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Naofen, a novel WD40-repeat protein, mediates spontaneous and tumor necrosis factor-induced apoptosis

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ABSTRACT

Naofen has recently been identified from the rat brain/spinal cord cDNA library as a substance reactive against an anti-shigatoxin (Stx)-2 antibody. Naofen mRNA is composed of 4620 nucleotides and encodes 1170 amino acids. Naofen contains four WD-repeat domains in its N-terminus and is ubiquitously distributed in many tissues of the rat. Tumor necrosis factor (TNF)-α enhanced the expression of naofen mRNA in HEK293 cells in a dose-dependent manner. Furthermore, naofen siRNA, which predominantly knocked down the expression of naofen mRNA, significantly reduced both TNF-α-induced caspase-3 activation and apoptosis in HEK293 cells. Overexpression of naofen in HEK293 cells (FLAG-NF) spontaneously induced caspase -3 activation and apoptosis, and showed extremely high susceptibility to TNF-α-induced apoptosis. These results indicated that naofen may function as a novel modulator activating caspase-3, and promoting TNF-α-stimulated apoptosis.

1. Introduction

WD40-repeat domain is a well-conserved domain in eukaryotes, consisting of two signature sites: a poorly conserved site A containing a glycine–histidine (GH) dipeptide, and a well-conserved site B containing a tryptophan–aspartic acid (WD) dipeptide [1,2]. Although WD40 repeats are separated by approximately 40 amino acids, the distance and the actual sequence of individual repeats are highly variable. The well-known WD40-repeat protein is the Gβ-subunit of heterotrimeric GTP-binding protein, which forms characteristic propeller-like structures [3,4]. WD40 repeats containing proteins play a variety of roles, including intracellular signaling, RNA processing and degradation, gene expression, vesicular traffic and fusion, cytoskeletal assembly and cell cycle (reviewed in Ref. [5]).

Apoptotic protease activating factor (Apaf)-1, a member of the WD40 family, is a crucial component of the mitochondrial apoptosis pathway. When cytochrome *c* binds, Apaf-1 becomes activated in the presence of dATP, resulting in the activation of caspase-9 [6]. Recently, Saeki et al. reported that another WD40-repeat protein, Monad, promoted apoptosis induced by TNF-α [7]; therefore, it is possible that some WD40-repeat proteins are involved in the apoptotic process. Naofen, a novel WD40-repeat protein, has been cloned from the brain/spinal cord cDNA library of rats using an antibody against shigatoxin type (Stx)-2. In the present study, we demonstrated that naofen may function as a novel modulator activating caspase-3, and promoting TNF-α-stimulated apoptosis.

2. Materials and methods

2.1. Materials

Trizol reagent, Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX were purchased from Invitrogen (Carlsbad, CA, USA); p3 × FLAG-CMV-14 vector, mouse anti-FLAG M2 antibody and cycloheximide (CHX) were from Sigma (St. Louis, MO, USA); pEGFP-C1 vector was from Clontech (Palo Alto, CA, USA); heat-inactivated fetal bovine serum, penicillin, streptomycin and trypsin–EDTA were from Gibco BRL (Grand Island, NY, USA); TaqMan® Gene Expression Assays for human naofen (WDR35, ID: Hs00918285_m1), human GAPDH (ID: Hs99999905_m1), human β-actin

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(ID: Hs99999903_m1), rat GAPDH (ID: Rn99999916_s1) and rat β-actin (ID: Rn00667869_m1) were from Applied Biosystems (Foster City, CA, USA). Anti-naofen peptide (anti-NF, amino acids 1104–1120) antibody was designed, produced and purified by the Peptide Institute (Osaka, Japan); goat anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-procaspase-3 antibody and anti-cleaved caspase-3 antibody were from Cell Signaling (Danvers, MA, USA).

