Carbon monoxide (CO)-releasing molecule-derived CO regulates tissue factor and plasminogen activator inhibitor type 1 in human endothelial cells

Title page

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Title: Carbon monoxide (CO)-releasing molecule-derived CO regulates tissue factor and plasminogen activator inhibitor type 1 in human endothelial cells

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Running title: CORM-2 modulates TF and PAI-1 expression in HUVECs

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Abstract

Introduction: Heme oxygenase-1 (HO-1) is the rate limiting enzyme that catalyzes the conversion of heme into biliverdin, free iron, and carbon monoxide (CO). The first human case of HO-1 deficiency showed abnormalities in blood coagulation and the fibrinolytic system. Thus, HO-1 or HO-1 products, such as CO, might regulate coagulation and the fibrinolytic system. This study examined whether tricarbonyldichlororuthenium (II) dimer (CORM-2), which liberates CO, modulates the expression of tissue factor (TF) and plasminogen activator inhibitor type 1 (PAI-1) in human umbilical vein endothelial cells (HUVECs), and TF expression in peripheral blood mononuclear cells (PBMCs). Additionally, we examined the mechanism by which CO exerts its effects.

Materials and Methods: HUVECs were pretreated with 50 μM CORM-2 for 3 hours, and stimulated with tumor necrosis factor-α (TNF-α, 10 ng/ml) for an additional 0-5 hours. PBMCs were pretreated with 50-100 μM CORM-2 for 1hour followed by stimulating with lipopolysaccharid (LPS, 10 ng/ml) for additional 0-9 hours. The mRNA and protein levels were determined by RT-PCR and western blotting, respectively.

Results: Pretreatment with CORM-2 significantly inhibited TNF-α-induced TF and PAI-1 up-regulation in HUVECs, and LPS-induced TF expression in PBMCs. CORM-2

inhibited TNF-α-induced activation of p38 MAPK, ERK1/2, JNK, and NF-κB signaling pathways in HUVECs.

Conclusions: CORM-2 suppresses TNF-α-induced TF and PAI-1 up-regulation, and MAPKs and NF-κB signaling pathways activation by TNF-α in HUVECs. CORM-2 suppresses LPS-induced TF up-regulation in PBMCs. Therefore, we envision that the antithrombotic activity of CORM-2 might be used as a pharmaceutical agent for the treatment of various inflammatory conditions.

Key words: CORM-2, TF, PAI-1, HUVECs

Abbreviations

CORM-2 = tricarbonyldichlororuthenium (II) dimer

CO = carbon monoxide

HO-1 = heme oxygenase-1

TF = tissue factor

PAI-1 = plasminogen activator inhibitor type 1

HUVECs = human umbilical vein endothelial cells

PBMCs= Peripheral blood mononuclear cells

TNF-α = Tumor necrosis factor-α

LPS=Lipopolysaccharide

DMSO = dimethyl sulfoxide

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

p38MAPK = p38 mitogen-activated protein kinase

ERK1/2 = extracellular signal-regulated kinase1/2

JNK = c-Jun N-terminal kinase

NF-κB = nuclear factor-kappa B

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase that catalyzes the formation of carbon monoxide (CO), biliverdin/bilirubin, and ferrous iron, is induced under conditions of oxidative stress and inflammation [1]. Recent analyses of HO-1 knockout mice and of human HO-1 deficiency have indicated that HO-1 is an important molecule in the host's defense against oxidative stress, and that HO-1 has potent anti-inflammatory properties [2, 3]. Moreover, Yachie A, *et al.* reported that the first human case of HO-1 deficiency showed endothelial cell injury and abnormalities in the coagulation/fibrinolytic system; plasma levels of the thrombin-antithrombin complex (TAT), plasmin-α_2 plasmin inhibitor complex (PIC), and thrombomodulin were significantly increased, respectively [3, 4]. Therefore, HO-1 or HO-1 products, such as CO, may be associated with the regulation of the coagulation/fibrinolytic system.

CO has long been known in biology and medicine as a toxic compound due to its ability to bind hemoglobin with a much higher affinity than oxygen [5]. Acute exposure to high concentrations of CO is one of the leading causes of fatal poisoning in industrialized countries. Despite its reputation as a toxic gas, CO endogenously produced by HO has essential physiological functions and is of vital importance for cellular hemostasis [6]. Moreover, anti-inflammatory, anti-apoptotic, anti-atherogenic,

and cytoprotective effects are just a few of the pharmacological actions attributed to exogenously applied CO in low concentrations in various models of disease [7, 8]. Numerous reports have demonstrated CO-dependent protection in animal models of inflammatory syndromes, including sepsis [9] and colitis [10]. Inhalation of CO significantly suppresses ischemic induction of PAI-1 expression and the accumulation of fibrin in mice [11]. CO provides protection in a murine model of sepsis through modulation of inflammatory cytokine production [12]. In addition, the absence of HO-1 in aortic allograft recipient mice results in 100% mortality within 4 days due to arterial thrombosis, and CO rescues HO-1-deficient recipients from thrombosis after transplantation [13]. Therefore, we predict that CO will be beneficial in situations in which inflammation plays a damaging role.

