophysiology.** Patch electrodes of 4–5 MΩ in resistance were fabricated by an automated puller (Sutter Instruments) and heat-polished by an iridium-wired microforge. Voltages were applied, and current signals were acquired through a high-impedance low-noise patch clamp amplifier (EPC9, HEKA Electronics), which was controlled by an A/D, D/A-converter (Digidata 1200, Axon Instruments) driven by the pClamp v.6 software (Axon Instruments). For long-time recordings, the current signals were low-pass-filtered at 100 Hz and digitized at 1 kHz by using PowerLab/400 (AD Instruments; sampling rate: 100 Hz) and analyzed by the software Chart v.5. The mean magnitude of recorded currents was evaluated as the average over 5–10 s. To construct the current-voltage relationships, rising ramp voltages (−100 - 100 mV, 2 s in duration) were applied to cells, and corresponding currents were sampled at 3 kHz after 1-kHz low-pass filtering and analyzed by the Clampfit v.9.2 software (Axon Instruments).

Drugs were rapidly applied onto cells by using a fast solution-change device driven by electromagnetic solenoid valves. All experiments were performed at room temperature (22–26°C).

**Material.** Fura-2 AM (WAKO), nifedipine (Calbiochem), and CPA (Calbiochem) were used for Ca^{2+} imaging. Indomethacin (Biomol), dexamethasone (Calbiochem), NF-κB cell-permeable inhibitory peptide SN-50 (Calbiochem), curcumin (Biomol), NFAT activation inhibitor III (NAI-III) (Calbiochem), FK506 (Fermentek), and cyclosporin A (Calbiochem) were added into culture medium. EZ-Link Sulfo-NHS-SS-Biotin (Pierce) and avidin beads (Sigma) were used for biotinylation assay.

Human Stealth siRNAs, TRPC1-HSS110981 (AUAAUUGAAGUCGGAAGCAGCAAGU, ACUGGGCUUUCGGACUUCUAAAU), TRPC1-HSS110982 (AUAAUAAGAGAACCCCUGGAAUCC, GCCAUUCCUGGUUUCGUCUUAU), and ORAI1-HSS131371 (ACCGAGUUGAAGUCGACGUUGGC, GCAACUGUCACAAUCUC-AACUGGU) (Invitrogen) were used for silencing according to the protocol: 10.220.33.2 on July 6, 2017 http://ajpgi.physiology.org/ Downloaded from

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**Fig. 1.** TNF-α induces cyclooxygenase (COX)-2 expression to stimulate PGE2 production in CCD-18Co myofibroblasts. A: time-dependent induction of COX-2 mRNA by TNF-α (50 ng/ml) treatment. COX-1 and COX-2 mRNAs were detected by RT-PCR. *P < 0.05 vs. untreated cells; n = 4–10. B: dose-dependent prostaglandin E2 (PGE2) production by TNF-α (50 ng/ml) treatment, in the absence or presence of BAPTA-AM (10 μM) or EGTA (1 mM); n = 4–15. *P < 0.05 against TNF-α alone with time-matched multiple-comparison test. C: time-dependent production of PGE2 by TNF-α (50 ng/ml) treatment. Indomethacin (INDO) 10 μM or dexamethasone (DEX) 100 nM was added simultaneously with TNF-α; n = 4–14. All data points with INDO or DEX after 12 h incubation are statistically different (**P < 0.01) from those with TNF-α alone (●).
manufacturer’s instructions. TIE3 antibody was kindly provided by Dr. D. J. Beech at University of Leeds, Leeds, UK.

Solutions. The normal external solution for Ca\(^{2+}\) imaging and whole cell patch-clamp experiments contained (in mM): 140 NaCl, 5 KCl, 1 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, 10 glucose (pH 7.4, adjusted with Tris base). For 20 or 100 mM, Ca\(^{2+}\) solutions contained (in mM) 20 or 100 CaCl\(_2\), 120 or 0 N-methyl D-glucamine-Cl, 10 HEPES, 10 glucose (pH 7.4, adjusted with Tris base). Pipette solution for whole cell recording contained (in mM): 120 CsOH, 120 aspartate, 20 CsCl, 2 MgCl\(_2\), 1 EGTA/0.3 CaCl\(_2\), 10 HEPES, 2 ATP, 10 glucose (adjusted to pH 7.2 with Tris base).