2.2. Rat brain/spinal cord cDNA library construction and naofen cloning

Total RNA was extracted from the brain/spinal cord of adult Wistar rats using Trizol reagent, and Poly(A)$^+$ RNA was purified with Oligotex-dT30 super (Takara, Japan). cDNA was synthesized from Poly(A)$^+$ RNA and double-stranded cDNA was ligated into ZAP express vector using the ZAP express cDNA® synthesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. About 1×10^6 plaques were screened using the purified rabbit polyclonal anti-Stx-2 antibody. After re-screening, positive clones (phage DNA) were converted to phagmid by in vivo excision using Exassit Helper phage. Phagmide DNA was isolated using the Wizard® Plus SV mini-preps (Promega, Madison, WI, USA) and their sequences were determined with T7 or T3 primer and BigDye terminator 3.1 using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

2.3. Quantitative real-time PCR (qPCR)

Total RNA (1 μg) extracted from rat tissues was reverse transcribed in the presence of random hexamers and oligo(dT)$_{20}$ primer using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). qPCR was performed with an ABI Step-one plus real-time PCR system (Applied Biosystems). Two microliters of cDNA was amplified using 1 μl TaqMan Gene Expression Assay and 10 μl TaqMan Gene Expression Master Mix in 20 μl reaction. The primers and probe for rat naofen were 5′-CGATTTCTGCATTTTGGCCACAA-3′ (forward primer), 5′-TCCAAGGGTGTGCCAATAGAATT-3′ (reverse primer) and 5′-CAAACTGAGGGTGATTTT-3′ (TaqMan MGB probe). The cycle threshold numbers (C_T) at which amplification entered the exponential phase were used as indicators of the relative amount of initial target RNA in each sample. Results are presented as ratios of naofen mRNA normalized to the internal control (GAPDH or β-actin) according to the $\Delta\Delta C_T$ method, as previously reported [8].

2.4. Immunoblotting assay

Tissues extirpated from rats were rinsed twice with ice-cold phosphate-buffered saline (PBS) and homogenized in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 10% [vol/vol] glycerol) containing a mixture of protease inhibitors (Complete™ tables; Roche Applied Sciences, Mannheim, Germany) by 10 strokes with a Dounce homogenizer. The homogenate was centrifuged for 30 min at 4 °C with 20,000*g* and the protein concentration of samples was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The same amount of sample was mixed with Laemmli denatured buffer, separated by SDS–PAGE and transferred to PVDF membranes (Immobilon-P, Millipore, USA). Blots were detected with anti-NF peptide antibody (1:200) or anti-GAPDH (1:1000), followed by peroxidase-conjugated anti-rabbit or rabbit anti-goat IgG (1:5000; Zymed Laboratories, San Francisco, CA, USA). Proteins were visualized using ECL Western blotting Reagent (GE Healthcare, Buckinghamshire, UK).

2.5. Cell lines

Human embryonic kidney (HEK) 293 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (complete medium) with 100 units/ml penicillin and 100 μg/ml streptomycin. For evaluating the effect of TNF-α (R&D Systems, MN, USA) in naofen expression, 3×10^5 HEK293 cells were seeded in a 6-well culture plate and treated with TNF-α (2.5–10 ng/ml) and CHX (0.5 μg/ml) for 24 h. CHX was used to block the NF-κB-mediated survival pathway induced by TNF-α [9]. Total RNA was extracted from cells using Trizol reagent, and relative naofen mRNA expression was quantified using qPCR as described above.

2.6. siRNA transfection and naofen knockdown

Silencer® Select Pre-designed siRNAs for naofen (NF-siRNA, siRNA ID: s33264) and a control-siRNA (negative control#1 siRNA, catalog #: 4390843) were purchased from Ambion (Austin, TX, USA). HEK293 cells were transfected with NF-siRNA using Lipofectamine™ RNAiMAX according to the manufacturer's protocol. After 24 h of transfection, total RNA was extracted and knockdown of naofen expression was verified at the mRNA level using qPCR. In order to evaluate the effect of naofen on TNF-α-induced caspase-3 activation, after 24 h of NF-siRNA (5nM) transfection, TNF-α (10 ng/ml) and CHX (0.5 μg/ml) were added to HEK293 cells and incubated for another 24 h. Caspase-3 activation was measured.