Transitional metal carbonyls have been identified as potential CO-releasing molecules (CO-RMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs [14, 15]. One novel member of the CO-RMs, tricarbonyldichlororuthenium (II) dimer (CORM-2), liberates CO in the presence of dimethyl sulfoxide (DMSO) [16]. It has been reported that CORM-2 exhibits anti-inflammatory actions in lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs) by decreasing LPS-induced production of reactive

oxygen species and nitric oxide [17]. Additionally, CORM-2 significantly suppresses elevated PAI-1 expression in HO-1 deficient cells [18]. In contrast, Nielsen *et al.* reported that CORM-2 significantly enhances the velocity of clot growth and strength, and attenuates protamine-mediated hypocoagulation/hyperfibrinolysis [19, 20]. CORM-2 has been recently demonstrated to diminish tissue-type plasminogen activator (tPA)-mediated fibrinolysis of plasma thrombi [21, 22]. Therefore, it is unclear whether CORM-2 has anti-inflammatory and anti-coagulation roles.

In this study, we examined whether CORM-2 modulates the expression of TF and PAI-1 in HUVECs, and the expression of TF in peripheral blood mononuclear cells (PBMCs). In addition, we investigated whether CORM-2 affects the MAPK and NF-κB signaling pathways.

Materials and methods

Materials

CORM-2 ($[Ru(CO)_3Cl_2]_2$) was obtained from Acros Organics (Geel, Belgium) and solubilized in DMSO. Inactive forms was $Ru(DMSO)_4Cl_2$ (iCORM-2), a molecule where the carbonyl groups have been replaced with DMSO. Tumor necrosis factor-α (TNF-α) was obtained from R&D systems (Minneapolis, MN, USA) and dissolved in 0.2-μm-filtered phosphate-buffered saline (PBS) + 1% Bovine serum albumin (BSA). SB203580, U0126, and SP600125 were purchased from Alexis Biochemicals Inc (San Diego, CA) and dissolved in DMSO. Anti-TF, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), phosho-p38 MAPK, phospho-ERK1/2, phospho-JNK, nuclear factor-kappa B (NF-κB, p65), IκBα, and Oct-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc (CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Nacalai Tesque Inc (Kyoto, Japan).

Cell Culture

HUVECs and associated medium, EGM-2 Bullet Kit, were purchased from TaKaRa

Bio Inc (Otsu, Japan). HUVECs were grown to confluence in EGM-2 that contained 2% fetal bovine serum at 37 ℃ in humidified atmosphere of 5% CO_2. The cells in this experiment were used within 3 to 8 passages. Cells were pretreated with CORM-2 (50 μM) for 3 hours followed by stimulating with TNF-α (10 ng/ml) for additional 0-5 hours. CORM-2 was dissolved in DMSO and then diluted in culture medium (0.05 % v/v, pH = 7.6). Cells were treated with 0.05 % DMSO (CORM-2 vehicle). Experiments were repeated with the negative controls iCORM-2 to assess whether the effects observed were due to the CO liberated by CORM-2 or caused by other components of the molecules. After stimulation, the cells were harvested separately.

PBMCs were isolated from heparinized peripheral blood by a density gradient centrifugation with Lymphoprep (Axis Shield, Oslo, Norway). PBMCs were suspended in RPMI 1640 medium (Invitrogen, California, USA). PBMCs were pretreated with 50-100 μM CORM-2 for 1 hour followed by stimulating with 10 ng/ml LPS for additional 0-9 hours.

Quantification of RNA

Total RNA was extracted from cultured cells by using Nucleo Spin®(NIPPON Genetics Inc, Tokyo, Japan). The cDNA was synthesized from total RNA by reverse transcription with the PrimeScript® RT-PCR Kit (TaKaRa Bio Inc, Otsu, Japan)

according to the manufacturer's instructions. The cDNA was subjected to the following PCR conditions: 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 30 s. The primers used in the reactions showed in Table1. PCR products were subjected to electrophoresis in 3 % agarose gel and densitometry was performed using Typhoon9200 (GE Healthcare Inc, Tokyo, Japan). Levels of mRNA were normalized to the concentration of GAPDH mRNA.

