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Ectodomain shedding of TNF-α is enhanced by nardilysin via activation of ADAM proteases

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is released from cells by proteolytic cleavage of a membrane-anchored precursor. The TNF-α-converting enzyme (TACE/ADAM17) is the major sheddase for ectodomain shedding of TNF-α. At present, however, it is poorly understood how its catalytic activity is regulated. Here, we show that nardilysin (*N*-arginine dibasic convertase; NRDc) enhanced TNF-α shedding. In a cell-based shedding assay, expression of NRDc synergistically enhanced TACE-induced TNF-α shedding. A peptide cleavage assay *in vitro* showed that recombinant NRDc enhances the cleavage of TNF-α induced by TACE. Notably, co-incubation of NRDc completely reversed the inhibitory effect of a physiological concentration of salt on TACE's activity *in vitro*. Overexpression of NRDc in TACE-deficient fibroblasts resulted in an increase in the amount of TNF-α released. Co-expression of NRDc with ADAM10 promoted the release compared with the sole expression of ADAM10. These results suggested that NRDc enhances TNF-α shedding through activation of not only TACE but ADAM10. Our results indicate the involvement of NRDc in ectodomain shedding of TNF-α, which may be a novel target for anti-inflammatory therapies.

Many membrane-anchored proteins are shed from the cell surface by proteolytic cleavage, a process referred to as ectodomain shedding. This process regulates the biological activities of structurally and functionally diverse proteins, including growth factors, cytokines, and receptors, as well as cell adhesion molecules [1,2]. A balance between the membrane-bound and soluble forms of these proteins appears to be strictly regulated in accordance with various *in vivo* situations. The proper cleavage of tumor necrosis factor-α (TNF-α), for instance, is required for the optimal development of inflammatory lesions [3,4].

ADAM (a disintegrin and metalloprotease) proteases, a family of membrane-anchored metalloproteases, are key components in ectodomain shedding [1,5]. Among them, TNF-α-converting enzyme (TACE/ADAM17) is the prototypical sheddase, which was initially identified as the protease that converts the membrane-anchored precursor of TNF-α to the soluble form [6,7]. Analyses of cells derived from TACE-deficient mice showed impaired shedding of TNF-α [6]. Moreover, ectodomain shedding of many other membrane proteins including transforming growth factor (TGF)-α [8], amyloid precursor protein (APP) [9], and Notch [10], was impaired in those cells, indicating essential roles of TACE in the shedding of multiple membrane proteins. TACE-deficient mice showed defects in multiple organs including the eye, hair, skin, intestine, lung, and heart [11]. Notably, mice lacking TGF-α showed similar defects in the eye, hair, and skin [11], while HB-EGF-deficient mice shared a similar heart defect in valvulogenesis with TACE-deficient mice [12]. These findings indicate that TACE-induced shedding is required for the *in vivo* biological functions of these EGF family ligands.

Nardilysin (EC 3.4.24.61, *N*-arginine dibasic convertase; NRDc) was initially cloned as a zinc metalloendopeptidase of the M16 family, which can selectively cleave the dibasic site *in vitro* [13]. Originally, we identified NRDc as a specific binding partner of HB-EGF [14]. We also have recently demonstrated that this metalloendopeptidase is a potent activator of the ectodomain shedding of HB-EGF [15]. Our results indicated that NRDc binds to and directly enhances the catalytic activity of TACE, and that metallopeptidase activity of NRDc is not required for the enhancement [15]. When cells were stimulated with phorbor ester, a general activator of ectodomain shedding, the formation of a complex between NRDc and TACE was promoted, suggesting the involvement of this interaction in the activation of ectodomain shedding [15]. We demonstrated that NRDc also enhances ectodomain shedding of APP (α-cleavage), which decreases the amount of amyloid β generated [16]. The results clearly show that

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the effect of NRDc on ectodomain shedding is not specific to HB-EGF. Furthermore, NRDc potentiated not only TACE, but ADAM9- and ADAM10-induced shedding of APP [16], suggesting that NRDc might be a general activator for ADAM proteases and ectodomain shedding. To further obtain evidence in support of this hypothesis, we examine the effect of NRDc on the cleavage of TNF-α, and demonstrate here the involvement of NRDc in ectodomain shedding of the inflammatory cytokine.