2.7. Estimation of caspase-3 activation

The activation of caspase-3 was determined using the Caspase Fluorometric assay kit (Medical & Biological Laboratories, Japan) according the manufacturer's instructions. In brief, cells were washed twice with cold PBS and lysed in the lysis buffer supplied with the kit. After incubation for 1 h on ice, cell lysates were centrifuged for 5 min at 4 °C with 1000*g*. Samples (50 μg protein) were mixed with 2× reaction buffer, followed by substrate addition: DEVD (Asp-Glu-Val-Asp)-AFC (7-amino-4-trifluoromethyl coumarin). After incubation for 2 h at 37 °C, free AFC cleaved by caspase from the substrate was quantified by Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) with excitation/emission (Ex/Em) = 400/505 nm.

2.8. Construction and establishment of naofen stable overexpressed cells

The entire coding region of rat naofen (NF) was amplified by PCR and inserted into the Not I-EcoR V restriction sites of p3 × FLAG-CMV-14 plasmid (pFLAG) or Xho I-Sac II sites of pEGFP-C1 vector (pGFP) to construct naofen-expressing plasmid: pFLAG-NF and pGFP-NF. Construction was further confirmed by sequencing. HEK293 cells were transfected with pFLAG-NF plasmid or its control (pFLAG) using Lipofectamine 2000, as suggested by the manufacturer. After 24 h of transfection, cells were selected with 500 μg/ml G-418 (Gibco BRL) at least for one week. Resistant clones were maintained in medium containing 100 μg/ml G-418. Cells were lysed in lysis buffer and the same amount of sample was separated by SDS- PAGE. Immunoblotting was carried out for naofen expression as above described with mouse anti-FLAG M2 antibody (1:2000) and anti-NF peptide antibody (1:200). In some experiments, FLAG-NF cells or its control (FLAG) cells were transfected with NF-siRNA. After 24 h of transfection, TNF-α and CHX were added and incubated for another 24 h. Expressions of FLAG-tagged naofen, caspase-3 activation and annexin V staining were examined in these cells.

2.9. Annexin V stain

In order to confirm whether overexpression of naofen definitely promotes TNF-α-induced apoptosis, both FLAG and FLAG-NF cells with/without NF-siRNA transfection were treated with TNF-α for 24 h, and then stained with an Annexin V apoptosis detection kit I (BD Biosciences, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were treated with trypsin–EDTA, and collected by centrifuge. After washing twice with cold PBS, 1×10^5 cells were resuspended in 100 μl binding buffer (supplied with the kit) and mixed with 5 μl annexin V-FITC and 5 μl propidium iodide (PI), followed by incubation for 15 min at room temperature in the dark. After adding 400 μl binding buffer, staining was quantified using a FACSCalibur flow cytometry system (BD Biosciences). For each experiment, 5000 events were counted, and data were obtained from three independent experiments.

2.10. Statistics

Results are expressed as means ± SEM and were analyzed for statistical significance with Kruskal–Wallis one-way ANOVA. $P < 0.05$ was considered to indicate significant differences.

3. Results

3.1. Identification of Naofen

While screening the rat brain/spinal cord cDNA library using anti-Stx-2 antibody, we found novel uncharacterized cDNA, designated as naofen (GenBank No. EF613262, NM_001099340). Naofen mRNA is composed of 4194 nucleotides and encodes a protein of 1170 amino acids with a calculated molecular mass of about 133 kD (GenBank No. NP_001092810). Naofen protein contains four WD40-repeat domains (residues 5–42, 60–99, 104–143 and 147–184) in its N-terminus, and shows 98.9% homology at the amino acid level with mice (GenBank No. BAL26663) and 92.7% with humans (GenBank No. NP_065830), respectively.

