We investigated the role of tumor necrosis factor-α (TNF-α) in activating the store-operated Ca\(^{2+}\) channels in endothelial cells via the expression of transient receptor potential channel (TRPC) isoforms. We observed that TNF-α exposure of human umbilical vein endothelial cells resulted in TRPC1 mRNA and protein expression, whereas it had no effect on TRPC3, TRPC4, or TRPC5 expression. The TRPC1 expression was associated with increased Ca\(^{2+}\) influx after intracellular Ca\(^{2+}\) store depletion with either thrombin or thapsigargin. We cloned the 5′-regulatory region of the human TRPC1 (hTRPC1) gene which contained a TATA box and CCAAT sequence close to the transcription initiation site. We also identified four nuclear factor-κB (NF-κB)-binding sites in the 5′-regulatory region. To address the contribution of NF-κB in the mechanism of TRPC1 expression, we determined the effects of TNF-α on expression of the reporter luciferase after transfection of hTRPC1 promoter-luciferase (hTRPC1-Pro-Luc) construct in the human dermal microvascular endothelial cell line. Reporter activity increased 4-fold at 4 h after TNF-α challenge. TNF-α-induced increase in reporter activity was markedly reduced by co-expression of either kinase-defective IKKβ kinase mutant or non-phosphorylatable IκB mutant. Treatment with NEMO-binding domain peptide, which prevents NF-κB activation by selectively inhibiting IKK interaction with IKK complex, also blocked the TNF-α-induced TRPC1 expression. Thus, TNF-α induces TRPC1 expression through an NF-κB-dependent pathway in endothelial cells, which can trigger augmented Ca\(^{2+}\) entry following Ca\(^{2+}\) store depletion. The augmented Ca\(^{2+}\) entry secondary to TRPC1 expression may be an important mechanism of endothelial injury induced by TNF-α.

An increase in [Ca\(^{2+}\)]\(i\), in endothelial cells can induce endothelial barrier dysfunction (1–6). Mediators such as thrombin, histamine, and oxidants mediate increased endothelial permeability by activating Ca\(^{2+}\)-sensitive signaling pathways (1–9). The increase in [Ca\(^{2+}\)]\(i\) occurs in two distinct phases: transient rise due to intracellular Ca\(^{2+}\) store depletion and second sustained phase due to Ca\(^{2+}\) entry into the cell from extracellular medium (9–11). We showed recently that Ca\(^{2+}\) entry through plasma membrane cation channels activated by Ca\(^{2+}\) store depletion is a critical determinant of increased endothelial permeability (3). Thrombin elicits an increase in [Ca\(^{2+}\)]\(i\), by activating sequential events involving the generation of inositol 1,4,5-trisphosphate, inositol 1,4,5-trisphosphate-induced ER\(^{1}\) store Ca\(^{2+}\) release, and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry in endothelial cells (3, 12).

Ca\(^{2+}\) influx due to endoplasmic stored Ca\(^{2+}\) depletion (i.e. capacitative Ca\(^{2+}\) entry) is mediated by store-operated cation channels (SOCs) (10). SOCs can be activated by inositol 1,4,5-trisphosphate-induced store depletion and inhibition of Ca\(^{2+}\)-ATPase with agents such as thapsigargin (Tg), cyclopiazonic acid, or dibenzoylmoquinone (10, 13, 14). Recent studies (3, 10, 14–16) have shown that the mammalian homologues of the transient receptor potential (TRP) gene family of channels function as SOC. TRP genes encode a superfamily of proteins with six transmembrane helices that are divided into three subfamilies: TRPC, TRPV, and TRPM (17). Members of the TRPC subfamily contain 700–1000 amino acids, and the seven subfamilies: TRPC, TRPV, and TRPM (17). Members of the TRPC subfamily contain 700–1000 amino acids, and the seven subfamilies: TRPC, TRPV, and TRPM (17). The TRPC1–7 are expressed in mammalian cells. Mammalian TRPCs are grouped into four subfamilies as follows. (i) The first consists of TRPC4 and TRPC5. Their activation is dependent on Ca\(^{2+}\) store depletion, and they have high Ca\(^{2+}\) selectivity as assessed by sensitivity to La\(^{3+}\) (10, 14). TRPC4 and TRPC5 are activated by G protein-coupled receptors and receptor tyrosine kinases coupled to phospholipase C. (ii) TRPC1 is closely related to TRPC4 and TRPC5; it forms SOCs but is a less Ca\(^{2+}\)-selective channel. (iii) TRPC3, TRPC6, and TRPC7 form store-independent non-selective cation channels that may be activated by diacylglycerol (10, 17); this type of store-dependent activation mechanism has been described for human TRPC3 (18). (iv) TRPC2 function is unclear, but it may be a pseudogene in humans (17).