Materials and methods

Plasmids. Expression plasmids for human NRDc (pcDNA3.1-hNRDc-V5), the enzymatically inactive mutant of human NRDc (pcDNA3.1-hNRDc E235A-V5), human TACE (pME18S-hTACE), and human ADAM10 (pME18S-hADAM10) were described previously [15,16]. The human cDNA for full-length TNF-α was cloned into pME18S-FLAG to generate pME18S-hTNF-α-FLAG. The human cDNA for NRDc was cloned into a lentivirus vector, pLenti6 (Invitrogen), to generate pLenti6-hNRDc.

Antibodies and reagents. The antibodies against V5 tag were obtained from Invitrogen; Flag tag (M2) from Sigma; TACE (C-15) from SantaCruz; ADAM10 from Calbiochem; Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) from Research Diagnostics. The mouse monoclonal antibody for NRDc (#23) was described previously [15]. Recombinant NRDc was synthesized using a Bac-to-Bac baculovirus expression system (Invitrogen) as described previously [15]. Recombinant NRDc lacking enzymatic activity (human NRDc-E235A) was similarly generated by a baculovirus expression system. Recombinant TACE was purchased from R&D.

Cell culture, transfections, and Western blot analysis. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified incubator with 5% CO_2. Mouse fibroblasts deficient in TACE activity (EC2 cells) were a gift from R. A. Black [17] and cultured in DMEM/F12 containing 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Transfections in COS7 cells were carried out using FuGENE 6 (Roche) for plasmids and siFECTOR (B-Bridge International) for siRNA, according to the manufacturer's instructions. Transfections in EC2 cells were carried out using a recombinant lentivirus vector. Briefly, pLenti6-hNRDc, in concert with PMDL, VSV-G, and RSV-REV, were transfected into 293T cells, and the recombinant lentivirus-containing supernatant was harvested. The culture medium of EC2 cells was replaced with the virus-containing supernatant, and the culture plates were centrifuged at 2500 rpm for 30 min. After a 24-h incubation, the supernatant was replaced with DMEM/F12 containing 5% fetal bovine serum. Western blot was conducted as described previously [15,16].

Peptide cleavage assay. The fluorescence-quenching peptide substrate of NRDc (Nma-GGFLR RVGK (Dnp) -NH2) was synthesized by Peptide Institute, Osaka. TACE substrate III, corresponding to the cleavage site of TNF-α, was obtained from Calbiochem. All in vitro peptide cleavage assays were performed as described previously [15,16].

Results and discussion

NRDc and TACE synergistically enhances ectodomain shedding of TNF-α

To examine the effect of NRDc on ectodomain shedding of TNF-α, the transmembrane precursor of human TNF-α was transiently introduced into COS7 cells with NRDc and/or TACE. The amount of the soluble form of TNF-α (sTNF-α) released in the conditioned medium was detected by ELISA. As expected, TACE expression in COS7 cells significantly enhanced the release of TNF-α into the conditioned medium. Addition of NRDc expression dramatically and time-dependently increased the release of TNF-α, although sole expression of NRDc had negligible effects (Fig. 1A).

We also examined the effect of an enzymatically inactive mutant of NRDc (Glu235 to Ala; E > A) [15] on ectodomain shedding of TNF-α. The precursor of TNF-α, a type II membrane protein, was N-terminally tagged with FLAG so that both the transmembrane precursor (tmTNF-α, 26 kDa and the N-terminal cytomembrane remnant of TNF-α (cTNF-α, 9 kDa) could be detected by anti-FLAG antibody in Western blotting (Fig. 1C). In co-expression experiments, similar to the wild-type, the mutant NRDc and TACE coordinately enhanced the production of sTNF-α (Fig. 1B) and cTNF-α (Fig. 1C), indicating that the metalloendopeptidase activity of NRDc is not required for the enhancement of TACE-induced ectodomain shedding of TNF-α. We have recently reported that the inactive NRDc, as well as wild-type NRDc, enhances TACE-induced HB-EGF shedding [15] and α-cleavage of APP [16], suggesting that ectodomain shedding of TNF-α is enhanced by NRDc through a similar molecular mechanism.

