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Reduction of tumor necrosis factor-alpha (TNF-α) related nuclear factor-kappaB (NF-κB) translocation but not inhibitor kappa-B (Iκ-B)-degradation by Rho protein inhibition in human endothelial cells

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Abstract

Degradation of inhibitor kappa-B (Iκ-B) followed by translocation of nuclear factor-kappaB (NF-κB) into the nucleus and activation of gene expression is essential in tumor necrosis factor-alpha (TNF-α)-signaling. In order to analyze the role of Rho proteins in TNF-α-induced NF-κB-activation in human umbilical cord vein endothelial cells (HUVEC) we used *Clostridium difficile* toxin B-10463 (TcdB-10463) which inactivates RhoA/Rac1/Cdc42 by glucosylation and *Clostridium botulinum* C3-toxin which inhibits RhoA/B/C by ADP-ribosylation. Exposure of HUVEC to 10 ng/mL TcdB-10463 or 2.5 μg/mL C3-toxin inhibited TNF-α (100 ng/mL)-induced expression of a NF-κB-dependent reporter gene. Moreover, preincubation of HUVEC with 10 ng/mL TcdB-10463 reduced TNF-α-related expression of interleukin-8 (IL-8), TNF-receptor associated factor-2 (TRAF2), and human inhibitor of apoptosis protein 1 (*hIAP1*)-mRNA. Blocking of Rho reduced NF-κB DNA-binding as shown by electrophoretic mobility shift assays. TcdB-10463 and C3-toxin blocked TNF-α-related nuclear translocation of NF-κB although Iκ-Bα/β was still degraded. In contrast, TcdB-10463 had no effect on IL-1β-related NF-κB-translocation and activation in HUVEC. Neither 1 μM Rho kinase inhibitor Y-27632 nor microfilament depolymerization by 50 ng/mL *C. botulinum* C2-toxin blocked TNF-α-induced degradation of Iκ-B, nuclear NF-κB translocation or expression of a NF-κB-dependent reporter gene. Therefore, TNF-α-related Iκ-B-degradation is Rho-independent in HUVEC, whereas a Rho protein-dependent signal is necessary to induce nuclear transport of NF-κB in these cells pointing to a novel and unique role of Rho in NF-κB-translocation.

Keywords: Transcription factors; NF-κB; TNF-α; Rho proteins; Clostridial toxin; Endothelium

1. Introduction

Endothelial cells are a primary target of tumor necrosis factor-alpha (TNF-α), a pleiotropic potent cytokine produced in response to inflammation, infection, and other environmental changes [1,2]. TNF-α elicits a broad spectrum of systemic and cellular responses, including fever, leukocyte activation, proliferation as well as apoptosis [2]. Expression of adhesion molecules (e.g. E-selectin, ICAM-1, VCAM-1), chemokines (IL-8) and coagulation factors (e.g. tissue factor, PAI-1) contribute significantly to the TNF-α-related endothelial inflammatory response [1,2]. Soluble TNF-α binds to two receptors, TNFR1 (55 kDa) and TNFR2 (75 kDa) which are both expressed on HUVEC. Most of the known TNF-α-responses occur *via* activation of TNFR1 [1,3–5]. Receptor activation results in clustering of preassembled TNFR-complexes and recruitment of adaptor proteins initiating multiple signaling pathways: cytocidal signals leading to programmed cell death may ensue with binding of death domain containing protein complexes (TRADD, TNFR1-associated protein with death domain; FADD, Fas-associated death domain) resulting in caspase 8-activation [1,3–5]. On the other hand, death-preventing, proinflammatory signals may arise from the same receptor *via* binding of TRADD, receptor interacting protein 1

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Abbreviations: HUVEC, human umbilical venous endothelial cells; TNF-α, tumor necrosis factor-alpha; IL-8, interleukin-8; TNFR, TNF-receptor; NF-κB, nuclear factor-kappaB; Iκ-B, inhibitor of NF-kappaB; *hIAP1*, human inhibitor of apoptosis protein 1; IKK, Iκ-B kinase; TRAF2, TNFR-associated factor 2; TcdB-10463, *Clostridium difficile* toxin B-10463; EMSA, electrophoretic mobility shift assay.