Separation of cytosol and nuclear extract

To separate nuclear and cytoplasmic protein fractions, cells were washed with cold PBS, lysed in buffer A (120 mM KCl, 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 1 mM dithiothreitol (DTT)) and incubated for 5 min on ice. Samples were centrifuged at 4℃ for 1 min at 10000 rpm. Remove supernatant and add buffer B (120 mM KCl, 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 1 mM DTT, 0.4% NonidetP-40, protease inhibitor cocktail solution (Roche Molecular Biochemicals)) to cells and incubate for 10 min on ice. The supernatant (cytosol extract) was obtained by centrifugation at 10000 rpm for 30 s. The pellets (nuclei) were resuspended in 200 μl of buffer C (120 mM KCl, 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 1 mM DTT, protease inhibitor cocktail solution) and centrifuged at 10000 rpm for 30 s at 4℃. The nuclei were then extracted in 20 μl of

buffer D (40 mM Tris-HCl (pH7.9), 0.8 M NaCl, 3 mM EDTA, 50% glycerol, 1 mM DTT, protease inhibitor cocktail solution) and incubated on ice for 15 min. Finally, the samples were centrifuged for 6 min at 15000 rpm, and the supernatants were collected and saved as the nuclear protein fraction. Samples were stored at -80℃.

Western bolt analysis

HUVECs were lysed in buffer comprising 50 mM Tris-HCl, pH7.5, 1 % BSA, 2 mM EDTA, 100 U/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 200 mM phenylmethanesulfonyl fluoride. Samples of cell lysate were electrophoresed on 10 % SDS/polyacrylamide gel, and transferred to nitrocellulose membrane Hypond™-P (GE Healthcare Inc, Tokyo, Japan). After being transferred to membranes, the samples were immunoblotted with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The protein bands were detected with Immobilon Western HRP Detection Substrate (Nihon Millipore Inc, Tokyo, Japan).

Cell viability using MTT assay

Cells were plated into the wells of 96-well microplates (Falcon, Becton Dickinson, NJ, USA). After incubation with or without CORM-2 or TNF-α, medium was changed to

normal culture medium. For cell viability assay, 10 µl of sterile, filtered MTT stock solutions in PBS (5 mg/ml) was added to each well. After three hours, the unreacted dye was removed, and the formazan crystals were dissolved with 150 µl of DMSO. After gentle agitation for five minutes, the absorbance was read at 570 nm using Microplate Reader (Tecan, Kanagawa, Japan). Cells incubated without treatment were considered as controls, and cell viability values were set at 100 %.

Statistical analysis

The mRNA expression data represent the mean ± SD of five independent experiments. Western blot image is representative of three different blots. Statistical analysis was performed using one-way ANOVA combined with Bonferroni test. A value of $p < 0.05$ was considered statistically significant.

Results

Pretreatment with CORM-2 suppressed TF and PAI-1 expression in TNF-α-stimulated HUVECs and TF expression in LPS -stimulated PBMCs.

After TNF-α stimulation, TF mRNA levels were approximately 6-fold (Fig 1B, $p<0.05$) and PAI-1 mRNA levels were approximately 2-fold (Fig. 1D, $p<0.05$) increased as compared to the control. Pretreatment of HUVECs with CORM-2 significantly decreased TF mRNA levels (approximately 80% suppression, $p<0.05$) (Fig. 1B), and PAI-1 mRNA levels (approximately 60% suppression, $p<0.05$) (Fig. 1D) as compared to the TNF-α-stimulated group. Similar to mRNA expression, pretreatment with CORM-2 inhibited TNF-α-induced TF and PAI-1 protein up-regulation (Fig. 1A and C). On the other hand, iCORM-2, which does not liberate CO, had no effect on TF and PAI-1 mRNA expression (Fig. 1B and D). Incubation with 50 μM CORM-2 or iCORM-2 did not affect cell viability according to MTT assays (data not shown).

In PBMCs, LPS increased approximately 10-fold TF mRNA expression (Fig. 2B, $p<0.05$). Pretreatment with CORM-2 significantly inhibited LPS-induced TF mRNA expression (50 μM: approximately 70% suppression, 100 μM; approximately 80% suppression, $p<0.05$ respectively) (Fig. 2B). Similarly to the mRNA expression, CORM-2 did not affect TF protein expression. CORM-2 significantly suppressed TF protein

expression induced by LPS (Fig. 2A).

Pretreatment with CORM-2 suppressed TNF-α stimulated Mitogen-activated protein kinases (MAPKs) Phosphorylation.

To determine whether pretreatment with CORM-2 affected MAPKs phosphorylation, the phosphorylation of p38 MAPK, ERK1/2, and JNK were assessed after TNF-α stimulation. TNF-α induced a transient phosphorylation of p38 MAPK and JNK, with maximal activation occurring after 10 min of stimulation. Additionally, TNF-α induced transient phosphorylation of ERK1/2 with maximal activation occurring after 30 min of stimulation (Fig. 3). On the other hand, pretreatment of CORM-2 inhibited phosphorylation of p38 MAPK, ERK1/2, and JNK (Fig. 3). Total expression of p38 MAPK, ERK1/2, and JNK remained unaffected at all time points examined.