Primary endothelial cells in culture express TRPC1–6 (3, 13, 14). Mouse aortic and lung endothelial cells express TRPC1, TRPC3, TRPC4, and TRPC6 (3, 14), but TRPC4 expression is a predominant isoform in mouse endothelial cells (3, 14). Deletion of TRPC4 in mice caused impairment in store-operated

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Ca2+ current and Ca2+ store release-activated Ca2+ influx in aortic and lung endothelial cells (3, 14). In TRPC4 knockout mice, the acetylcholine-induced endothelium-dependent smooth muscle relaxation was reduced (14). Increased microvascular permeability caused by thrombin receptor activation was impaired in TRPC4 knockout mice (3). A number of studies showed that TRPC expression patterns in human vascular endothelial cells differ from the mouse (13, 19–21). Groeschner et al. (19) showed by RT-PCR the expression of TRPC1, TRPC3, and TRPC4 transcripts in human umbilical vein endothelial cells (HUVEC). Moore et al. (20) showed the expression of TRPC1 in human pulmonary artery endothelial cells (HPAEC), and TRPC1-mediated Ca2+ influx caused endothelial cell shape change. Inhibition of TRPC1 expression by transfecting TRPC1-specific antisense oligonucleotide reduced the thapsigargin-induced increase in [Ca2+]o, as well as store-operated Ca2+ entry current in HPAEC (20). We observed that human vascular endothelial cells (HUVEC, HPAEC, and human dermal microvessel endothelial cell line (HMEC)) express TRPC1 and, to a lesser extent, TRPC3 and TRPC4 (21). We also showed that increased expression of TRPC1 in HMEC resulted in increased thrombin-induced Ca2+ influx as well as store-operated cationic current compared with controls (21).

Although previous studies have established that TRPC1 is a critical component of SOC in human vascular endothelial cells, the regulation of TRPC1 expression is not known. We showed by the genomic data base analysis of the 5′-regulatory region of human TRPC1 (hTRPC1) the presence of multiple binding sites for transcription factor NF-κB. Therefore, we addressed the role of TNF-α, the pro-inflammatory cytokine known to activate NF-κB (22), in inducing the expression of TRPC1 in human vascular endothelial cells. We showed that TNF-α increased the NF-κB-dependent expression of TRPC1 and that this resulted in an augmented store-operated Ca2+ influx. Thus, TRPC1-mediated Ca2+ influx in endothelial cells may play an important role in signaling endothelial cell activation and injury subsequent to sepsis-induced vascular inflammation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Endothelial growth medium (EGM-2) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Cell-permeable NEMO-binding domain (NBD) synthetic peptides (wild type, drqikiw- and reverse, 5′-GAGAGCTGACAGTCCAAGC-3′) (23). c-Myc monodomain antibody (anti-TRPC1 Ab) was raised in rabbits using hTRPC1 COOH-terminal synthetic peptide (QLYDKGYTSKEQKDC) (23). c-Myc monoclonal antibody was obtained from Clontech (Palo Alto, CA). TRIZol reagent, Taq DNA polymerase, LipofectAMINE, LipofectAMINE Plus reagent, MCDB 131, Opti-MEM I, TOPO cloning kit, and restriction enzymes were from Invitrogen. BAC Clone RPC1-11C was obtained from GenBank accession number U31110. The hTRPC1 cDNA was obtained by RT reaction performed using 1 μg of DNAase-treated total RNA isolated from HUVEC. The middle portion of an 840-bp hTRPC1 was amplified using the following primers: forward, 5′-ATATATCGATTTGGGCC-3′, and reverse, 5′-TGTAGGATGGAATGCATCCCA-3′. The 5′-end of hTRPC1 was amplified using the following forward primer, 5′-ATATATATGATCC-3′, and reverse, 5′-AGAATCTCAATGAGCGAGG-3′. The 3′-end of hTRPC1 was obtained by PCR using the following primers: forward, 5′-AGAATCTGGTATAACTGAG-3′, and reverse, 5′-GACTAATTTCTGATTA-3′. The PCR-amplified fragments for the 5′- and 3′-ends were 865 and 1027 bp, respectively.