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(RIP1), and TNFR-associated factor 2 (TRAF2), with subsequent activation of the IKK-complex [1,3–6]. IKKα and IKKβ then phosphorylate Iκ-Bα and Iκ-Bβ on critical serine residues. Phosphorylation is followed by ubiquitination and degradation of Iκ-B molecules allowing translocation of NF-κB into the nucleus. Moreover, TNF-α-stimulation activates several kinase-dependent signaling pathways in endothelial cells, including c-*jun* NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 and extracellular signal regulated kinase (ERK) as well as phosphatidylinositol-3 (PI-3) kinase /Akt [1,3–6].

Several lines of evidence point to a central role of Rho proteins in TNF-α signaling [7–11]. Rho proteins belong to the Ras superfamily of small GTP binding proteins and are central regulators of the microfilament system and several important signaling pathways [12,13]. These proteins are activated following stimulation with TNF-α leading to actomyosin-mediated retraction of endothelial cells [9,10]. Moreover, TNF-α activated Rac in a PI-3 kinase-dependent manner in Rat-2 fibroblasts and L929 cells [7,11].

We made use of clostridial toxins which selectively modify Rho GTPases to analyze the role of Rho proteins in TNF-α-signaling in human endothelial cells [14,15]. These toxins turned out to be effective tools to study the role of small GTP-binding proteins in cellular processes [15]. *Clostridium difficile* toxin B-10463 (TcdB-10463), a single-chained 270 kDa molecule which easily enters cells by receptor-mediated endocytosis, inactivates Rho proteins by glucosylation at threonine 37 (Rho) [16] or threonine 35 (Rac/Cdc42) [17]. *Clostridium botulinum* C3-toxin inhibits RhoA, RhoB and RhoC by ADP-ribosylation at asparagine 41 [18]. In previous studies using these toxins we demonstrated TcdB-10463-dependent glucosylation of Rho proteins in endothelial cells accompanied by loss of endothelial barrier function [19] and decreased PKC-activation [20]. Moreover, using TcdB-10463, we showed that LPS-induced activation of IL-8 expression in human endothelial cells needs both, a Rho protein dependent and independent pathway [21].

In this study we show that intact Rho proteins are essential for TNF-α-dependent activation of NF-κB in cultured human endothelial cells. Rho protein inhibition did not block TNF-α induced degradation of Iκ-Bα or Iκ-Bβ but reduced NF-κB translocation, DNA-binding and NF-κB-dependent gene transcription.

2. Materials and methods

2.1. Antibodies and reagents

MCDB131, FCS, trypsin-EDTA-solution, and antibiotics were from Gibco. TNF-α was obtained from R&D Systems. [^{32}P]γATP was purchased from Amersham Pharmacia Biotech. All other chemicals used were analytical grade and obtained from commercial sources. Antibodies used in Western blotting for human p65 Rel, Iκ-Bα, Iκ-Bβ, and TRAF2 were obtained from Santa Cruz Biotechnology.

2.2. Preparation of bacterial toxins

C. difficile toxin B-10463 was purified as described previously [16,22]. *C. botulinum* C2-toxin and *C. botulinum* C3-toxin were kindly provided by Prof. K. Aktories, Department of Pharmacology and Toxicology, Albert-Ludwigs-University.

2.3. Preparation of human umbilical cord vein endothelial cells (HUVEC)

Cells were isolated from umbilical cord veins and identified as described previously [20,21,23,24]. Briefly, cells obtained from collagenase digestions were washed, resuspended in MCDB131/5% FCS and seeded into 6-, 24- or 96-well plates. Only confluent monolayers of primary cultures were used.