Statistical analysis. The results are expressed as the means ± SE. The data were accumulated under each experimental condition from at least four independent experiments. For [Ca\(^{2+}\)]\(_i\) measurements, the typical time course of Ca\(^{2+}\) response represents the average from more than 30 cells from the same batch of cells. The same protocol was repeated for at least four different batches of cells for each experimental condition, and the pooled data were averaged (shown as histograms) and subjected to statistical analysis. Statistical significance was evaluated by Student’s t-test, and Dunnett’s test was employed for multiple comparisons. The value of \(P < 0.05\) was considered to be significant.

RESULTS

TNF-\(\alpha\) induces COX-2 expression and promotes PGE\(_2\) production in CCD-18Co cells. CCD-18Co human colonic cells exhibit many properties typical of gastrointestinal subepithelial myofibroblasts. CCD-18Co cells are capable of actively producing PGE\(_2\), a potent mediator of immune and inflammatory responses (11). The stimulatory effects of proinflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) on PGE\(_2\) production are well documented in various types of cells (14, 22). Therefore, in the first step of this study, we explored the effects of TNF-\(\alpha\) on COX mRNA expression and PGE\(_2\) production in CCD-18Co myofibroblasts.

As shown in Fig. 1A, at the mRNA level, the expression of COX-1 was constitutive in CCD-18Co and unresponsive to...
TNF-α (50 ng/ml) treatment (open bars in Fig. 1Ab). In contrast, that of COX-2 was progressively upregulated by TNF-α treatment (solid bars in Fig. 1Ab). Only 1 h after the addition of TNF-α in culture medium, a significant elevation in COX-2 mRNA expression was detected. In parallel with the induction of COX-2, TNF-α significantly increased the rate of PGE2 production in time- and dose-dependent manners (● in Fig. 1, B and C).

It has been reported that PGE2 production stimulated by TNF-α may be Ca2+ dependent (25, 47). We thus tested the effects of extra- and intracellular Ca2+ buffering with EGTA (1 mM) and BAPTA-AM (10 μM), respectively. Both procedures suppressed TNF-α-induced PGE2 production (Fig. 1B). In addition, consistent with previous reports, the enhancing effects of TNF-α on PGE2 production were almost completely blocked by the simultaneous addition of anti-inflammatory agents, indomethacin (INDO, 10 μM) or dexamethasone (DEX, 100 nM) (Fig. 1C) (14).

FIG. 3. Negative correlation between TRPC1 expression and TNF-α-induced PGE2 production. Aa: representative immunoblots for TRPC1 protein in CCD-18Co cells transfected with control siRNA (siCon) or TRPC1-siRNA (siC1: HSS110981 or HSS110982) for 24 h. After transfection, the cells were treated with TNF-α (50 ng/ml) for another 24 h. Ab: histograms summarizing the effects of siRNA on TNF-α-induced PGE2 production. The same protocol as in Aa was used for siRNA transfection and subsequent TNF-α treatment; n = 4–5; *P < 0.05 vs. siCon-transfected cells. B: representative immunoblots for TRPC1 protein in CCD-18Co cells transfected with empty vector or human trpc1 cDNA (a) and the summary of their effects on TNF-α-induced PGE2 production (b). *#P < 0.05 with respect to corresponding experimental conditions in empty vector-transfected cells; n = 4–5. C: representative store-operated [Ca2+]i responses (a) and their averaged magnitudes (b) in CCD-18Co cells transfected with siCon, siCon plus TNF-α (50 ng/ml) or siC1 plus TNF-α (50 ng/ml). The transfection protocol was the same as in A; n = 4; *P < 0.05.
Importantly, the \([\text{Ca}^{2+}]_i\) rise induced by store depletion was significantly suppressed by the so-called SOC inhibitors Gd\(^{3+}\) (5 \(\mu\)M) and SK&F96365 (1 \(\mu\)M), respectively (Fig. 2A, a and b). These results indicate that TNF-\(\alpha\) treatment upregulates a store-dependent \(\text{Ca}^{2+}\) influx.