**Cloning of myc-TRPC1**—RT-PCR method was used to clone full-length coding region of hTRPC1. The primers for PCR amplification was designed from the hTRPC1 sequence (GenBank accession number U31110). The hTRPC1 cDNA was obtained by RT reaction performed using 1 μg of DNAase-treated total RNA isolated from HUVEC. The middle portion of an 840-bp hTRPC1 was amplified using the following primers: forward, 5′-ATATATCGATTTGGGCC-3′, and reverse, 5′-TGTAGGATGGAATGCATCCCA-3′. The 5′-end of hTRPC1 was amplified using the following forward primer, 5′-ATATATATGATCC-3′, and reverse, 5′-AGAATCTCAATGAGCGAGG-3′. The 3′-end of hTRPC1 was obtained by PCR using the following primers: forward, 5′-AGAATCTGGTATAACTGAG-3′, and reverse, 5′-GACTAATTTCTGATTA-3′. The PCR-amplified fragments for the 5′- and 3′-ends were 865 and 1027 bp, respectively. The overlapping region was 212 bp between 5′-end and middle portion and 130 bp between 3′-end and middle portion. The 5′- and middle portion were first ligated by PCR using the high fidelity Pfu DNA polymerase (Roche Applied Science), and the DNA fragment obtained was PCR-ligated again with the 3′-end to obtain the full-length coding sequence for hTRPC1. The DNA obtained was cloned into pCR3.1-TOPO cloning vector (Invitrogen), and the orientation was checked by restriction analysis. The PCR-amplified DNA was sequenced by the New England BioLabs (Woburn, MA). Furthermore, to obtain NH2-terminal Myc-tagged TRPC1 cDNA, the full-length hTRPC1 was PCR-amplified and subcloned into pCMV-Myc mammalian expression vector (Clontech). In this PCR, the following primers were used: forward, 5′-ATAATACTGAGCAGGCGCCTG-3′, and reverse 5′-ATAATACTGAGCAGGCGCCTG-3′. The PCR-amplified fragment was double-digested with SaI and NotI and then the digested products were ligated to obtain Myc-TRPC1 construct.

**Cloning of human TRPC1 Promoter**—About a 3.2-kb size hTRPC1 5′-regulatory sequence region was obtained from human genomic data base (GenBank accession number U31110). The promoter containing the 5′-end was ligated into pGL2 vector using the primer set: forward, 5′-CTGACATTTGGCTATAATGGGCAAGC-3′, and reverse, 5′-AATGGAGACGCAGACGGTGCGGTGGG-3′. The 2.1-kb hTRPC1 5′-regulatory region (starting from −1685 to +440) was amplified using Pfu DNA polymerase (Roche Applied Science). PCR-am-
plified hTRPC1 5′-regulatory region was sequenced to determine correct nucleotide sequence. We also PCR-amplified the hTRPC1 5′-regulatory region using DNA isolated from HUVEC using DNAseI reagent. Before PCR amplification, DNA isolated from HUVEC, digested with NotI to generate the 12-kb DNA fragment which contained the hTRPC1 5′-regulatory region, was used as a template for PCR amplification. We obtained an ~2-kb size fragment from this PCR. This fragment was sequenced and aligned with human genomic sequence (see Fig. 4A) and also compared with PCR product obtained using BAC clone as template. The PCR products were cloned into TOPO cloning vector (pCR2.1, Invitrogen) and used for nucleotide sequencing. Primer walking method was used to sequence the cloned DNA fragment. The sequencing was done by the commercial facility (ACGT, Northbrook, IL). We observed that 106 bp (from −991 to −885) were absent in HUVEC-DNA PCR fragment. Moreover, the sequence (−991 to −885) did not reveal the presence of any putative transcription factor binding sites. Therefore, we cloned the PCR fragment, ~2 kb, obtained using HUVEC-DNA as template into pGL2 basic vector and used for transfection studies (see Fig. 5). This cloned DNA fragment contains the sequence −1685 to +440 but lacks the sequence between −991 and −885. We also prepared hTRPC1-Pro-Luc expression constructs using PCR method. The DNA fragment that contains the sequence (−1685 to +440) in pCR2.1 vector was PCR-amplified for preparing different hTRPC1-Pro-Luc constructs. The following primers were used for preparing constructs: hTRPC1-Pro-Luc constructs. The following primers were used for preparing constructs: hTRPC1-Pro-Luc (−1685 to +440), forward, 5′-ATAAACTGGACTGAGATACACAC′; hTRPC1-Pro-Luc (−1220 to +440), forward 5′-ATAAATACGCTAGCTCCTCTC′-3′, and hTRPC1-Pro-Luc (−1685 to +440), forward 5′-ATAAATACGCTAGCTCCTCTC′-3′. All three PCR products were double-digested with MluI and XhoI and inserted into pGL2-basic vector. These constructs sequences were verified by sequencing.