2.4. Western blotting

Stimulated endothelial cells were collected, washed, and lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% IGEPAL CA-650). Cell lysates were briefly centrifuged, endothelial nuclei were harvested and lysed in Tris–HCl 50 mM, pH 7.4, containing 1% Triton X-100, EDTA 0.25 mM, PMSF 1 mM, and protease inhibitors. Lysates were centrifuged and pellets resuspended in gel-loading buffer, sonified, and boiled. About 40 μg cytosolic or nuclear protein per lane were separated on a 10% SDS–PAGE and blotted on Hybond-ECL membranes (Amersham Pharmacia Biotech). Membranes were blocked, washed and exposed to rabbit polyclonal antibodies (p65 Rel, Iκ-Bα, Iκ-Bβ) or mouse antibody (TRAF2). Lipofectamin (2.5 μg/mL) was used to enhance uptake of *C. botulinum* C3-toxin (2.5 μg/mL) into HUVEC as described elsewhere [25]. Detection was performed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) or by visualization of IRDye800-labeled secondary antibodies (Odyssey infrared imaging system, LI-COR Inc.).

2.5. Electrophoretic mobility shift assay (EMSA)

After preincubation with 10 ng/mL TcdB-10463 for 1 hr HUVEC-monolayers were stimulated with 100 ng/mL TNF-α for 30 min and nuclear protein was isolated as described [23]. The consensus NF-κB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG-3′) (Promega) was end-labeled with [^{32}P]γATP using T4 polynucleotide kinase (Bioline). Unincorporated nucleotides were separated over a Sephadex G-25 spin column (Pharmacia). EMSA binding reactions were performed by first preincubating 5 μg of nuclear extract with 1 μg of poly(dI-dC) in

binding buffer for 20 min. Approximately 10,000 cpm (0.2 ng) of ^{32}P-labeled DNA probe was added. The reaction mixture was subjected to electrophoresis on 7% native acrylamide gels before vacuum drying and exposing to a storage phosphor screen for quantification and documentation (PhosporImager, Molecular Dynamics). Competition experiments were performed as above except that 100-fold excess of unlabelled competitor DNA was added to the incubation mixtures.

2.6. NF-κB reporter gene assay

Six NF-κB DNA binding sites (5′-GGG GAC TTT CCC T-3′) were inserted into the SmaI site in a pGL3basic vector (Promega). Downstream of this NF-κB binding regions a minimal β-globin promoter (containing a TATA box) was inserted into the XhoI/HindIII sites followed by the luciferase gene (pGL3.BG.6κB). HUVEC were transiently transfected with 2 μg NF-κB reporter plasmid. Transfected HUVEC were stimulated, harvested in reporter lysis buffer (Promega), and total protein was measured. Luciferase-assay was performed using a commercial kit (Promega). Luminescence was measured in a Lumat LB 9501 luminometer (Berthold). Relative luminescence readings were normalized to total protein and expressed as fold activation relative to control ± SEM. A control plasmid was created by inserting six mutated NK-κB sites (5′-GGC CAC TTT CCC T-3′) into the same vector (pGL3.BG.6κB.mut) as described recently [23,24].

2.7. Northern blot

Briefly, RNA was extracted, cDNA probes were labeled with [α-^{32}P]-dCTP (>3000 Ci/mmol) by random priming (Rediprime DNA labeling system, Amersham Pharmacia Biotech), and added to the prehybridization chambers before incubation for 12–16 hr at 42°. Human *hIAP*1-cDNA probe was a friendly gift of Dr. R. de Martin (Dept. of Vascular Biology and Thrombosis Research, VIRCC, University of Vienna). The 598 bp cDNA fragment of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was obtained as previously described [21,23,24].

2.8. Interleukin-8 (IL-8) ELISA

HUVEC monolayers were stimulated for 12 hr as indicated in a humidified atmosphere. After incubation supernatants were collected, centrifuged and processed for IL-8-quantification using a commercial ELISA (R&D Systems).