Enhancement of TACE-induced peptide cleavage by NRDc

Recombinant NRDc protein was produced in a baculovirus–insect cell system as described previously [15]. The recombinant form of enzymatically inactive NRDc was similarly produced and its purity was confirmed by Coomassie blue staining (Fig. 2A). A cleavage assay using a fluorescence-quenching peptide substrate containing a dibasic site demonstrated that the mutant NRDc (E > A) completely lost its enzymatic activity (Fig. 2B). NRDc

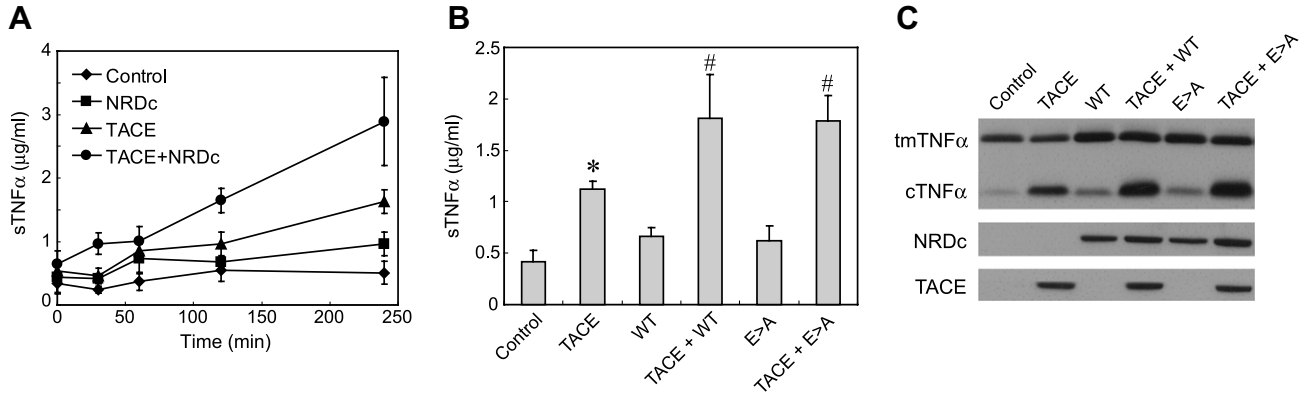

Fig. 1. NRDc and TACE coordinately enhance ectodomain shedding of TNF-α. (A) The expression vector encoding TNF-α was co-transfected into COS7 cells with either a control vector, expression vector for NRDc, and/or TACE as indicated. The culture medium was changed to DMEM with 0.1% BSA 24-h post-transfection, and the conditioned medium was collected at the indicated time point after the change. The amount of TNF-α secreted in the conditioned medium was measured by ELISA (PeproTech, London, UK) following the manufacturer's instructions. (B,C) The expression vector encoding TNF-α, N-terminally tagged with FLAG, was co-transfected into COS7 cells with either a control vector (Control), expression vector for NRDc (WT), enzymatically inactive mutant of NRDc (E > A), and/or TACE. Both NRDcs are tagged with V5 at the C-terminus. The culture medium of transfected cells was changed to DMEM with 0.1% BSA 24 h after transfection. The conditioned medium and total cell lysate were collected after an additional 4-h incubation. The amount of shed TNF-α in the conditioned medium was determined by ELISA (B). Results are means ± standard deviation (SD) for three independent experiments. The asterisk (*), representing $p < 0.05$ (Student's t test), indicates significant differences between control cells and TACE-transfected cells. The pound sign (#), representing $p < 0.01$, indicates significant differences between TACE-transfected cells and TACE/NRDc-transfected cells. Western blot analyses with anti-FLAG (TNF-α), anti-V5 (NRDc), or anti-TACE antibodies were done for cell lysates (C). Anti-FLAG antibody detected the transmembrane precursor (tmTNF-α) and N-terminal cytosolic remnant of TNF-α (cTNF-α) in the cell lysates.

