Nuclear factor erythroid 2-related factor 2 (NRF2) is a negative regulator of tissue plasminogen activator synthesis in cultured human vascular endothelial EA.hy926 cells

Tsutomu Takahashi1,*, Tsuyoshi Nakano1,*, Go Katoh1, Yo Shinoda1, Chika Yamamoto2, Eiko Yoshida3, Toshiyuki Kaji3 and Yasuyuki Fujiwara1

1Department of Environmental Health, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji 192-0392, Japan
2Department of Environmental Health, Faculty of Pharmaceutical Sciences, Toho University, Funabashi 274-8510, Japan
3Department of Environmental Health, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda 278-8510, Japan

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ABSTRACT — Blood coagulation and the fibrinolytic system contribute to vascular lesions. Fibrinolysis in normal circulating blood strongly depends on the balance between tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) secreted from vascular endothelial cells; however, the mechanisms by which endothelial fibrinolysis is regulated remain to be fully understood. In the present study, human vascular endothelial EA.hy926 cells were transfected with small interfering RNA for nuclear factor erythroid 2-related factor 2 (NRF2) and the expression of t-PA and PAI-1 and fibrinolytic activity in the conditioned medium were examined. EA.hy926 cells were also treated with sulforaphane, an NRF2 activator, and fibrinolytic activity was examined to confirm the NRF2 signaling pathway’s effect. Enhanced fibrinolytic activity in the conditioned medium was observed in association with increased expression and secretion levels of t-PA in NRF2 knockdown EA.hy926 cells. However, sulforaphane inhibited fibrinolytic activity and t-PA synthesis in EA.hy926 cells without any cell damage. The expression level of PAI-1 did not change in either NRF2 knockdown or sulforaphane treated cells. These results suggest that transcription factor NRF2 may play a role in down-regulating endothelial t-PA synthesis and fibrinolytic activity.

Key words: NRF2, Plasminogen activator, Fibrinolysis, Human endothelial cell line EA.hy926, Vascular

INTRODUCTION

Fibrinolysis is the process by which plasmin degrades fibrin in vascular tissue. Plasminogen is the precursor to plasmin and can be converted to plasmin in the blood by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) secreted from vascular endothelial cells (Levin and Loskutoff, 1982; Collen, 2001). Because u-PA lacks fibrin binding ability whereas t-PA activity increases markedly upon binding to fibrin (Hoylaerts et al., 1982; Rånbys, 1982; Zamarron et al., 1984), t-PA rather than u-PA is likely more important for fibrinolytic activity in the blood. Plasminogen activation is further controlled by plasminogen activator inhibitor type 1 (PAI-1), which inhibits t-PA’s effect (van Mourik et al., 1984; Gelehrter and Szynycz-Laszuk, 1986). Because vascular endothelial cells express and secrete not only t-PA but also PAI-1, endothelial fibrinolytic activity appears to strongly depend on the balance between t-PA and PAI-1. The activated protein kinase C and cyclic AMP (cAMP) are involved in stimulation and suppression, respectively, of endothelial t-PA and PAI-1 synthesis (Francis and Neely, 1989; Kooistra et al., 1991); however, the intracellular signaling pathways that are involved in the endothelial t-PA and PAI-1 synthesis are not yet completely understood.

t-PA and PAI-1 secretion from vascular endothelial cells is influenced by not only various endogenous fac-
tors including thrombin (Levin et al., 1984; Gelehrter and Sznycer-Laszuk, 1986), endothelin (Kaji et al., 1992a; Yamamoto et al., 1992, 1993a), cytokines (Schleef et al., 1988) and growth factors (Kaji et al., 1994; Yamamoto et al., 1994) but also heavy metals such as lead (Kaji et al., 1992b) and cadmium (Yamamoto et al., 1993b). Recently, we have also shown that copper diethyldithiocarbamate, which is a copper complex that exhibits interesting biological activities including activation of nuclear factor erythroid 2-related factor 2 (NRF2) (Fujie et al., 2016), suppresses the fibrinolytic activity of human coronary endothelial cells by down-regulating t-PA synthesis (Fujie et al., 2017). Although transcription factor NRF2 mainly regulates the gene expression of antioxidiant and phase II xenobiotic metabolizing enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) (Kensler et al., 2007), the role of NRF2 in regulating endothelial fibrinolytic activity remains unclear. Because cadmium and lead also induce NRF2 pathway activation in vascular endothelial cells (Shinkai and Kaji, 2012; Shinkai et al., 2016), we hypothesized that NRF2 pathway activation may affect the fibrinolytic system of vascular endothelial cells.