2.9. Release of lactate dehydrogenase (LDH)

LDH-activity was determined as described in [19–21,23,24].

2.10. Statistical methods

A one-way ANOVA was used for data of Figs. 1, 2A and C. Main effects were then compared by a F-probability test. $P < 0.05$ was considered to be significant.

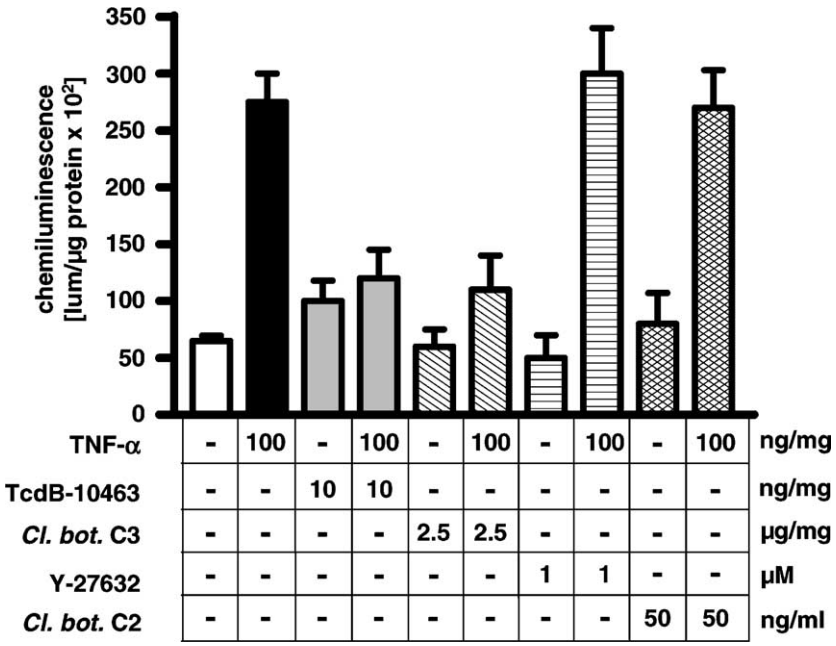

Fig. 1. Inhibition of Rho, Rac, Cdc42 by TcdB-10463 or RhoA/B/C by *C. botulinum* C3-toxin reduced TNF-α-dependent gene expression in HUVEC. HUVEC transiently transfected with a NF-κB-luciferase reporter construct were exposed to 100 ng/mL TNF-α for 12 hr or pretreated with 10 ng/mL TcdB-10463 (1 hr), *C. botulinum* C3-toxin (2.5 μg/mL), Rho kinase inhibitor Y-27632 (1 μM, 15 min), or F-actin depolymerizing *C. botulinum* C2-toxin (50 ng/mL, 2 hr) as indicated, and the expression of the reporter gene was measured by chemiluminescence assay. Rho inhibition (TcdB-10463, C3-toxin), but not blocking of Rho kinase (Y-27632) or F-actin depolymerization (C2-toxin) reduced TNF-α-induced expression of a NF-κB-dependent reporter gene. Data presented are mean ± SEM of four separate experiments.