MAPK Inhibitors Affected TNF-α-induced TF and PAI-1 Protein Expression

To confirm the role of p38 MAPK, ERK, or JNK in the repression of TF and PAI-1 expression by CORM-2, HUVECs were pre-incubated with p38 MAPK inhibitor (SB203580, 20 μM), ERK1/2 inhibitor (U0126, 20 μM), or JNK inhibitor (SP600125, 20 μM) for 1 hour each. HUVECs were stimulated with 10 ng/ml TNF-α, and TF and PAI-1

protein expressions were analyzed by western blots. The p38 MAPK inhibitor, SB203580, and JNK inhibitor, SP600125, strongly inhibited TNF-α-induced TF protein up-regulation. In contrast, the ERK1/2 inhibitor U0126 did not inhibit TNF-α-induced TF protein up-regulation (Fig. 4). TNF-α-induced PAI-1 protein up-regulation was suppressed by the p38 MAPK inhibitor, ERK inhibitor, and JNK inhibitor (Fig. 4).

Pretreatment with CORM-2 Inhibited NF-κB Activation in TNF-α-stimulated HUVECs.

To determine whether pretreatment with CORM-2 could inhibit NF-κB transcriptional activation, nuclear NF-κB (p65) was detected by western blot. Moreover, IκBα and phosphorylated IκBα in cytosolic extracts were determined by western blots. TNF-α promoted translocation of NF-κB (p65) into the nucleus (Fig. 5B). In contrast, pretreatment with CORM-2 reduced NF-κB (p65) translocation to the nucleus after TNF-α stimulation (Fig. 5B). Although TNF-α induced IκBα degradation and phosphorylation of IκBα, pretreatment with CORM-2 inhibited TNF-α-induced degradation of IκBα and phosphorylation of IκBα (Fig. 5A).

Discussion

We demonstrated that CORM-2 suppresses TNF-α-induced TF and PAI-1 up-regulation in HUVECs and LPS-induced TF up-regulation in PBMCs. Additionally, pretreatment with CORM-2 suppressed MAPKs and NF-κB signaling pathways activation by TNF-α. Thus, we suggest that CORM-2 might provide a therapeutic avenue for the hypercoagulable state associated with inflammation diseases.

TF combines with factor VIIa to activate factor X leading to thrombosis. The majority of cases of systemic inflammatory response syndrome, disseminated intravascular coagulation, and sepsis are accompanied by hyperactivation of TF in circulating monocytes and damaged tissue. Induction of TF expression on monocytes and endothelial cells is central to the development of septic coagulopathy. Early modulation of TF activity is presently a therapeutic strategy in systemic inflammatory response syndrome patients [23]. On the other hand, PAI-1 plays an important role in the regulation of fibrinolysis and proteolysis by inhibiting tissue-type plasminogen activator and urokinase-type plasminogen activator [24]. Studies in animal models have indicated that increased PAI-1 activity enhances thrombosis, and antibodies against PAI-1 prevent the progression of thrombosis [25]. Our study showed that pretreatment with CORM-2 suppresses the TNF-α-induced TF and PAI-1 up-regulation in HUVECs.

Furthermore, we showed that LPS-induced TF mRNA and protein up-regulation on PBMCs were significantly inhibited by CORM-2. The negative control (iCORM-2), which does not liberate CO, did not affect TF and PAI-1 expression. Therefore, CO released from CORM-2 might inhibit TNF-α-induced TF and PAI-1 expression in HUVECs and LPS- induced TF expression in PBMCs.

MAPKs constitute a family of proline-directed serine/threonine kinases that include at least three distinct cascades: p38 MAPK, ERK, and JNK. MAPKs function as cellular signal transducers, and play a central role in the control of cellular proliferation, the production of inflammatory cytokines [26, 27], and regulating TF and PAI-1 expression [28, 29]. In our study, phosphorylation of p38 MAPK, ERK1/2, and JNK enhanced by TNF-α and pretreatment with CORM-2 suppressed phosphorylation of p38 MAPK, ERK1/2, and JNK. The p38 MAPK and JNK inhibitors strongly inhibited TNF-α-induced TF and PAI-1 protein up-regulation. The ERK1/2 inhibitor suppressed TNF-α-induced PAI-1 protein up-regulation, but did not inhibit TF protein up-regulation. A previous study indicated that pretreatment of HUVECs with an ERK inhibitor (U0126, 10 μM) did not significantly inhibit TF induction by TNF-α [30]. In contrast, another study showed that ERK activation by TNF-α increased TF expression in endothelial cells [31]. Further studies may be necessary to examine whether ERK1/2

activation induces TF expression in HUVECs using dominant negative mutants of ERK1/2.