Although there is a report that NRF2 knockdown in mouse embryonic fibroblast stimulates the PAI-1 protein synthesis (Zagotta et al., 2013), investigation using vascular endothelial cells is necessary to evaluate the involvement of the NRF2 pathway in fibrinolytic activity in the blood. In the present study, we then investigated the effects of NRF2 knockdown and sulforaphane (an NRF2 activator) treatment on t-PA and PAI-1 synthesis in human vascular endothelial EA.hy926 cells in culture.

**MATERIALS AND METHODS**

**Cell culture**

A permanent human endothelial cell line, EA.hy926 cells (ATCC, Manassas, VA, USA) was cultured in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France; 10% FBS-DMEM) in a humidified 5% CO₂ atmosphere at 37°C.

**siRNA transfection**

Double-strand control interfering RNA (siRNA) and NRF2 siRNA (CAAACUGACAGAAGUUGACAAU- UAU) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA USA), respectively. EA.hy926 cells were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, double-strand siRNA solution (5 pmol: final siRNA used per well) was added to RNAiMAX transfection reagent and incubated for 10 min at room temperature to allow siRNA/RNAiMAX complex formation. After the incubation, the siRNA/RNAiMAX complex was added to a suspension of EA.hy926 cells (2.5 × 10⁶ cells/mL) and seeded in 400 µL aliquots per well in 24-well culture plates (5 × 10⁴ cells/cm²). After 24 hr incubation, EA.hy926 cells were further incubated for 24 hr in fresh 10% FBS-DMEM.

**Real-time RT-PCR analysis**

Total RNA extraction from cultured cells and subsequent real-time RT-PCR analysis were performed as described previously (Takahashi et al., 2018). Briefly, after incubation, the culture medium was removed and the cell layer was washed twice with cold Dulbecco’s phosphate-buffered saline (D-PBS, Wako Pure Chemical Industries) and 300 µL cold ISOGEN II reagent (Nippon Gene, Tokyo, Japan) was added to each culture well. Cells were collected by scraping and homogenized by pipetting. The RNA quality was ensured by spectrophotometric analysis (OD260/280) using the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). The reverse transcriptome was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) and GeneAmp PCR system 9700 (Thermo Fisher Scientific). Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) using 0.5 µM primers and LightCycler 96 (Roche, Tokyo, Japan). The thermal treatment was 95°C for 10 min and 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The PCR primers (Table 1) were purchased from Star Oligo Rikaken (Aichi, Japan). The fold change for each gene was assessed after normalization of the intensity value to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Determination of t-PA**

The conditioned medium of EA.hy926 cells transfected with siRNA or treated with sulforaphane (10 µM) (LKT Laboratories, St. Paul, MN, USA) in 24-well culture plates was used to determine t-PA using an enzymelinked immunosorbent assay kit (Assaypro LLC, St. Charles, MO, USA), after which the cell layer was analyzed for DNA content using fluorometry (Kissane and Robins, 1958) to normalize the t-PA content in the conditioned medium per µg DNA.

**Fibrin zymography**

Fibrin zymography was performed as previously...
reported (Yamamoto et al., 2005; Nsimba et al., 2013). The conditioned medium of EA.hy926 cells transfected with siRNA or treated with sulforaphane (5 and 10 µM) in 24-well culture plates was harvested and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on a 7.5% slab gel with a 4.5% stacking gel. The gel containing fibrin was prepared by mixing plasminogen-rich fibrinogen (1.5 mg/mL) from bovine plasma (Sigma-Aldrich) with thrombin (10 NIH U/mL) from human plasma (Sigma-Aldrich). The conditioned medium was incubated with 0.125 M Tris-HCl buffer solution containing 4% SDS, 20% glycerol and 0.002% bromophenol blue at 37°C for 1 hr under a non-reducing condition. After SDS-polyacrylamide gel electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 hr, incubated at 37°C for 24 hr in 0.1 M glycine-NaOH buffer solution (pH 8.3) and then stained with Coomasie brilliant blue. Finally, the gel was destained with 7.5% acetic acid to detect the lytic zones that indicate t-PA activity.

**Western blotting analysis**

EA.hy926 cells were seeded in 6-well culture plates at a density of 5 × 10⁴ cell/cm² and cultured for 24 hr in 10% FBS-DMEM. The medium was discarded and the cells were treated with sulforaphane (10 µM) at 37°C for 24 hr in fresh 10% FBS-DMEM. After treatment, the cell layers were washed twice with cold D-PBS, lysed with RIPA buffer [1 mM Tris-HCl (pH7.4), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM PMSF] and centrifuged at 15,000 × g at 4°C for 5 min. The supernatant was collected and the protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Merck KGaA, Darmstadt, Germany) and visualized using primary antibodies against NRF2 (Novus Biologicals, Littleton, CO, USA), β-actin (Wako Pure Chemical Industries) and horseradish peroxidase (HRP) -coupled secondary antibodies; anti-rabbit IgG-HRP antibody (Thermo Fisher Scientific) and anti-mouse IgG-HRP antibody (GE Healthcare Japan, Tokyo, Japan).