**TRPC1 cDNA Transfection—**Myc-TRPC1 expression construct was transfected into the HUVEC using LipofectAMINE Plus reagent in serum-free Dulbecco’s modified Eagle’s medium according to the manufacturer’s instructions (Invitrogen). HUVEC grown on 25-mm glass coverslips or 35-mm culture dishes were transfected with either 1 μg/ml Myc-TRPC1 cDNA or vector alone. DNA/LipofectAMINE plus mixture was incubated with cells for 4 h in serum-free Dulbecco’s modified Eagle’s medium, and complete growth medium was added and then incubated in a culture incubator. At 72 h after transfection, cells were used for experiments. Immunoblotting with anti-Myc antibody was performed to assess the Myc-TRPC1 expression in HUVEC (Fig. 2E).

**Expression of Reporter Construct—**HMEC grown to 50% confluency in 6-well culture plates were used for reporter constructs transfection (25). Plasmid DNA mixtures containing 1 μg of hTRPC1 promoter-luciferase (hTRPC1-Pro-Luc) in pGL2 vector and 0.035 μg of pRL/TK (Promega) were transfected using LipofectAMINE as described (25). LipofectAMINE-DNA complexes were diluted with 0.8 ml of Opti-MEM I before being added to HMEC and prewashed two times with Opti-MEM I for 2–4 h. To end transfection, 2 ml of MCDB131 medium supplemented with 10% FBS was added to each well.

**Dual Luciferase Reporter Assay—**At 48 h after transfection, the cells were incubated in growth medium for 2 h and then stimulated with TNF-α. Following stimulation, cells were lysed, and 20 μl of lysate (500 μl total) was assayed for gene expression (25). Firefly (P. pyralis) and sea pansy (R. reniformis) luciferase activity were assayed by the Dual Luciferase Reagent Assay System (Promega). Protein concentrations were determined using Bio-Rad reagents.

**Cytoplasmic Ca2+ Measurement—**The cytoplasmic Ca2+ concentration ([Ca2+]i) in single endothelial cells was measured by Fura-2 fluorescence imaging (6). Cells grown on 25-mm diameter glass coverslips were washed twice in Hank’s balanced salt solution (HBSS) and loaded with 3 μM fura-2/AM for 20 min at 37 °C. Cells were then washed twice in HBSS and imaged by using an Attofluor RatioVision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) equipped with a Zeiss Axiovert S100 inverted microscope and F-Fluor ×40, 1.3 NA oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emission at 520 nm ± 5-nm intervals. The 334/380 nm fluorescence ratio has been used to represent changes in [Ca2+]i.

**Statistical Analysis—**Statistical comparisons were made using two-tailed Student’s t test. Experimental values were reported as means ± S.E. Differences in mean values were considered significant at p < 0.05.

**RESULTS**

**TNF-α Induces TRPC1 Expression in Endothelial Cells—**We determined the effects of TNF-α on the expression of TRPC isoforms in HUVEC. We exposed HUVEC monolayers to TNF-α and determined TRPC expressions by RT-PCR (see details under “Experimental Procedures”). TNF-α increased TRPC1 expression in HUVEC which was dependent on the duration of
TNF-α exposure (Fig. 1A). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression did not change after TNF-α treatment (Fig. 1, A and B). TRPC1 expression levels were compared with GAPDH. At 2 h after TNF-α, the transcript level increased 2-fold, and at 4 h the expression level reached a maximum of 4-6-fold increases (Fig. 1B, bottom panel). TRPC1 transcript expression started to decrease at 6 h after TNF-α exposure (Fig. 1A). We also determined the expression of other TRPCs (TRPC3, TRPC4, and TRPC5). Expression of TRPC3, TRPC4, and TRPC5 was not altered by TNF-α exposure (Fig. 1A). We tested the PCR conditions to assess the expression of TRPC3, TRPC4, and TRPC5 using HEK295 cells; in this cell type we observed the expression of TRPC3, TRPC4, and TRPC5 (data not shown).

To address whether the increase in TRPC1 mRNA was due to an increase in the transcription, we incubated HUVEC with actinomycin D and exposed the cells to TNF-α. Total RNA was isolated to determine the TRPC1 mRNA expression by RT-PCR. We observed that actinomycin D blocked the expression of TRPC1 mRNA expression (Fig. 1B).