NF-κB is a rapidly acting transcription factor involved in inflammatory reactions and coagulation/fibrinolytic systems; it regulates the expression of cytokines, TF, and PAI-1 [27, 32]. NF-κB is a heterodimer composed primarily of a 50 kDa DNA binding subunit and a 65 kDa transactivator (p65 or Rel A). NF-κB is retained in the cytoplasm associated with a family of inhibitory proteins termed IκB. In response to inflammatory stimuli such as TNF-α and LPS, the IκBs are rapidly phosphorylated, releasing NF-κB and undergoing ubiquitination and proteolysis by the 26S proteosome [33]. In the present study, TNF-α induced translocation of NF-κB (p65) into the nucleus. In contrast, pretreatment with CORM-2 suppressed TNF-α induced NF-κB translocation to the nucleus. Thus, we consider that pretreatment with CORM-2 might suppress TNF-α-induced TF and PAI-1 up-regulation via regulating TNF-α signaling pathways in HUVECs.

It has been reported that CORM-2 and CO confer anti-inflammatory effects by interfering with NF-κB activation [12, 34]. On the other hand, inhalation of CO for 1 h has no anti-inflammatory effects in a systemic inflammation model in humans [35]. CORM-2 increases the velocity of thrombus growth and strength in hemophilia A,

hemophilia B, and factor VII-deficient plasmas [36]. Furthermore, CORM-2 enhances coagulation and markedly attenuates fibrinolysis in human plasma [37]. In contrast, enhanced induction of HO-1 and the administration of CORM-2 inhibit thrombus formation and affect the protein C system in sepsis [38]. CORM-2 reduces HMGB1 release in macrophages and septic animal models [9, 39]. Our study showed that CORM-2 moderates TNF-α-induced TF and PAI-1 up-regulation. We consider that CORM-2 might have benefit in situations in which inflammation plays a damaging role. However, acute exposure to high concentrations of CO is one of the leading causes of fatal poisoning in industrialized countries. Further research is thus needed to understand the effect of CORM-2 in inflammatory states. Whether direct application of CO by pharmacologic administration in humans will provide a safe and effective modality for treatment of human diseases is not yet known. We believe that further research is needed to understand the pharmacokinetics and toxicology of CO application in humans.

In conclusion, our study indicated that pretreatment of CORM-2, a CO-releasing molecule, suppresses TNF-α-induced TF and PAI-1 up-regulation in HUVECs, and LPS-induced TF up-regulation in PBMCs. CORM-2 regulates the activation of MAPKs and NF-κB signaling pathways. Therefore, we suggest that CORM-2 could constitute a

novel anti-coagulative therapy in various inflammatory diseases.

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Conflict of interest

None declared.

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Figures and Legends

Figure 1 Effects of pretreatment with CORM-2 on TF and PAI-1 expression in TNF-α-stimulated HUVECs. HUVECs were pretreated with CORM-2 at a concentration of 50 μM for 3 h, and stimulated with 10 ng/ml TNF-α for additional 0-5 h. TF (A) and PAI-1(C) mRNA levels were determined by RT-PCR. TF (B) and PAI-1 (D) protein levels were analyzed by western blotting. All values are expressed as means ± SD (n=5).

Figure 2 Effects of pretreatment with CORM-2 on TF expression in LPS-stimulated PBMCs. PBMCs were pretreated with CORM-2 at concentration of 50 -100 μM for 3 h, and stimulated with 10 ng/ml LPS for additional 0-9 h. (A) TF protein levels were analyzed by western blotting. (B) TF mRNA levels were determined by RT-PCR. All values are expressed as means ± SD (n=5).

Figure 3 Effects of pretreatment with CORM-2 on TNF-α stimulated mitogen-activated protein kinases (MAPKs) phosphorylation. HUVECs were pretreated with CORM-2 at a concentration of 50 μM for 3 h, and stimulated with 10 ng/ml TNF-α for additional 0-1 h. Phosphorylations of p38 MAPK, ERK1/2, and JNK were analyzed by western blots. A

representative image from three experiments is shown.

Figure 4 Effects of MAPK inhibitors on TF and PAI-1 protein expression in TNF-α-stimulated HUVECs. HUVECs were pre-incubated with p38 MAPK inhibitor SB203580 (20 μM), ERK1/2 inhibitor U0126 (20 μM), or JNK inhibitor SP600125 (20 μM) for 1 h and then stimulated with TNF-α for 5 h. TF and PAI-1 protein expression were analyzed by western blots. A representative image from three experiments is shown. Control: unstimulated cells.

Figure 5 Effect of CORM-2 pretreatment on NF-kB activation in TNF-α stimulated HUVECs. HUVECs were pretreated with CORM-2 at a concentration of 50 μM for 3 h, and stimulated with 10 ng/ml TNF-α for an additional 0-5 h. Nuclear and cytoplasmic proteins of HUVEC were extracted. (A) Nuclear NF-κB (p65) was detected by western blot. (B) IκBα and phosphorylated IκBα in cytosolic extracts were determined by western blots. A representative image from three experiments is shown.