It has been suggested that several members of TRPC channels contribute to SOC in other types of cells (1, 41, 46). We therefore examined the expression profile of TRPC mRNAs in CCD-18Co myofibroblasts. The RT-PCR analysis detected five TRPC mRNAs in the CCD-18Co, i.e., TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6. Of these isoforms, only the expression of TRPC1 mRNA was significantly enhanced by TNF-\(\alpha\) treatment (Fig. 2B). Consistent with this observation, both total and biotin-labeled surface levels of TRPC1 protein were significantly increased by TNF-\(\alpha\) treatment of more than 18 h (Fig. 2C). A similar time course of the enhancing effects of TNF-\(\alpha\) on store-dependent \(\text{Ca}^{2+}\) influx was observed (\(\Delta\)ratio 340/380; 0.13 \(\pm\) 0.04 at 0 h, 0.62 \(\pm\) 0.08 at 18 h, and 1.09 \(\pm\) 0.20 at 28 h; \(P < 0.05\) with Dunnett’s test). In contrast, the expression level of the ER \(\text{Ca}^{2+}\)-sensor/signal protein STIM1 (Fig. 2C) or a SOC pore-forming molecule Orai1 (Supplemental Fig. S2A) remained unchanged after TNF-\(\alpha\) treatment. These observations suggest that augmentation of store-dependent \(\text{Ca}^{2+}\) influx may be associated in some way with the increased protein expression of TRPC1, which is presumably responsible for TNF-\(\alpha\)-induced PGE\(_2\) production.

TRPC1-associated \(\text{Ca}^{2+}\) influx negatively regulates TNF-\(\alpha\)-induced PGE\(_2\) production in CCD-18Co cells. To more directly corroborate the involvement of TRPC1-associated \(\text{Ca}^{2+}\) influx in TNF-\(\alpha\)-induced PGE\(_2\) production, stealth siRNA (siC1) was employed to specifically eliminate TRPC1 protein expression in CCD-18Co cells. As shown in Fig. 3A, two distinct siC1s employed successfully decreased the expression...
level of TRPC1 protein, whereas control siRNA (siCon) exerted no inhibitory effect at the same concentration (Fig. 3Aa). With the same transfection protocol, the basal production of PGE2 was not affected by either of the two siC1s, but TNF-α-induced PGE2 production was enhanced by them more than twofold (Fig. 3Ab). Overexpression of TRPC1 in CCD-18Co cells (Fig. 3Ba) produced the opposite consequence, i.e., significant suppression of TNF-α-induced PGE2 production (Fig. 3Bb). These results strongly suggest that upregulation of TRPC1 may negatively regulate PGE2 production during TNF-α treatment.

To confirm that TRPC1 actually contributes to store-dependent Ca2+ influx in CCD-18Co, we next tested the effect of siC1. Compared with siCon, specific knockdown of TRPC1 by siC1 greatly attenuated the TNF-α-induced enhancement of store-dependent Ca2+ influx (Fig. 3C). In addition, we tested the consequence of the knockdown of Orai1 by its specific siRNA, but no significant suppression was observed for the enhancement of the Ca2+ influx by TNF-α pretreatment (Supplemental Fig. S2). These results lend a strong support to the essential contribution of TRPC1 to TNF-α-induced enhancement of Ca2+ influx in CCD-18Co cells.