TNF-α Increases TRPC1 Protein Expression and Augments the Store-operated Ca2+ Influx—We studied the TRPC1 protein expression response to TNF-α stimulation. HUVECs were treated with TNF-α for different times, and TRPC1 protein expression was measured by immunoblotting with anti-TRPC1 antibody (Ab). TRPC1 protein expression was increased ~3-
NF-κB Activation-dependent TRPC1 Expression

To characterize the basis of Ca\(^{2+}\) influx in TNF-α-treated cells, we depleted ER-stored Ca\(^{2+}\) with Tg and measured the Ca\(^{2+}\) influx. Addition of Tg produced a peak increase in [Ca\(^{2+}\)]\(_i\), followed by a slow decline to the plateau level in the control cells. The rise in Ca\(^{2+}\) level persisted more than 30 min in TNF-α-treated cells (Fig. 3A; although the data in the figure show only up to 16 min). We also measured the Tg response in the absence of extracellular Ca\(^{2+}\). In control cells, Tg produced a peak increase in [Ca\(^{2+}\)]\(_i\), that rapidly returned to basal levels, and there was additional peak increase in [Ca\(^{2+}\)]\(_i\), after addition of extracellular Ca\(^{2+}\), which persisted for ~30 min (Fig. 3B; tracing shows only up to 10 min). In TNF-α-stimulated cells, the initial peak increase was similar to controls, but the Ca\(^{2+}\) influx was 2-fold greater than control (Fig. 3B).

**Identification of cis-Acting Elements in hTRPC1 Promoter**—We analyzed the hTRPC1 5′-regulatory region DNA sequence using TESS (www.cbil.upenn.edu/cgi-bin/tess/tess3?bin/tess33?_if = 1&RF = WELCOME) and TFsearch programs (www.cbrc.jp/research/db/TFSEARCH.html). The hTRPC1 promoter contains conventional TATA and CAAT box upstream of the transcription initiation sites (Fig. 4). The promoter/enhancer region contains AP1 and AP2 transcription factors in several locations upstream of transcription initiation sites. The proximal promoter region also contains three (TA)\(_n\) repeats. We also observed the presence of numerous potential cis-acting elements including NF-κB, AP1, AP2, NFAT, PU.1/Spi-B, STAT-1, C/EBP, and OCT-1 (Fig. 4, A and B) in this promoter. Interestingly, the hTRPC1 promoter contained four NF-κB consensus sites (Fig. 4, A and B).

As TNF-α induces expression of genes by activating NF-κB signals in endothelial cells (22, 24, 26, 27), we addressed the possibility that NF-κB is involved in the mechanism of TRPC1 expression in endothelial cells. We cloned the proximal hTRPC1 5′-regulatory region, which contains three NF-κB consensus binding sequences (−1685 to +440) (Figs. 4 and 5) (see details under “Experimental Procedures” and Fig. 5). We also made hTRPC1-Pro-Luc expression constructs lacking the 5′-UTR sequence (−1 to +440) and the sequences −1685 to −1221 (which contains an NF-κB site) (Fig. 5).

**TNF-α Induces Expression of hTRPC1 Promoter**—We transfected hTRPC1-Pro-Luc constructs into HMEC and studied the effects of TNF-α on the reporter activity (see details under “Experimental Procedures”). At 48 h after transfection, cells were challenged with TNF-α for 4 h, and the reporter activity was measured to assess TRPC1 expression. The schematics of the expression constructs are shown in Fig. 5. Transfection of the construct-1 (−1685 to +440) in HMEC showed ~2-fold induction of Luc activity over control response to TNF-α. Deletion of a major portion of the 5′-UTR (−1 to +440) (construct-2) showed a 4–5-fold increase in Luc activity (Fig. 5), suggesting that 5′-UTR may act as a repressor. Expression of the construct-3 (deletion of −1685 to −1221 and +1 to +440) showed only basal Luc activity, indicating the −1685 to −1221

![Figure 3](image_url)

**Figure 3.** TNF-α exposure increases Tg-stimulated Ca\(^{2+}\) influx in endothelial cells. HUVEC grown on glass coverslips were incubated with or without TNF-α for 18 h in medium containing 1% FBS and then loaded with fura 2-AM for 30 min at 37°C. A, fura 2-AM-loaded cells were incubated with nominal Ca\(^{2+}\) (1.26 mM) (data not shown). To determine whether ectopic TRPC1 expression in HUVEC can mimic TNF-α-induced TRPC1 expression, we transfected Myc-TRPC1 cDNA in HUVEC and studied thrombin response (see details under “Experimental Procedures”). We observed TRPC1 expression in the Myc-TRPC1 cDNA transfected cells (Fig. 2E). In these cells, the thrombin-induced increase in [Ca\(^{2+}\)]\(_i\) was significantly greater compared with vector alone transfected HUVEC (Fig. 2E). We did not observe any difference between the control and vector alone transfected cells (data not shown).