Table1 Sequences of oligonucleotide primers used for polymerase chain reaction.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
TF	CTCGGACAGCCAACAATTCAGAGT	TGTTCGGGAGGGAATCACTGCTTGAACACT
PAI-1	GAGGTGCCTCTCTCTGCCCTCACCAACATT	AGCCTGAAACTGTCTGAACATGTCG
GAPDH	CGTCATGGGTGTGAACCATGAGAAG	GCATGGACTGTGGTCATGAGTCCTT

TF: tissue factor, PAI-1: plasminogen activator inhibitor type 1
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Figure 1 Effects of pretreatment with CORM-2 on TF and PAI-1 expression in TNF-α-stimulated HUVECs.

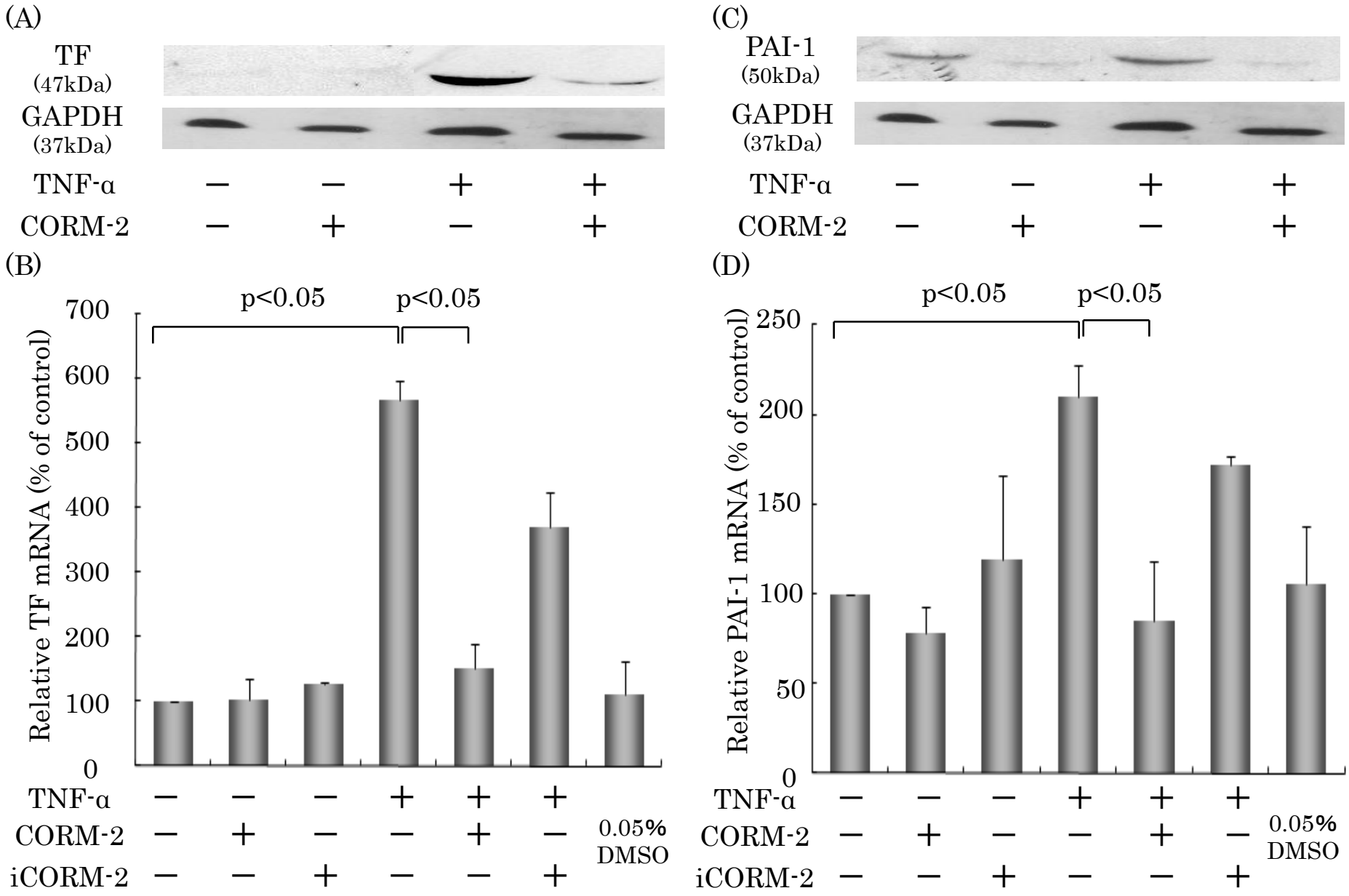

Figure 2 Effects of pretreatment with CORM-2 on TF expression in LPS-stimulated PBMCs.