Nonselective cationic conductance induced by a store-depleting agent thapsigargin in CCD-18Co cells. It has often been a matter of debate that store-dependent Ca2+ influx actually reflects the induction of Ca2+-permeable membrane conductance. We therefore attempted the direct recording of ionic currents accompanying store depletion by the patch-clamp technique. As demonstrated in Fig. 4A, a noisy long-lasting inward current (or conductance) with double voltage-dependent rectifications (Fig. 4B) was elicited in response to a store-depleting agent thapsigargin (TG; 2 μM) at a holding potential of −60 mV. When the external concentration of Ca2+ was suddenly elevated to 20 or 100 mM, only small inward conduction of Ca2+ was observed (Fig. 4A, a and b). The reversal potential of TG-induced current was shifted from −1.65 ± 2.6 (n = 8) (Fig. 4B) to −37.35 ± 0.72 mV (n = 4) by changing the ionic composition of the bath from normal to 100 mM Ca2+-containing conditions, suggesting that the channel underlying is non-

Fig. 5. Suppression of TRPC1-associated Ca2+ influx by T1E3 antibody augments TNF-α-induced COX-2 expression. A: representative [Ca2+]i responses (a) and their averaged magnitudes (b) in CCD-18Co cells pretreated with TNF-α (50 ng/ml) for 24 h; n = 4. The cells were incubated with T1 (1:200) or T1E3 (1:200) antibodies for 24 h before [Ca2+]i measurement. B: representative immunoblots of COX-2 protein (top) and its averaged expression level (bottom) in the absence and presence of T1E3 antibody or SK&F96365 (1 mM) with TNF-α (50 ng/ml) treatment; n = 4–6. C: effect of T1E3 antibody on TNF-α (50 ng/ml)-induced PGE2 production in untreated or stimulated CCD-18Co cells evaluated from 4 individual experiments. *P < 0.05.
selective cationic and would have, if any, a very low Ca\(^{2+}\) permeability (P_{Ca}/P_{Na} \approx 0.1; estimated on the basis of independence principle; see e.g., Ref. 17).

Pretreatment with 50 ng/ml of TNF-\(\alpha\) enhanced the magnitude of TG-induced current almost threefold (Fig. 4, Ac and Ca), which was specifically counteracted by the knockdown of TRPC1 expression with its specific siRNA (Fig. 4Ca) and greatly suppressed by Gd\(^{3+}\) at its concentration (5 \(\mu M\)) to block store-dependent Ca\(^{2+}\) influx (Fig. 4Cb vs. Fig. 2Ab).

The above-described nature of TG-induced inward current in CCD-18Co cells is reminiscent of that observed for heterologously expressed TRPC1 (34). However, the rapid time course of activation by TG and nonselectivity over cations with a very low Ca\(^{2+}\) permeability suggest that the current activated by TG in CCD-18Co cells may reflect a Ca\(^{2+}\)-sensitive cationic conductance rather than a Ca\(^{2+}\)-permeable one directly responsible for store-dependent Ca\(^{2+}\) influx, which may just mirror a [Ca\(^{2+}\)]\(_i\) rise caused by Ca\(^{2+}\)-releasing and store-depleting actions of TG (16). Although we could not obtain the compelling evidence for the direct contribution of TRPC1 to store-dependent Ca\(^{2+}\) influx as a SOC pore-forming subunit, the above results are still consistent with the primary role of TRPC1 in regulating store-dependent Ca\(^{2+}\) influx in CCD-18Co cells.