![Diagram](image_url)
NF-κB Activation-dependent TRPC1 Expression

**Fig. 4. Analysis of hTRPC1 5' -regulatory sequence.** A, the putative nucleotide sequence identified in the regulatory region of the hTRPC1 gene is shown. DNA sequence was obtained from human genomic data base (see details under “Experimental Procedures”). Nucleotides are numbered relative to the transcription start site as +1. Potential consensus sequences for transcription factor binding sites are in boldface and underlined. 106-bp sequences (−991 to −885) were absent in the hTRPC1 promoter cloned using HUVEC-DNA as template for PCR amplification (underlined with thin line). The hTRPC1 gene sequence reveals the presence of a large 5'-untranslated sequence (+1 to +794). B, this figure summarizes the different transcription factor binding sites in the hTRPC1 5'-regulatory sequence. The bent arrow indicates the transcription initiation site.

**TNF-α Activation of NF-κB Induces TRPC1 Expression—**To address the role of NF-κB activation in the mechanism of TRPC1 expression, we first transfected dominant negative IKKβ mutant with TRPC1-Pro-Luc in HMEC, and we studied TNF-α-induced TRPC1 expression. In controls (i.e. cells transfected with only TRPC1-Pro-Luc), the TNF-α-induced reporter expression was time-dependent. The reporter activity was maximal (−5-fold over basal) at 4 h after TNF-α stimulation (Fig.
6A). In dominant negative IKKβ-transfected cells, TNF-α-induced reporter expression was inhibited by ~60% (Fig. 6A), indicating that NF-κB activation is important in the mechanism of TRPC1 expression.

IkB phosphorylation and subsequent degradation promotes NF-κB translocation to the nucleus to initiate gene transcription (22). To study whether the IkB phosphorylation step is critical for the activation of NF-κB, we transfected the TRPC1-Pro-Luc construct with the non-phosphorylatable IkBo dominant negative mutant (IkBo-DN) in HMEC and measured TNF-α-induced TRPC1 expression. IkBo-DN expression with TRPC1-Pro-Luc reduced by ~80% the TNF-α-induced TRPC1 expression (Fig. 6B).

Previous studies have shown that interaction of IKKγ/ NEMO with the IKK complex is critical for the activation of the IKK complex and the subsequent activation of NF-κB (24, 28). A cell-permeable synthetic peptide (NEMO-binding domain peptide (NBD peptide)) corresponding to the NEMO amino-terminal α-helical region was shown to block TNF-α-induced NF-κB activation (24). To address further whether NF-κB signaling is necessary for TRPC1 expression, we pretreated HUVEC with Wt-NBD peptide or Mt-NBD peptide, and we measured the TNF-α-activated response. In Mt-NBD peptide-treated cells, TNF-α-induced TRPC1 mRNA expression was not significantly altered compared with controls (i.e. in the absence of peptide) (Fig. 6C, top); however, in Wt-NBD peptide- incubated cells, TNF-α-induced TRPC1 mRNA expression was markedly reduced (Fig. 6C). In Wt-NBD peptide-treated cells, TRPC1 expression was reduced >70% at 4 h after TNF-α stimulation, whereas the Mt-NBD peptide had no effect on TRPC1 expression (Fig. 6C, middle). As a positive control, we also measured TNF-α-induced ICAM-1 expression in the presence of NBD peptides. In Wt-NBD peptide-treated cells, ICAM-1 expression was blocked compared with mutant peptide-exposed cells (Fig. 6C, bottom). GAPDH expression was not altered by the TNF-α exposure.

**DISCUSSION**

In the present study, we demonstrate for the first time that TNF-α, the pro-inflammatory cytokine, increases the expression of store-operated Ca²⁺ channel in human vascular endothelial cells. TNF-α treatment induced the expression of TRPC1 without altering the expression of other TRPC isoforms (TRPC3–5) in HUVEC. The TRPC1 expression was associated with increased Ca²⁺ entry in response to either thrombin or Tg. We also showed by cloning the hTRPC1 promoter and its expression that the TNF-α-induced TRPC1 expression was dependent on NF-κB activation.