3.2. Tissue distribution of naofen mRNA

Distribution of naofen mRNA was examined in various rat tissues using qPCR. The naofen transcript was expressed in all tissues examined, and the expression amounts differed greatly among the tissues. As shown in Fig. 1A, naofen transcription was most abundant in the testis, and then in the brain, and was also present at lower levels in numerous other tissues, including the kidney, liver and spleen when normalized with GAPDH as an internal control. A similar pattern of naofen mRNA expression was also confirmed using β-actin as an internal control (data not shown).

To determine whether naofen is exactly expressed at the protein level, we performed immunoblotting analysis using anti-NF peptide antibody. Naofen, as a protein of approximately 130 kDa, was expressed in the testis at a high level and in the liver at a lower level (Fig. 1B), which is consistent with naofen mRNA expression.

3.3. Effect of TNF-α in naofen expression and function of naofen in TNF-α-induced caspase-3 activation

Because anti-rat NF peptide antibody did not respond to human naofen, we did not reliably estimate the increase of naofen protein induced by TNF-α in HEK293 cells; however, TNF-α greatly increased naofen mRNA expression in a dose-dependent manner in HEK293 cells (Fig. 2A). Expression of naofen mRNA was increased within 24 h, and then diminished towards the control level for a longer incubation period (Fig. 2B). In order to study the effect of naofen on TNF-α-induced caspase-3 activation, NF-siRNA, which

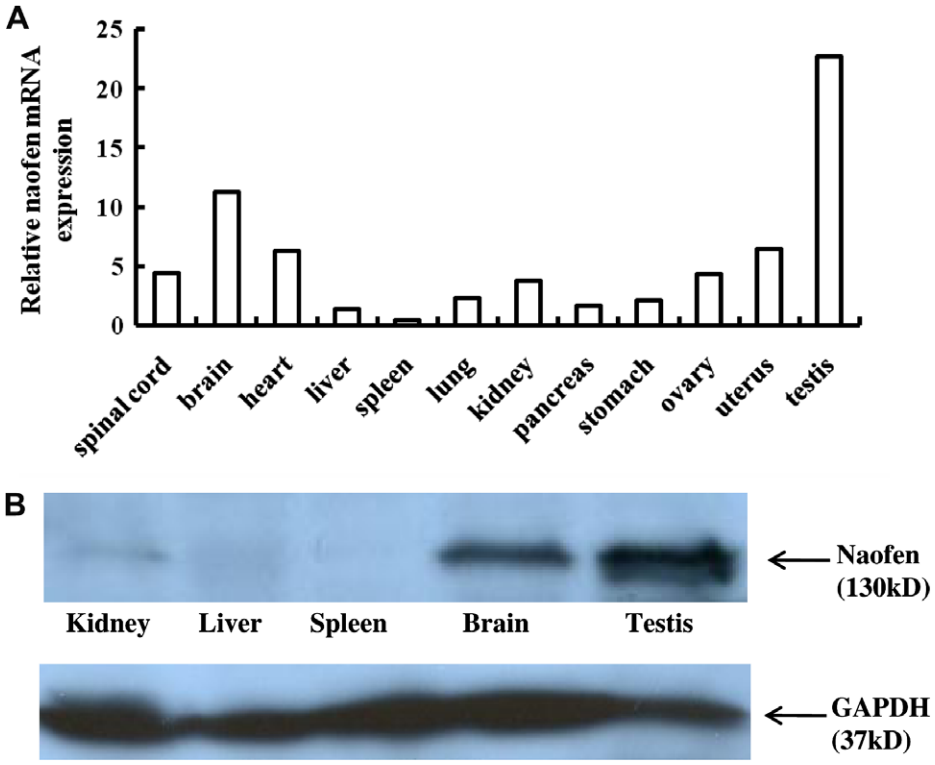

Fig. 1. (A) Quantitative real-time PCR (qPCR) analysis of naofen in rat tissues. qPCR was performed using TaqMan Gene Expression Assay for naofen, as described in Materials and methods. Results are presented as ratios of naofen mRNA normalized to internal GAPDH. (B) Immunoblotting assay for naofen. Rat tissue lysates were analyzed for naofen expression with anti-naofen peptide antibody. GAPDH was used as an internal control.