**Fig. 6.** Effects of NF-\(\kappa\)B and nuclear factor of activated T-cell proteins (NFAT) inhibitors on TNF-\(\alpha\)-induced COX-2/PGE\(_2\) and TRPC1-mediated signalings. A and B: Western blots of COX-2 (a) and PGE\(_2\) production (b) in untreated or TNF-\(\alpha\)-stimulated CCD-18Co cells. NF-\(\kappa\)B inhibitors (A): curcumin (curc; 20, 100 \(\mu M\)); SN-50 cell permeable inhibitor peptide (SN-50; 2 \(\mu M\)). Calcineurin/NFAT inhibitors B: NFAT activation inhibitor III (NAI-III; 1 \(\mu M\)) and Tacrolimus (FK506; 1 \(\mu M\)) were added simultaneously with TNF-\(\alpha\) (50 ng/ml) for 24 h; \(n = 3–5\). C: Western blots of TRPC1 protein (a) and the magnitudes of CPA-induced Ca\(^{2+}\) influx (b) in untreated or TNF-\(\alpha\)-stimulated CCD-18Co cells with various drugs. The protocol for drug application was the same as in A or B; \(n = 3\); *\(P < 0.05\).
Inhibition of TRPC1-associated store-dependent Ca\textsuperscript{2+} influx by T1E3-antibody also accelerates TNF-\alpha-induced COX-2 upregulation and PGE\textsubscript{2} production. The results of the siRNA experiments suggest that increased expression of TRPC1 (and enhanced SOC activity) parallels with the decreased PGE\textsubscript{2} production. However, it does not necessarily mean that TRPC1-mediated Ca\textsuperscript{2+} influx directly regulates PGE\textsubscript{2} production. We therefore examined the effects of direct blockade of the Ca\textsuperscript{2+} influx by a functional antibody T1E3, which reportedly inhibits Ca\textsuperscript{2+} influx through TRPC1 channel from the cell exterior (42).

As shown in Fig. 5A, 24-h pretreatment with T1E3 antibody (diluted 1:200) significantly reduced store-dependent Ca\textsuperscript{2+} influx by \textasciitilde40%. Strikingly, this antibody enhanced the expression of COX-2 and production of PGE\textsubscript{2} induced by TNF-\alpha treatment (Fig. 5, B and C). In contrast, another antibody (T1) raised against the 15 amino acids in the second intracellular loop of TRPC1 protein ('QLYDKGYSKEQKDC') was totally ineffective when applied externally and could not prevent the TNF-\alpha-induced enhancement of store-operated Ca\textsuperscript{2+} influx, COX-2 expression, or PGE\textsubscript{2} production (Fig. 5, A–C). Similar to T1E3 antibody, a nonspecific SOC inhibitor SK&F96365 (1 \mu M) produced enhancing effects on TNF-\alpha-induced COX-2 expression (Fig. 5B). These results strongly suggest that, during TNF-\alpha stimulation, TRPC1-associated Ca\textsuperscript{2+} influx exerts a negative feedback on COX-2 induction, thereby limiting the extent of PGE\textsubscript{2} production.

Involvement of NF-\kappaB- and calcineurin-NFAT-signaling in TNF-\alpha-induced COX-2-dependent PGE\textsubscript{2} production. Elevated [Ca\textsuperscript{2+}]\textsubscript{i} activates downstream transcriptional activities such as NFAT and NF-\kappaB. In colon carcinoma cell as well as other types of cells, calcineurin-NFAT- and NF-\kappaB-mediated signalings have been implicated in the transcriptional control of COX-2 and TRPC1 (5, 7, 13). We therefore explored the possible link between TNF-\alpha-enhanced Ca\textsuperscript{2+} influx and COX-2-dependent PGE\textsubscript{2} production in CCD-18Co cells, in terms of these two transcription factors by applying their inhibitors simultaneously with TNF-\alpha.

![Figure 7](http://example.com/fig7.png)

**Figure 7.** TRPC1 suppression alters subcellular localization of I\kappaB\textsuperscript{\alpha} and NF-\kappaB (subunit p65). A: representative immunoblots for I\kappaB\textsuperscript{\alpha}, NF-\kappaB (subunit p65), and NFAT3 proteins in the cytoplasmic and nuclear fractions of untreated or TNF-\alpha-stimulated CCD-18Co cells. To confirm the equal loading and specificity of samples, blots were re-probed for \beta-actin or lamin B. NAI-III (1 \mu M), T1 antibody (1:2000), T1E3 antibody (1:200), or EGTA (1 mM) were applied simultaneously with TNF-\alpha (50 ng/ml) for 24 h. Control siRNA (siCon) or TRPC1-siRNA (siC1: HSS110982) was transfected for 24 h before TNF-\alpha (50 ng/ml) treatment. B: altered localization of NF-\kappaB/p65 and NFAT3. Histograms show the relative level of NF-\kappaB/p65 and NFAT3 protein to a nuclear marker lamin; n = 4–6; *P < 0.05.
As summarized in Fig. 6, inhibitors for NF-κB (curcumin, SN-50) significantly suppressed TNF-α-induced COX-2 expression and PGE2 production (Fig. 6A). In contrast, the inhibition of NFAT activity by NAI-III peptide potentiated it (Fig. 6B), as observed for the effects of TRPC1 inhibition by its siRNA or antibody. A similar extent of potentiation was also observed with a calcineurin inhibitor FK506 (Fig. 6B).