We have demonstrated previously that the increase in intracellular Ca²⁺ in endothelial cells regulates vascular permeability in both in vitro and in vivo models (1–3, 29). A growing body of evidence shows that the TRPC gene family of channels functions as SOC in mammalian cells (10, 17). We showed recently that TRPC1 is the predominant isoform expressed in human vascular endothelial cells (21). Expression of TRPC1 in endothelial cells increased the Ca²⁺ influx upon ER Ca²⁺ store depletion (21). We have also shown that 2 h of TNF-α exposure of endothelial cells increased the thrombin-induced Ca²⁺ influx (30). Thus, in the present study, we addressed the possibility that TNF-α increases the expression of TRPC1 in human vascular endothelial cells and can thereby regulate Ca²⁺ influx.

We showed that TNF-α exposure caused an increase in TRPC1 mRNA and protein levels in HUVEC. TNF-α-induced increase in TRPC1 mRNA expression was prevented by the RNA polymerase II inhibitor actinomycin D. We observed that the TRPC1 mRNA expression peaked at 4 h after TNF-α treatment, and TRPC1 protein expression was increased >3-fold at 18 h after TNF-α exposure. The delayed time period of TRPC1 protein expression after TNF-α treatment in HUVEC is similar in its kinetics to the thrombin-activated synthesis of the proteinase-activated receptor-1 (PAR-1) in endothelial cells (25, 31). We addressed the functional significance of the increased TRPC1 expression by measuring the store-operated Ca²⁺ influx in control and TNF-α-treated HUVEC. ER Ca²⁺-store depletion with either thrombin or Tg augmented the Ca²⁺ influx in TNF-α-treated cells compared with control cells. Thus, the TNF-α-induced increase in TRPC1 expression is capable of activating Ca²⁺ influx following Ca²⁺ store depletion in human vascular endothelial cells.

Previous studies (22) showed that the mechanism of TNF-α-induced vascular injury involves expression of ICAM-1, VCAM-1, and E-selectin in vascular endothelial cells. TNF-α increases the expression of these cell surface adhesion proteins by activating the transcription factor NF-κB (22). To address mechanisms of TNF-α-induced TRPC1 expression in endothelial cells, we analyzed the 5′-regulatory region of the hTRPC1 gene sequence in the genomic data base. The hTRPC1 5′-regulatory region was shown to contain four NF-κB consensus binding sites (Fig. 4).

To address the TNF-α-induced TRPC1 promoter activation in endothelial cells, we cloned the hTRPC1-proximal promoter
cells were stimulated with TNF-α (1000 units/ml) and lysed at the indicated times. Firefly and sea pansy luciferase activity was measured. Values are shown as mean ± S.E. from four experiments with triplicate transfection each time point. * indicates the difference from corresponding control transfection (i.e. in the absence of IKKβ-DN expression) with TNF-α stimulation (p < 0.05). B, dominant negative IκBα mutant inhibits TNF-α-induced TRPC1 expression. HMEC were transfected with hTRPC1-Pro-Luc (-1686 to +40) construct with pRL/TK plasmid and in the presence of different concentrations of IκBα-DN CDNA as described in A. At 48 h after transfection, cells were stimulated with TNF-α (1000 units/ml) for 4 h. After this treatment, cells were lysed, and reporter activity was measured. Cell lysates (50 μg of protein) were immunoblotted with anti-IκBα antibody to assess the expression of IκBα-DN. Values are shown as mean ± S.E. from four experiments with triplicate transfection assay. * indicates the difference from the control (i.e. TNF-α-stimulated in the absence of IκBα-DN expression) (p < 0.05); ** indicates the difference from control (p < 0.001). IB, immunoblot. C, NEMO-binding domain peptide inhibits TNF-α-induced expression of TRPC1 and ICAM-1 in endothelial cells. HUVEC grown to confluence were incubated with cell-permeable wild type-NBD (WT-NBD) or mutant NBD (Mt-NBD) peptides (250 μM) for 2 h in serum-free medium. Cells were then challenged with TNF-α for the indicated times. Total RNA was isolated, and RT-PCR was performed for the expression of TRPC1 and ICAM-1 (see details under “Experimental Procedures”). Expression of GAPDH was determined for each sample to normalize the expression level of TRPC1 and ICAM-1. The experiment was repeated four times with triplicates. Top panel shows the TRPC1 expression in the presence of WT-NBD or Mt-NBD peptide. The middle panel shows expression of TRPC1 at 4 h after TNF-α stimulation as the fold induction (i.e. ratio of TRPC1 to GAPDH). * indicates the difference from corresponding TNF-α-stimulated control (p < 0.005). The bottom panel shows the inhibition of TNF-α-induced ICAM-1 mRNA expression in HUVEC by WT-NBD peptide.