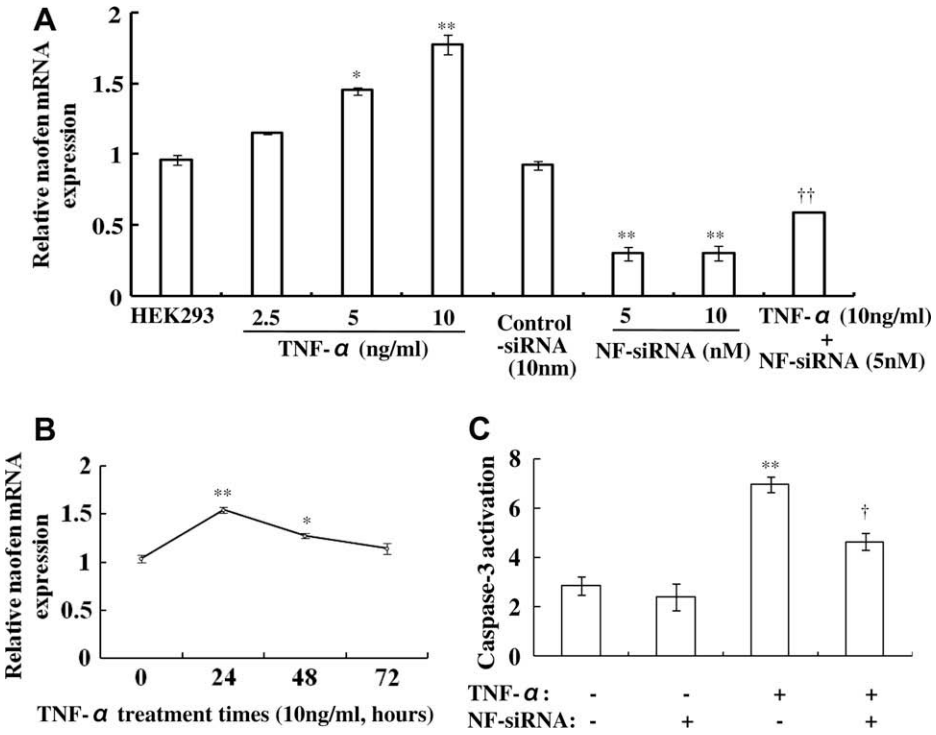

Fig. 2. (A) HEK293 cells were treated with either a combination of TNF-α and CHX (0.5 μg/ml) or NF-siRNA for 24 h. Total RNA was extracted with Trizol reagent and the relative mRNA expression of naofen was quantified using qPCR. GAPDH was used as an internal control. In order to examine the effect of NF-siRNA in naofen expression induced by TNF-α, HEK293 cells were transfected with NF-siRNA (5nM) transfection using Lipofectamine RNAiMAX. After 24 h transfection, TNF-α (10 ng/ml) and CHX (0.5 μg/ml) were added and incubated for another 24 h. Naofen mRNA was measured with qPCR. (B) Time course of TNF-α (10 ng/ml) on naofen mRNA expression. (C) Effect of naofen knockdown in TNF-induced caspase-3 activation. HEK293 cells were treated as described above. Cells were lysed in lysis buffer and caspase-3 was activated. Data are shown as means ± SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$, vs. HEK293; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$vs. without NF-siRNA.

predominantly diminished the expression of naofen mRNA (Fig. 2A), was employed. As expected, NF-siRNA significantly inhibited TNF-α-induced caspase-3 activation (Fig. 2C).