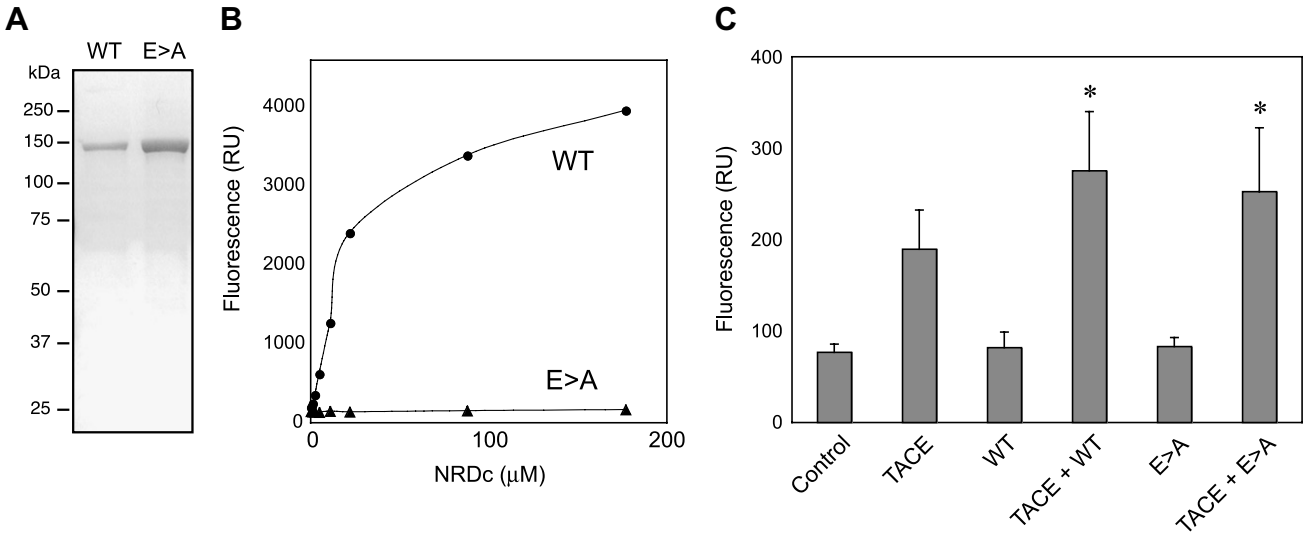

Fig. 2. NRDc potentiates catalytic activity of TACE in the peptide cleavage assay. (A) Recombinant NRDc (WT) and an enzymatically inactive mutant of NRDc (E > A) were synthesized using a baculovirus expression system. The purified proteins were separated by SDS–PAGE and subjected to Coomassie blue staining. (B) Enzymatic activity of the indicated concentration of NRDc (WT) and inactive mutant of NRDc (E > A) was measured by a cleavage assay with a fluorescence-quenching peptide substrate for NRDc (Nma-GGFLR RVGK (Dnp)-NH2). Note that the inactive mutant of NRDc lost all activity. (C) A fluorescence-quenching peptide substrate corresponding to the cleavage site of TNF-α (250 μM) was incubated with recombinant TACE (5 μg/ml), NRDc (WT: 25 μg/ml), or enzymatically inactive NRDc (E > A: 25 μg/ml) as indicated. After 15 h of incubation at 37 °C, fluorescence intensity (340/430) was measured. Data represent means ± SD for three independent experiments. The asterisk (*), $p < 0.01$ (Student's t test).

directly binds to TACE [15]. Peptide cleavage assays *in vitro* using peptides corresponding to the cleavage sites of HB-EGF and APP have demonstrated that the catalytic activity of TACE is activated by NRDc [15,16]. To determine whether the direct binding of NRDc to TACE is sufficient for TACE's activation in the cleavage of TNF-α, we performed an *in vitro* cleavage assay using an internally quenched fluorogenic peptide substrate corresponding to the cleavage site of TNF-α. While TACE increased the amount of fluorescence detected, addition of NRDc to TACE significantly strengthened the fluorescence signal, indicating an enhancement of the cleavage by NRDc (Fig. 2C). Similar to the results obtained in the cell-based shedding assay, the enzymatically inactive mutant of NRDc also enhanced TACE-induced TNF-α peptide cleavage. These results indicate that NRDc markedly activated the catalytic activity of TACE via direct binding, independent of the metalloendopeptidase activity of NRDc.