We also tested whether the inhibition of these transcription factors affect the enhancing effects of TNF-α on TRPC1 protein expression and Ca2+-influx in CCD-18Co cells. Whereas NF-κB inhibitors (curcumin and SN-50) strongly counteracted the enhancement of TRPC1 expression and Ca2+-influx by TNF-α, the inhibitors of calcineurin-NFAT signaling (FK506 or NAI-III) were almost without effect (Fig. 6C).

These results together suggest that, in CCD-18Co cells, activation of NF-κB signaling is essential for TNF-α-induced TRPC1 upregulation, COX-2 expression, and PGE2 production. In contrast, activation of NFAT is rather modulatory, acting as a negative feedback regulator to limit the COX-2 upregulation and PGE2 production induced by TNF-α at the downstream of TRPC1/SOC signaling.

Inhibition of TRPC1-mediated Ca2+-influx accelerates NF-κB signaling. We finally examined whether there is a cross talk between TRPC1-associated Ca2+-influx/NFAT and TNF-α/NFAT signalings. For this purpose, we tested the effects of T1E3 antibody and NFAT inhibitor (NAI-III) on IκBα degradation and subsequent NF-κB/p65 nuclear translocation by immunoblot analysis.

Twenty-four-hour pretreatment of CCD-18Co cells with TNF-α caused the degradation of IκBα and paralleled decrease and increase in the cytoplasmic and nuclear levels of NF-κB/p65 protein, respectively (Fig. 7, Aa and Ba). The same procedure also facilitated the nuclear translocation of NFAT3, the major isoform assigned to TNF-α-induced COX-2 expression in other cell types (28, 43) (Fig. 7, Ab and Bb). When T1E3 antibody was cotreated with TNF-α for 24 h, the extent of TNF-α-induced degradation of IκBα was as well as that of the nuclear translocation of NF-κB/p65 protein was further enhanced (Fig. 7, Aa and Ba). Essentially the same results were obtained with siRNA knockdown of TRPC1 (Fig. 7, Ab and Bb), which conversely attenuated the TNF-α-induced nuclear translocation of NFAT3 (Fig. 7, Ab and Bb). In contrast, the inhibition of NFAT signaling by NAI-III exerted only marginal effects on TNF-α-induced IκB degradation or NF-κB translocation (Fig. 7, Aa and Ba), despite its clear enhancing actions on COX-2 expression and PGE2 production (Fig. 6). In aggregate, these results can most straightforwardly suggest that activation of TRPC1-associated Ca2+-influx may counteract the IκB degradation and NF-κB/p65 nuclear translocation induced by TNF-α via a pathway independent of NFAT signaling.

Finally, somewhat unexpectedly from the above results, rigorous deprivation of extracellular Ca2+ (by adding 1 mM EGTA to Ca2+-free external solution) rather resulted in significant inhibition of NF-κB/p65 translocation to the nucleus under TNF-α stimulation (Fig. 7, Aa and Ba). These results raise the idea that, in addition to the degradation of NF-κB/IκB complex, the inductive effects of TNF-α on COX-2/PGE2 production (Fig. 1) may require additional Ca2+-influx through an unknown pathway(s) distinct from the TRPC1-associated one.