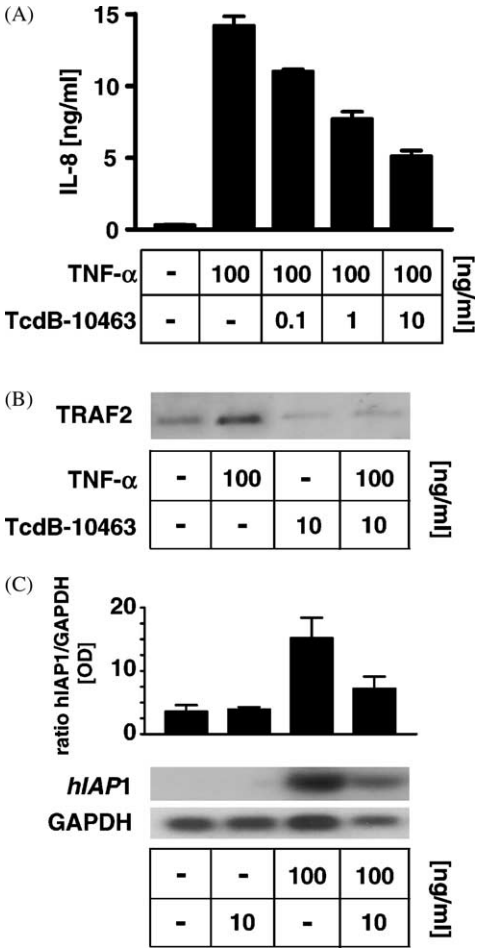

Fig. 2. TcdB-10463 reduced the expression of IL-8, TRAF2 and *hIAP1*. (A) Preexposure of HUVEC to TcdB-10463 (0.1–10 ng/mL) 2 hr before stimulation with 100 ng/mL TNF-α for 16 hr reduced dose-dependently TNF-α-induced IL-8-expression as shown by ELISA. (B) Inactivation of Rho proteins by TcdB-10463 as indicated reduced TNF-α-dependent expression of TRAF2 as shown by Western blot. (C) TNF-α-related expression of *hIAP1*-mRNA was reduced in HUVEC pretreated with 10 ng/mL TcdB-104653 as shown by Northern blot. Data presented in (A) and (C) are mean ± SEM of four separate experiments. Representative gels (B, C, one of three, four separate experiments, respectively) are shown in (B) and (C).

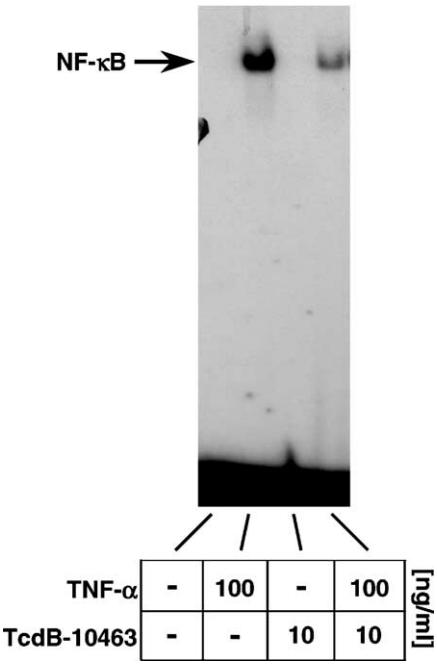

Fig. 3. Following pretreatment of HUVEC with 10 ng/mL TcdB-10463 or 100 ng/mL TNF-α alone or both together as indicated, binding of NF-κB to radiolabeled consensus-oligonucleotides was analyzed by EMSA. Note that TcdB-10463-induced inactivation of Rho proteins resulted in a substantial decrease of NF-κB-binding to the consensus oligonucleotide. A representative gel (one of three separate experiments) is shown.

3. Results

Inactivation of RhoA, Rac1 and Cdc42 by preincubation of HUVEC with 10 ng/mL TcdB-10463 for 1 hr or inhibition of RhoA/B/C by lipofected *C. botulinum* C3-toxin (2.5 μg/mL) both reduced the TNF-α (100 ng/mL, 12 hr)-related, NF-κB-dependent transcription of a luciferase reporter gene transfected into HUVEC (Fig. 1). Although Rho inhibition induced profound alterations of the endothelial microfilament system neither inhibition of p160ROCK/Rho kinase (1 μM Y-27632) or depolymerization of F-actin (*C. botulinum* C2-toxin 50 ng/mL) showed a significant effect on TNF-α-dependent reporter gene expression within the time and dose frame tested (Fig. 1). Furthermore, TNF-α-dependent expression of several proteins regulating different important endothelial cell functions was reduced in HUVEC with blocked Rho proteins: TcdB-10463-preexposure reduced the expression of the proinflammatory cytokine IL-8 (Fig. 2A), anti-apoptotic *hIAP1* (Fig. 2C) as well as TNFR-adaptor protein TRAF2 (Fig. 2B). Within the dose and time frame studied, no significant LDH release was noted, i.e. there was no evidence of overt cell damage (data not shown).