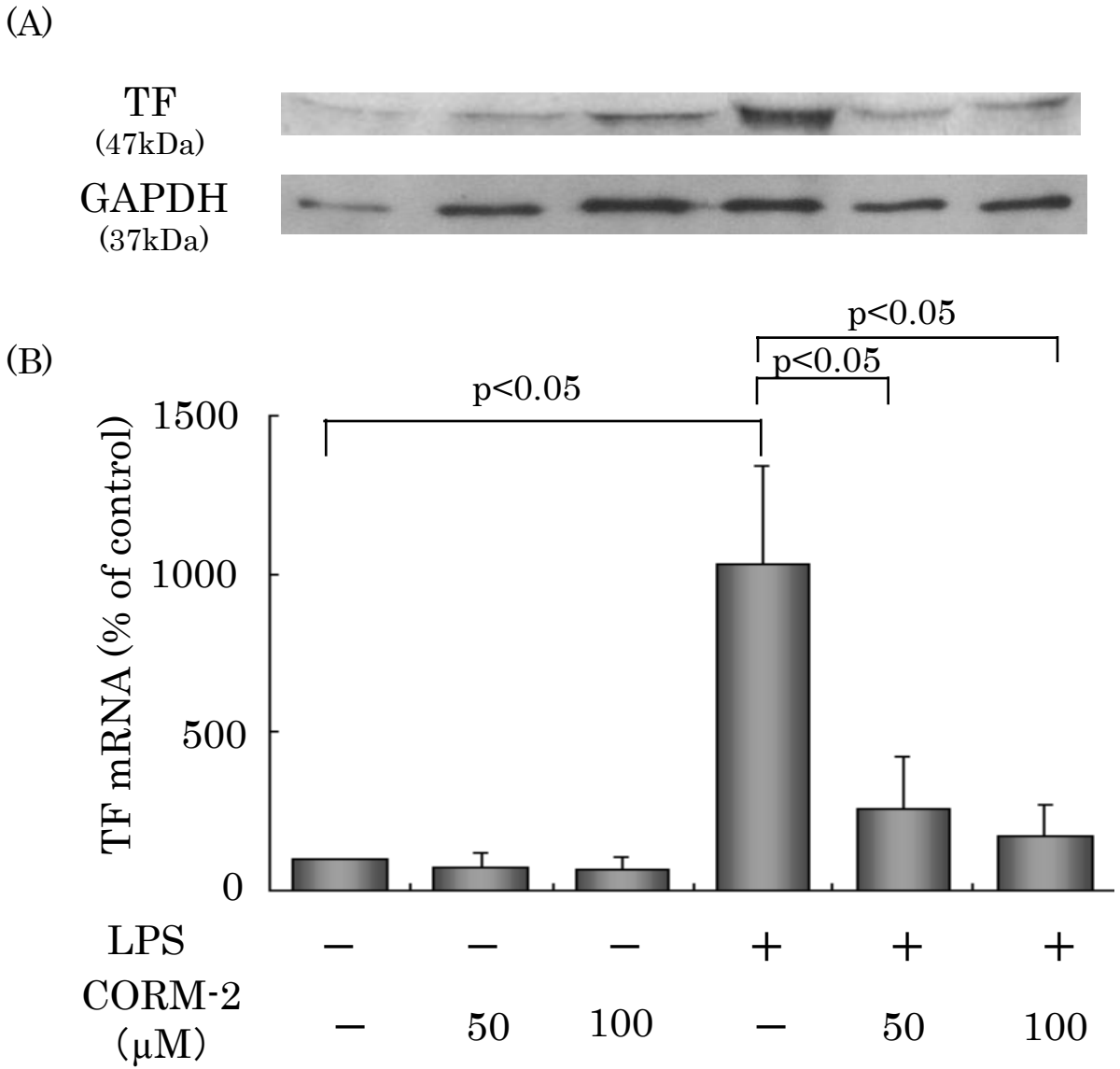

Figure 3 Effects of pretreatment with CORM-2 on TNF-α stimulated mitogen-activated protein kinases (MAPKs) phosphorylation.