Co-existence of NRDc overcomes the inhibitory effect of NaCl on TACE activity

TACE activity is regulated by salt concentrations: it is much less active in the presence of physiological concentrations of salt [18]. Therefore, all previous studies with recombinant TACE have been performed with little or no salt present. To examine the effect of salt on NRDc's ability to potentiate TACE activity, we measured the cleavage of a synthetic peptide substrate containing the cleavage site of TNF-α by TACE in the absence or presence of NRDc. While NaCl dose-dependently inhibited the cleavage by TACE, co-incubation of NRDc completely reversed this inhibitory effect (Fig. 3). This finding suggests that full activation of TACE under physiological conditions is regulated by NRDc.

NRDc enhances TNF-α shedding through activation of not only TACE but ADAM10

TACE was originally identified as a sheddase for TNF-α [6,7]. While TACE-deficient cells showed a marked reduction in TNF-α release, a significant amount of soluble TNF-α is released into the medium by those cells (Fig. 4A and B) [6,19]. These results indicate the existence of other cleaving enzymes responsible for TNF-α shedding. To determine if TACE is essential for the ability of NRDc to enhance ectodomain shedding, the effect of NRDc on TNF-α shedding was

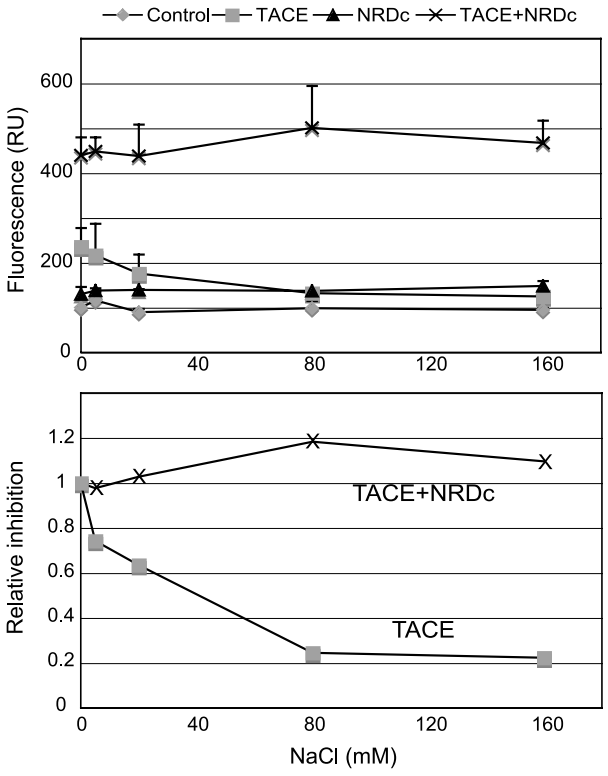

Fig. 3. Co-existence of NRDc overcomes the inhibitory effect of NaCl on TACE activity. A fluorescence-quenching peptide substrate corresponding to the cleavage site of TNF-α (10 μM) was incubated with either buffer (control), recombinant TACE (0.5 μg/ml), NRDc (30 μg/ml) or TACE (0.5 μg/ml) in concert with NRDc (30 μg/ml) in the presence of the indicated concentration of NaCl. After 15 h of incubation at 37 °C, fluorescence intensity (340/460 nm) was measured. Data represent means ± SD for three independent experiments (upper panel). The relative inhibitory effect of NaCl over peptide cleavage in the absence of NaCl is shown (lower panel).