Binding-capacity of NF-κB to corresponding consensus-oligonucleotides was reduced in endothelial cells pretreated with 10 ng/mL TcdB-10463 before TNF-α-stimulation as shown by EMSA (Fig. 3).

Therefore, the effect of Rho protein-inhibition on TNF-α-dependent translocation of NF-κB-p65/RelA into the nucleus and on degradation of Iκ-B molecules was analyzed in detail. Western blot analysis of nuclear extracts of HUVEC showed a substantial reduction of TNF-α-triggered p65/RelA translocation into the nucleus of TcdB-10463-treated endothelial cells (Fig. 4A). Interestingly, in cytosolic extracts of these cells exposed to TcdB-10463 degradation of Iκ-Bα and Iκ-Bβ was still detected (Fig. 4A). Moreover, inhibition of RhoA, B, and C by using lipofected *C. botulinum* C3-toxin also blocked TNF-α-related translocation of NF-κB into the nucleus without impairing degradation of Iκ-B (Fig. 4B). Since Rho protein-inhibition disturbed endothelial cell microfilament organization we depolymerized F-actin by using *C. botulinum* C2-toxin. However, treatment of HUVEC with *C. botulinum* C2-toxin displayed no effect on TNF-α-induced translocation of NF-κB (Fig. 4C) although typical morphological alterations were noted (data not shown).