Fig. 6. A, dominant negative IKKβ mutant (IKKβ-DN) inhibits TNF-α-induced TRPC1 expression. HMEC were transfected with hTRPC1-Pro-Luc (-1686 to +40) construct together with pRL/TK plasmid as described under “Experimental Procedures.” At 48 h after transfection, employing two different strategies (see details under “Experimental Procedures”). The DNA sequencing of the cloned products showed that the DNA fragment obtained using HUVEC-DNA as template for PCR lacked 106-bp nucleotide sequences (from −991 to −885) (see Fig. 4). We did not note any putative transcription factor binding sites in the sequence −991 to −885. Because this sequence was obtained from endothelial cells, we cloned the hTRPC1 promoter using HUVEC-DNA and used this strategy for further studies. We transfected hTRPC1-Pro-Luc expression constructs in HMEC and studied the expression of the reporter in response to TNF-α. Three different hTRPC1-Pro-Luc expression constructs were used in this study. Transfection of the construct lacking 5'-UTR region (+41 to +440 deleted) showed a 4–6-fold increase in reporter activity over basal in response to TNF-α. The construct retaining a large portion of 5'-UTR (sequence +1 to +440) showed minimal reporter activity in response to TNF-α. This finding may be the result of formation of a hairpin loop structure because of the presence of inverted GC-rich sequences (32, 33). Transfection of the construct (−1220 to +40) lacking one of the NF-κB sites (sequence −1545 to −1534) and 5'-UTR sequences (+41 to +440) showed minimal reporter activity. Thus, the NF-κB consensus site in position between −1545 and −1534 may be critical for TRPC1 expression in response to TNF-α. NF-κB is composed of homodimers and heterodimers of five different proteins (p50, p52, p65 [RelA], Rely, and c-Rel) (22, 23, 26, 27), which in turn phosphorylate serine residues 32 and 36 of IκBα and serine residues 19 and 23 of IκBβ, respectively (22, 26, 27). Phosphorylation of IκBα and IκBβ leads to proteolytic degradation of IκBα and dissociation of NF-κB, and NF-κB translocates to the nucleus to induce gene transcription (22, 26, 27). We investigated the involvement of NF-κB signaling in the transcriptional regulation of TRPC1 by expressing the kinase-defective mutant of IKKβ. Co-transfection of hTRPC1-Pro-Luc with kinase-defective IKKβ markedly
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Reduced the TNF-α-induced expression of the reporter in HMEC. In addition, co-expression of non-phosphorylatable IkBα mutant prevented the TNF-α-induced TRPC1 expression. These results indicate that NF-κB signaling is necessary for TNF-α-induced TRPC1 expression in endothelial cells.

May et al. (24) have shown that NF-κB activation can also be prevented by disrupting IkB kinase complex formation (i.e. by inhibiting IKKγ interaction with IKKα and IKKβ). These workers, using the regulatory protein NEMO (i.e. IKKγ) binding domain inhibitory peptide (24), showed that cell-permeable NEMO binding domain peptide (NBD peptide) blocked TNF-α-induced NF-κB activation in endothelial cells and the phorbol ester-induced inflammation in mouse model (24). Therefore, we studied the effects of cell-permeable wild type (Wt) and mutant (Mt) NBD peptides in the expression of TRPC1 in endothelial cells. Wt-NBD peptide pretreatment prevented ~75% of the TNF-α-induced TRPC1 expression, whereas the Mt peptide had no significant effect. The effect of NBD peptides on ICAM-1 expression was also studied as a control because the TNF-α-induced ICAM-1 expression in endothelial cells is primarily dependent on NF-κB activation (34, 35). Wt-NBD peptide treatment prevented ICAM-1 expression whereas the Mt-NBD peptide had no significant effect. Thus, NF-κB-induced TRPC1 expression in endothelial cells is critically dependent on NF-κB activation.

In summary, we showed that the hTRPC1 promoter is enriched in NF-κB consensus binding sites. We provide evidence that TNF-α induces NF-κB-dependent TRPC1 expression in endothelial cells which resulted in an increase in store-operated Ca²⁺ influx. Thus, Ca²⁺ influx induced by TRPC1 expression may play an important role in signaling endothelial activation and injury during inflammation.

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