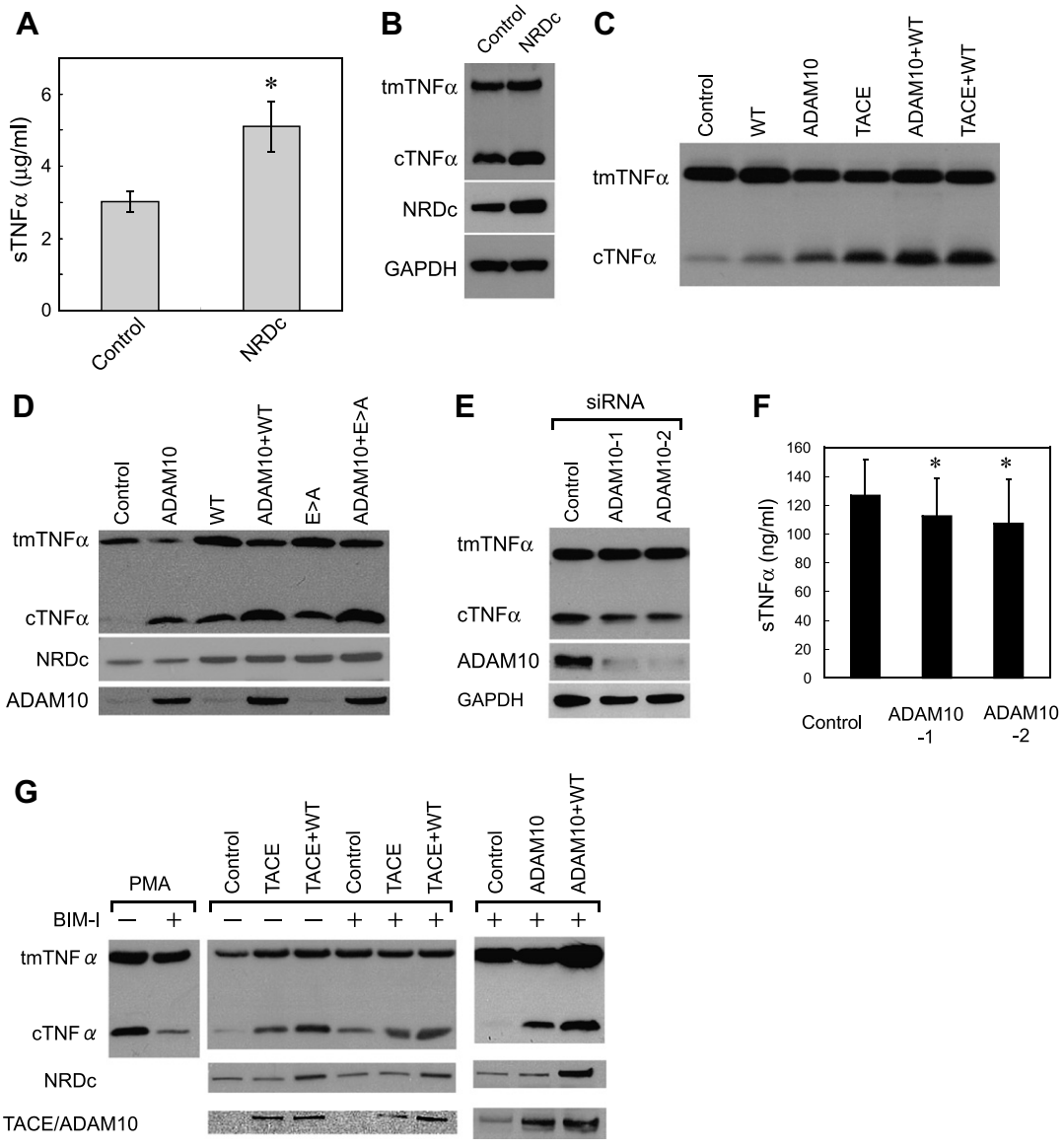

Fig. 4. NRDc potentiates ADAM10-induced TNF-α shedding. (A,B) Fibroblasts deficient in TACE activity (EC2) stably expressing FLAG-TNF-α were transfected with either empty (control) or recombinant lentivirus vector for NRDc (NRDc) as described in Materials and methods. The conditioned medium and total cell lysate were collected, and the amount of sTNF-α was measured by ELISA (A). Data represent means ± SD for five independent experiments. The asterisk (*), $p < 0.01$ (Student's t test). Total cell lysate was separated by SDS–PAGE, followed by Western blotting with either anti-FLAG (tmTNF-α and cTNF-α), anti-NRDc (#23), or anti-GAPDH antibody (B). (C,D) FLAG-TNF-α was co-transfected into COS7 cells with combinations of expression vectors as indicated. Total cell lysate was collected and analyzed as described in the legend of Fig. 1C. (E,F) FLAG-TNF-α was co-transfected into COS7 cells with either two different siRNAs against ADAM10 (ADAM10-1, ADAM10-2) or with non-silencing control siRNA (control). The culture medium was renewed after 24 h of incubation. After an additional 24 h of incubation, the conditioned medium and cell lysate were collected. Expression levels of tmTNF-α and cTNF-α (top panel), ADAM10 (middle panel), and GAPDH (bottom panel) in total cell lysate were determined by Western blot analysis (E), and the amount of sTNF-α in the conditioned medium was measured by ELISA (F). Data represent means ± standard error for three independent experiments. The asterisk (*), representing $p < 0.05$ (Student's t test). (G) FLAG-TNF-α was co-transfected into COS7 cells with combinations of expression vectors as indicated. The culture medium was renewed 20 h after transfection. Total cell lysate were collected after an additional 4-h incubation in the presence or absence of bisindolylmaleimide-I (BIM-I; 1 μM; Calbiochem), followed by Western blotting with antibodies as indicated.