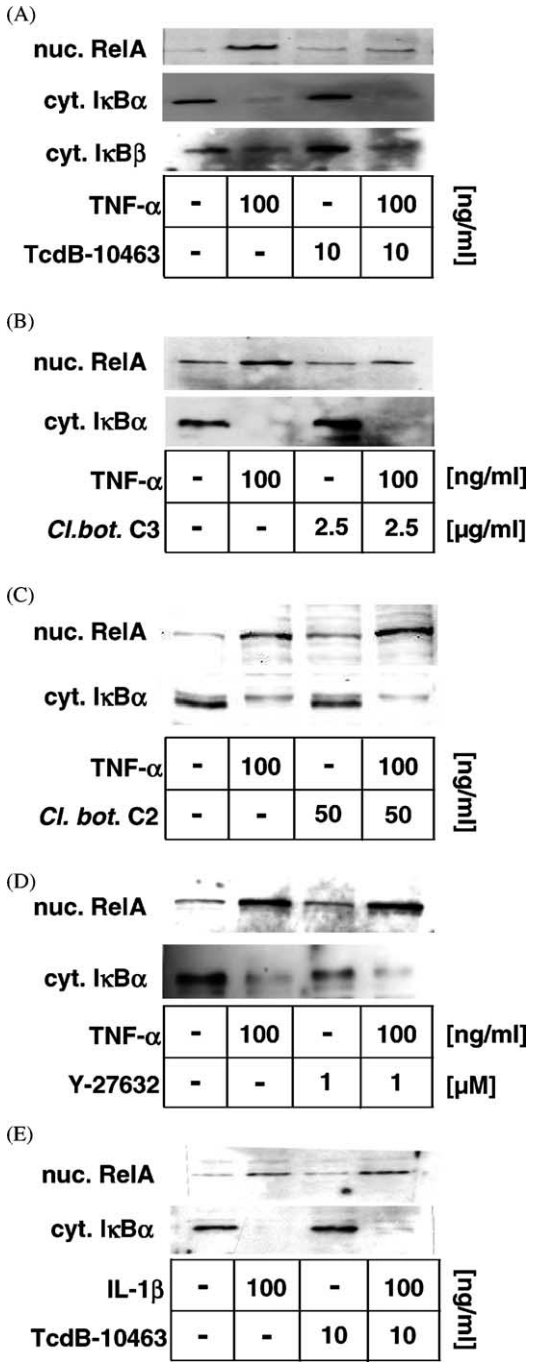

Fig. 4. Rho activity contributed to TNF-α-dependent nuclear NF-κB translocation independently from Iκ-B-degradation. Following pretreatment of HUVEC with 100 ng/mL TNF-α or 10 ng/mL TcdB-10463 (A), 100 ng/mL TNF-α or 2.5 μg/mL *C. botulinum* C3-toxin (B), 100 ng/mL TNF-α or 50 ng/mL *C. botulinum* C2-toxin (C), 100 ng/mL TNF-α or 1 μg/mL ROCK inhibitor Y-27632 (D), alone or in combination, cytosolic and nuclear extracts were prepared, and subjected to Western blot analysis developed with antibodies against p65/RelA, Iκ-Bα or β. Note that in cells without Rho function (A, B) TNF-α-related nuclear p65/RelA-translocation is significantly reduced although Iκ-B proteins are still degraded. Depolymerization of microfilaments (*C. botulinum* C2-toxin, C) or inhibition of ROCK kinase (Y-27632, D) before TNF-α-stimulation did not reduce TNF-α-dependent NF-κB translocation or Iκ-Bα degradation. In contrast, Rho inhibition displayed no effect on IL-1β-induced nuclear translocation of NF-κB (E). Representative gels (one of four separate experiments for A, one of three separate experiments for B–E) are shown.

Furthermore, TNF-α-dependent Iκ-Bα-degradation as well as TNF-α-induced translocation of NF-κB into endothelial cell nucleus seemed to be independent on Rho kinase activity (no effect of 1 μM Rho kinase inhibitor Y-27632) (Fig. 4D). In contrast, Rho protein-inhibition neither blocked IL-1β-induced Iκ-Bα-degradation (Fig. 4E) and nuclear translocation of NF-κB, nor IL-1β-dependent expression of a NF-κB-dependent reporter gene in human endothelial cells (data not shown).

4. Discussion

The results presented indicate that inhibition of Rho proteins by clostridial toxins reduced TNF-α-dependent gene expression in cultured human endothelial cells. Although cytosolic inhibitors of NF-κB, Iκ-Bα and Iκ-Bβ, were still degraded in TNF-α-stimulated cells without Rho function, no nuclear translocation of p65 Rel was noted. Therefore, an additional, Rho-dependent signal may contribute to TNF-α-induced nuclear NF-κB-translocation in human endothelial cells.

The study of Rho protein function used to be difficult. Available tools such as *C. botulinum* C3-toxin which ADP ribosylates Rho proteins at asparagine 41, poorly enter mammalian cells and constitutively activated p21Rho must be microinjected into target cells [15,18]. Overexpression of GTPases has been useful to study Rho protein function, although this method suffers some limitations [13]. TcdB-10463 which glucosylates Rho proteins at threonine 35/37 thereby rendering them functionally inactive, easily enters mammalian cells and turned out to be a highly selective and powerful alternative tool to study Rho protein function [14–16]. Previous studies using this toxin confirmed the requirement of Rho proteins for maintenance of endothelial barrier function [19], PKC-activation and translocation [20], phospholipase D activation [26], and myosin light chain phosphorylation [27]. Using TcdB-10463, we recently demonstrated that LPS induced two parallel signaling pathways (one Rho/tyrosine kinase-dependent and one Rho-independent, MKK6/p38 MAPK-depending) leading to IL-8 expression in human endothelial cells [21].