3.4. Effect of naofen overexpression on TNF-α-induced caspase-3 activation

Compared to FLAG cells, FLAG-NF cells, in which the expression of FLAG-tagged naofen was examined by immunoblotting using anti-FLAG antibody and anti-NF peptide antibody (data not shown), showed an obvious increase of caspase-3 activation. TNF-α treatment significantly enhanced caspase-3 activation in FLAG cells. Furthermore, TNF-α-induced caspase-3 activation in FLAG-NF cells was greatly increased compared to in FLAG cells (Fig. 3A). NF-siRNA was used to verify the effect of naofen in caspase-3 activation. NF-siRNA significantly diminished caspase-3 activities in either FLAG-NF cells or both TNF-α treated FLAG and FLAG-NF cells. Likewise, similar results were obtained in HEK293 cells transfected with pGFP-NF plasmid (data not shown). In addition, naofen promoted the activation of caspase-3 in these cells, as demonstrated by the processing of procaspase-3 and the cleavage of caspase-3 (Fig. 3B).

3.5. Apoptosis determination

As shown in Fig. 3C, compared to FLAG cells, FLAG-NF cells showed a significant increase of apoptotic cell populations (annexin V-positive and PI-negative). Furthermore, TNF-α significantly elevated apoptotic cell populations in FLAG cells, and showed a marked effect on FLAG-NF cells. In FLAG cells, NF-siRNA significantly diminished TNF-α-induced apoptotic cell populations, but not without TNF-α treatment; however, NF-siRNA significantly diminished apoptotic cell populations in FLAG-NF cells either with or without TNF-α treatment.

4. Discussion

To date, a large number of WD40-repeat proteins have been identified, with most containing four to eight WD40 repeats and having important roles in a variety of cellular functions from cell proliferation and different cell signal transduction pathways, to cell apoptosis. Among WD40-repeat proteins, Apaf-1 is composed of 13 WD40 repeats, which usually function as an autoregulatory region to keep Apaf-1 in an inactive state [10]; however, naofen, which contained four WD40 repeats in its N-terminus, was different from Apaf-1. Naofen was also unlike Monad, which contains two WD40-repeat domains (from our research, Monad should contain four WD40-repeat domains, not two), but the function of WD40 repeats in naofen remains to be elucidated in the future. Since STX-2 may release TNF-α, and both Stx-2 and TNF-α may induce apoptosis in many cells [11–15], we investigated whether naofen may participate in apoptosis induced by TNF-α. TNF-α significantly elevated the expression of naofen mRNA in a dose-dependent fashion in HEK293 cells, and simultaneously increased caspase-3 activation. Furthermore, NF-siRNA, which predominantly inhibited the expression of naofen mRNA, significantly inhibited TNF-α-induced caspase-3 activation in HEK293 cells, indicating that naofen may be involved in TNF-α-induced caspase-3 activation.

In order to clarify the precise role of naofen, we have studied the effect of naofen overexpression in a TNF-α-induced apoptotic process. Cloned FLAG-NF cells elicited an obviously greater increase of caspase-3 activation and higher frequency of spontaneous apoptosis than FLAG cells. The action of naofen-induced spontaneous