tested in mouse fibroblasts deficient in TACE activity (TACE−/−) [17]. TACE−/− cells were transfected with NRDc via a lenti virus-based vector, which resulted in an increase of NRDc protein expression by 1.7 times compared to the endogenous level (Fig. 4B, second panel). Notably, overexpression of NRDc significantly increased the amount of sTNF-α in the conditioned medium (Fig. 4A) and cTNF-α in cell lysates (Fig. 4B) even in TACE activity-deficient cells. These results indicated that NRDc enhances the constitutive shedding of TNF-α at least partially in a TACE-independent manner.

We have previously demonstrated that NRDc enhances α-cleavage of APP not only by TACE but also by ADAM9 or ADAM10 [16]. To test whether ADAM10 is involved in NRDc-enhanced shedding of TNF-α, a co-expression experiment with ADAM10 and NRDc was performed. While the sole expression of ADAM10 had a weak effect on TNF-α shedding, cells transfected with ADAM10 and NRDc showed significantly enhanced shedding of TNF-α (Fig. 4C). The enzymatic activity of NRDc was not required for the enhancement of ADAM10-induced TNF-α shedding, similar to the case of TACE (Fig. 4D). To confirm the involvement of ADAM10 in ectodomain shedding of TNF-α, the effect of two distinct small interfering RNA duplexes (siRNA) against ADAM10 on constitutive TNF-α shedding was examined in COS7 cells. An approximately 90% reduction in the amount of endogenous ADAM10 protein was accompanied with a weak but significant reduction in levels of

cTNF-α (Fig. 4E) and sTNF-α (Fig. 4F). These results suggested that NRDc enhances TNF-α shedding through activation of not only TACE but other ADAMs, such as ADAM10 at least in COS7 cells. Several substrates, such as amyloid precursor protein [9,20], CD44 [21], and Notch ligands [22,23], have been reported to be shed by both TACE and ADAM10. The molecular mechanism by which the substrate specificity of ADAM proteases is determined has been poorly understood. We demonstrate here that NRDc dramatically enhanced ADAM10-induced TNF-α shedding, although sole expression of ADAM10 had little effect on the cleavage. These results suggest that the level of NRDc expression can modulate the substrate specificity of ADAM proteases.

Activation of protein kinase C is not required for the effect of NRDc on TNF-α shedding

To exclude the possibility that NRDc indirectly activates ADAM proteases through protein kinase C (PKC), we have examined the effect of bisindolylmaleimide-I (BIM-I), an inhibitor of PKC, on the process of TNF-α shedding. While a pre-treatment of BIM-I efficiently blocked PMA-activated TNF-α shedding (Fig. 4G, lanes 1 and 2), the inhibitor had no effect on the increase of TNF-α shedding by exogenous expression of TACE or ADAM10. Furthermore, NRDc enhanced the TACE- or ADAM10-induced shedding in the presence of BIM-I, indicating that PKC activation is not required for the enhancement of TNF-α shedding by NRDc (Fig. 4G). These results support the notion that NRDc directly enhances the catalytic activity of ADAM proteases.

Our findings reveal a novel mechanism of how ectodomain shedding of TNF-α is regulated. A full accounting of the role of NRDc in ectodomain shedding will require both genetic gain-of-function and loss-of-function study in mice. Future knowledge of NRDc-ADAM proteases interaction might lead to new anti-inflammatory therapies.

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