We now showed that inactivation of Rho, Rac and Cdc42 proteins by TcdB-10463 [15] reduced TNF-α-induced nuclear NF-κB translocation, DNA-binding and gene transcription. Moreover, blocking of RhoA, B, and C by *C. botulinum* C3-toxin displayed the same results. Therefore, this effect can be assigned to Rho. Using transfected dominant-negative mutants of RhoA, Perona *et al.* [8] noted as well an inhibition of TNF-α-dependent NF-κB-activation. TNF-α-stimulation resulted in activation of the multiprotein IKK complex which phosphorylates Iκ-B molecules, thereby targeting Iκ-B for ubiquitination-dependent degradation [3,6]. Interestingly, inhibition of Rho GTPases in TNF-α-stimulated endothelial cells seems not to interfere with the IKK-pathway of Iκ-B-degradation.

This is in line with the recent report of Cammarano and Minden [29] showing that Rac stimulates NF-κB by activation of IKKβ whereas Cdc42 and Rho activate NF-κB without stimulating either IKKα or IKKβ. However, using ectopic expressed mutants of Rho GTPases TNF-α-dependent degradation of Iκ-B molecules was blocked by inactive Rho mutants in some cell lines suggesting that Rho proteins participate in the activation of Iκ-B degradation in these cells [8]. Since different cells (cell lines vs. primary isolated cells) and experimental approaches for analysis of Rho function (transient transfection of Rho proteins vs. toxin-mediated inactivation by covalent modification) were used in these studies it is unclear how far these inconsistent results depend on cellular specificity or experimental conditions used [8,28,29]. However, a second, Rho-dependent signal may be necessary for TNF-α-induced NF-κB-translocation in human endothelial cells.

Besides masking of the nuclear import sequence by Iκ-B molecules phosphorylation of NF-κB may regulate its translocation into the nucleus in HUVEC as shown for *Drosophila* Rel protein Dorsal [30]. Since treatment of endothelial cells with Rho kinase inhibitor Y-27632 did not reduce TNF-α-dependent translocation of NF-κB into the nucleus nor TNF-α dependent expression of a reporter gene it seems unlikely that this kinase participated in the system tested. Although Wang and Baldwin [31] demonstrated TNF-α-dependent phosphorylation of Rel/p65 on serine 529 within the transactivation domain which did not affect nuclear translocation in HeLa cells it can not be ruled out that Rho protein-activated pathways may contribute to NF-κB-translocation. Furthermore, since TcdB-10463-treatment did not block TNF-α-dependent activation of NF-κB but reduced transcription factor activity related to genotoxic stress in HeLa cells a stimulus- and cell-type-specific regulation must be considered [28].

Rho protein inhibition induced marked alterations of the endothelial microfilament system and reduced the F-actin content in endothelial cells [19]. We used *C. botulinum* C2-induced depolymerization of endothelial F-actin [32,33] to control for microfilament-dependent effects on NF-κB-activation. *C. botulinum* C2-toxin had no effect on TNF-α-induced NF-κB-translocation or reporter gene expression indicating that Rho protein-specific, microfilament-independent signaling seems to participate in NF-κB translocation. Overall, TNF-α related Iκ-B degradation in endothelial cells was Rho-independent, but NF-κB-translocation was Rho-dependent.

In contrast, Rho inhibition did not reduce IL-1β-induced NF-κB-translocation or gene expression in human endothelial cells. Although IL-1β as well as TNF-α activated NF-κB, different adaptor molecules are recruited to activated receptors and result in divergent signaling pathways [34]. However, there is evidence that Rho proteins participate in the regulation of IL-1β-related activation of NF-κB in fibroblasts and HeLa cells [35].

In conclusion, the study presented demonstrates that inactivation of Rho proteins reduced TNF-α-dependent NF-κB-activation and NF-κB-dependent gene transcription in human endothelial cells. By taking advantage of TcdB-10463 and *C. botulinum* C3-toxin which inhibit different Rho GTPases RhoA/B/C proteins were identified as the responsible GTPases for TNF-α-dependent NF-κB-activation. Although Iκ-B molecules were degraded in TNF-α-treated cells with inactivated Rho proteins, NF-κB did not translocate into cells nucleus. Further studies are required to identify the Rho-dependent molecular mechanism participating in TNF-α-induced NF-κB-activation in endothelial cells.

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