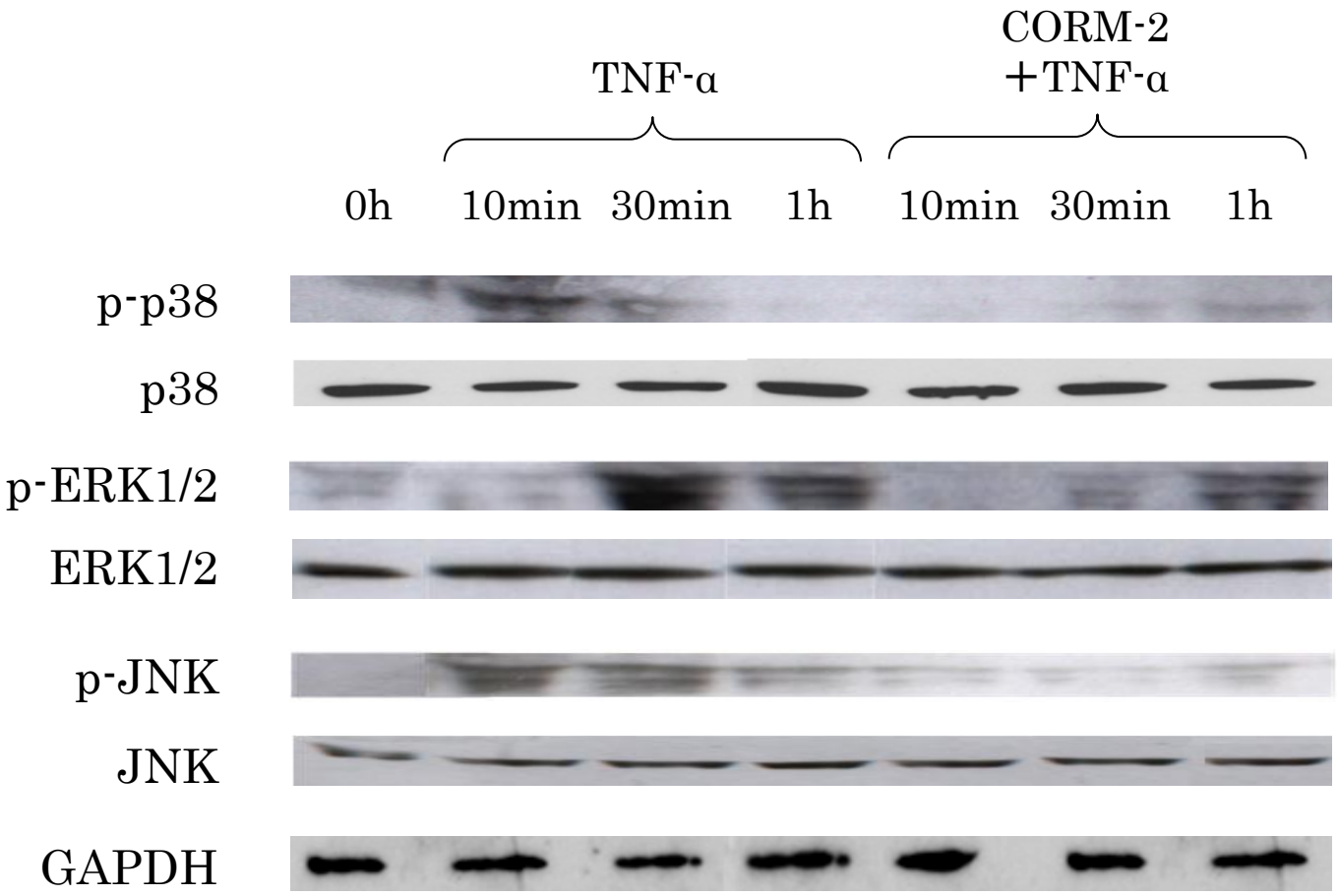

Figure 4 Effects of MAPK inhibitors on TF and PAI-1 protein expression in TNF-α stimulated HUVECs

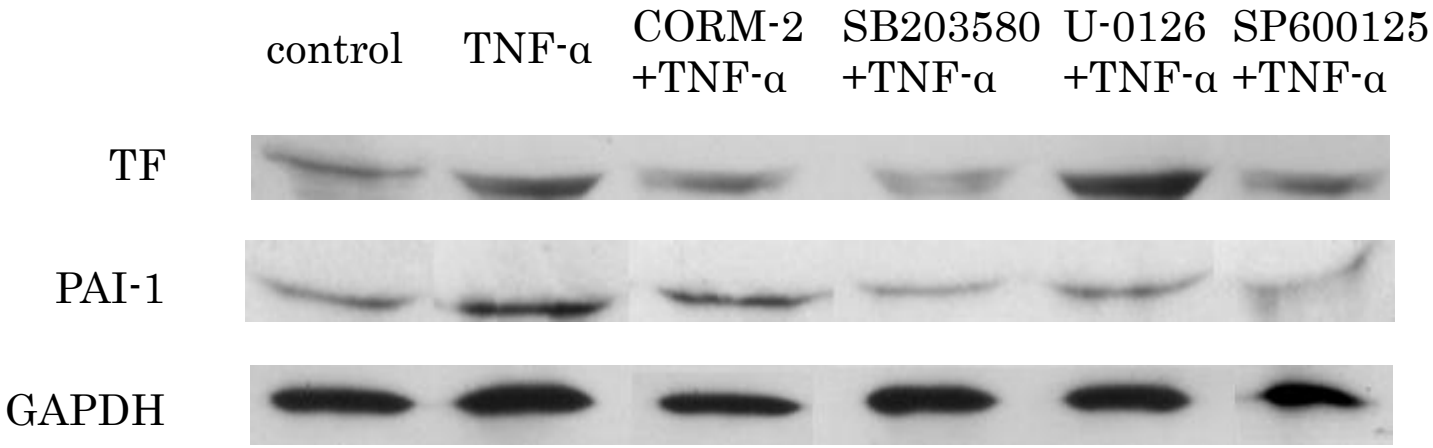

Figure 5 Effect of CORM-2 pretreatment on NF-κB activation in TNF-α stimulated HUVECs