DISCUSSION

The principal finding of this study is that PGE2 production in CCD-18Co cells, which is stimulated by the major inflammatory cytokine TNF-α, is negatively regulated by TRPC1-associated Ca2+-influx. This is supported by the specific knockdown or overexpression of TRPC1 protein or direct blockade of TRPC1-associated Ca2+-influx by T1E3 antibody (Figs. 3 and 5). Part of the TRPC1-mediated negative regulation seems to occur through activation of NFAT signaling, as siRNA knockdown of TRPC1 inhibited the nuclear translocation of NFAT3 (Fig. 7Bb) and calcineurin/NFAT inhibitors significantly potentiated the induction of COX-2 and subsequent PGE2 production during TNF-α treatment (Fig. 6). However, more direct inhibition of NF-κB signaling through TRPC1-associated Ca2+-influx may also be involved therein, as evidenced by NFAT-independent facilitating effects of T1E3 antibody and siRNA knockdown of TRPC1 on IκBα degradation and NF-κB/p65 nuclear translocation (Fig. 7). In addition, other lines of evidence indicate that, in CCD-18Co cells, TNF-α can induce the expression of COX-2 and TRPC1 proteins via a NF-κB-dependent signaling pathway (Figs. 1, 2, and 6), and the former appears to require Ca2+ from a source distinct from TRPC1-associated Ca2+-entry pathway (Figs. 1B and 7Bc). These observations collectively suggest that Ca2+-mediated signalings play multiple roles in regulating COX-2/PGE2 production in CCD-18Co cells, and that enhanced Ca2+-influx attributable to increased TRPC1 expression may act as an effective negative feedback mechanism that limits TNF-α-induced PGE2 production both directly and indirectly through NF-κB signaling (for summary, see Fig. 8).

Consultation with previous studies reveals that both cox-2 and trpc1 genes are subject to transcriptional control of NF-κB (23, 29). In fact, there are several consensus motifs present for NF-κB binding in the 5′-regulatory regions of these genes.
Ca^2+ receptor agonist involved, which may activate differential simplistically be ascribed to differences in the cell type and in these genes (Supplemental Fig. S3 suggests the existence of consensus motifs for NFAT binding (PP2A) activity to prevent the nuclear translocation of NF-κB dimeric proteins. Thus the role of the Ca^2+ was significantly inhibited by vigorous Ca^2+ influx that stimulates the phosphatase 2A (PP2A) activity to prevent the nuclear translocation of NF-κB signaling via inhibition of NF-κB activation. This is associated with enhanced store-operated Ca^2+ influx that stimulates the phosphatase 2A (PP2A) activity to prevent the nuclear translocation of NF-κB dimeric proteins. Thus the role of the Ca^2+ influx is stabiliza- tion of the PP2A complex, which in turn leads to dephosphorylation of IκB proteins and inhibition of NF-κB signaling (20). Because PP2A is ubiquitously expressed and intimately involved in intestinal mucosal homeostasis (20, 21), this enzyme may also participate in TRPC1/SOC-mediated inhibition of NF-κB signaling in CCD-18Co cells (Fig. 8).

The observed Ca^2+ dependence of TNF-α-induced PGE2 production (Fig. 1) favors the involvement of Ca^2+-dependent process(es) therein. Because the NFAT-mediated pathway is inhibitory to COX-2 expression/PGE2 production, it is reasonable to assume that some steps in activating NF-κB signaling may be Ca^2+ dependent and facilitatory. In agreement with this possibility, the rate-limiting step of NF-κB activation, i.e., degradation of IκB and subsequent nuclear translocation of NF-κB p65 protein, was significantly inhibited by vigorous Ca^2+ chelation with EGTA (Fig. 7, Ac and Be). It has been reported that activation of Ca^2+-dependent transcription pathways depend differently on the magnitude and pattern of [Ca^2+]i elevation. For example, in lymphocytes, NFAT is activated in response to a small but sustained [Ca^2+]i elevation or [Ca^2+]i oscillations at high frequency, whereas a large transient rise in [Ca^2+]i is sufficient for the activation of NF-κB. These differences may be accounted for by fast switching of NFAT activation/inactivation cycle by the calcineurin/kinase system vs. rather slow reversal of NF-κB activation attributable to proteolytic regulation of IκB proteins by the ubiquitin/proteasome-mediated system (18, 39).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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