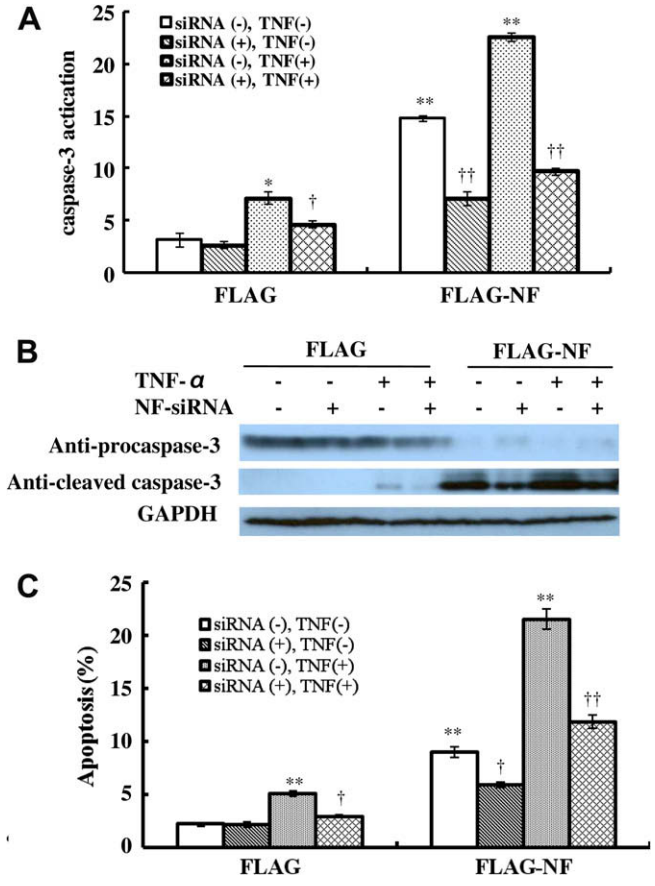

Fig. 3. HEK293 cells stably overexpressing naofen (FLAG-NF) and its control (FLAG) were transfected with NF-siRNA. After 24 h transfection, TNF-α (10 ng/ml) was added and incubated for another 24 h. (A) Relative caspase-3 activation was examined. (B) Naofen activates spontaneous or TNF-α-induced procaspase-3 processing. Cell lysates were analyzed by immunoblotting using anti-procaspase-3 or cleaved anti-caspase-3 antibody. One representative result from three independent experiments is shown. (C) Naofen potentiates spontaneous or TNF-α-induced apoptosis. Apoptosis was performed using flow cytometry analysis with annexin V-FITC and PI staining. The results are expressed as average values with SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. FLAG; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ vs. without NF-siRNA.

apoptosis was different from Monad, which only promoted TNF-α-induced apoptosis, and did not affect spontaneous apoptosis [7]. Moreover, FLAG-NF cells showed markedly higher susceptibility to TNF-α-induced apoptosis, suggesting that increased expression of naofen promoted cell apoptosis induced by TNF-α. Thus, to control TNF-α-induced apoptosis in such pathological conditions, it may be important to regulate naofen production. If increased naofen expression may be involved in TNF-α-induced apoptosis, the knockdown of naofen should inhibit cell apoptosis induced by TNF-α. As expected, NF-siRNA blocked TNF-α-induced caspase-3 activation and apoptosis in FLAG cells and FLAG-NF cells; however, NF-siRNA did not affect caspase-3 activation in HEK293 cells and apoptosis in FLAG cells. These results may be explained because, under normal conditions, there is a rather loser expression of naofen, and knockdown of naofen did not affect the lower activation of caspase-3 and apoptosis in HEK293. To exclude the possibility that the effect of naofen on caspase-3 activation may be due to the fusion of FLAG and naofen, we further studied the effect of another naofen fusion protein (GFP-tagged naofen) on caspase-3 activation and obtained similar results. These results indicated that caspase activation could be attributed to increased levels of naofen, not to the fusion of naofen and FLAG.

In the normal tissues examined, naofen was most highly expressed in the testis and poorly in the spleen, but even in poor naofen-expressing organs, the pathological status influenced/enhanced the amount of naofen. As shown previously in deoxycorticosterone acetate-salt hypertensive rats, aortic endothelial cells elicited an increase in the expression of naofen, declining the GCHI expression, resulting in reduced relaxation mediated by nitric oxide [16]. Although further experimental evidence is necessary, naofen was most markedly expressed in the testis, indicating that naofen may be involved in the apoptosis of germ cells during spermatogenesis. In conclusion, naofen may function as a novel modulator activating caspase-3, and promote TNF-α-stimulated apoptosis.

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