**Cell viability assay**

Twenty-four hours after treatment of sulforaphane (2, 5 and 10 µM), cell viability was measured using MTT assay as described previously (Yamada et al., 2018).

**Statistical analysis**

The data are expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance using Student’s t-test with Statcel3 (OMS, Tokyo, Japan) when possible. P values less than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

To examine the involvement of NRF2 in regulating t-PA and PAI-1 synthesis, EA.hy926 cells were transfected with NRF2 siRNA. In the transfected cells, NRF2 mRNA expression was much lower than that of the control siRNA transfected cells (Fig. 1A, left panel). Additionally, the expression levels of NRF2-target genes NQO1 and HO-1 were also significantly lower in cells transfected with NRF2 siRNA (Fig. 1A, middle and right panels). These results indicate that NRF2 transcription activity was effectively down-regulated in EA.hy926 cells transfected with NRF2 siRNA. However, both t-PA mRNA expression and protein secretion were significantly increased in NRF2 knockdown EA.hy926 cells (Figs. 1B and C). Figure 1D shows the fibrin zymography of the conditioned medium of EA.hy926 cells transfected with control or NRF2 siRNAs. Lower lytic zones, which indicate the fibrinolytic activity of free t-PA, were observed in broad bands. Therefore, NRF2 knockdown increased endothelial t-PA activity in the conditioned medium. These data suggest that NRF2 knockdown enhances endothelial fibrinolytic activity by inducing t-PA synthesis.

NRF2 signaling activity is repressed by Kelch-like ECH associated protein 1 (KEAP1) by facilitating NRF2...
polyubiquitination and subsequent proteasomal degradation. Sulforaphane, a naturally occurring NRF2 activator, binds to KEAP1 and disrupts the degradation process, which enables NRF2 accumulation and translocation into the nucleus (Suzuki et al., 2013). Because NRF2 knockdown selectively increased t-PA synthesis, we next examined whether NRF2 activator sulforaphane selectively inhibits t-PA synthesis in EA.hy926 cells. Figure 2A shows the NRF2 protein level in EA.hy926 cells treated with sulforaphane at 10 µM for 24 hr. Sulforaphane significantly increased the NRF2 protein level. As shown in Fig. 2B, NQO1 and HO-1 mRNA expression levels were also increased by sulforaphane treatment, which indicates that sulforaphane activated the NRF2 pathway in EA.hy926 cells. At that time, both t-PA mRNA expression and t-PA protein secretion were significantly decreased by sulforaphane treatment (Figs. 2C and D). As shown in Fig. 2E, sulforaphane also attenuated endothelial t-PA fibrinolytic activity in the conditioned medium. Additionally, the cell viability was not changed by sulforaphane at 10 µM and less (data not shown). These results indicated that NRF2 activator sulforaphane inhibits endothelial fibrinolytic activity in EA.hy926 cells by inhibiting t-PA synthesis without any cell damage.

Figure 3 shows the effects of NRF2 knockdown and sulforaphane on u-PA and PAI-1 mRNA expression in EA.hy926 cells. u-PA mRNA and PAI-1 mRNA expression levels were not changed in EA.hy926 cells trans-

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Fig. 1. Effect of NRF2 knockdown on the fibrinolytic activity of t-PA in EA.hy926 cells. Control siRNA or NRF2 siRNA transfected EA.hy926 cells were incubated at 37°C for 24 hr in fresh 10% FBS-DMEM. [A] The expression levels of NRF2, NQO1 and HO-1 mRNAs in EA.hy926 cells transfected with control or NRF2 siRNAs. Values are means ± S.D. of three samples. Significantly different from the corresponding control siRNA, *p < 0.05; **p < 0.01. [B] The expression level of t-PA mRNA in EA.hy926 cells transfected with control or NRF2 siRNAs. Values are mean ± S.D. of three samples. **Significantly different from the control siRNA, p < 0.01. [C] The secretion level of t-PA in the conditioned medium of EA.hy926 cells transfected with control or NRF2 siRNAs. Values are mean ± S.D. of four samples. *Significantly different from the control siRNA, p < 0.05. [D] Fibrin zymography of the conditioned medium of EA.hy926 cells transfected with control or NRF2 siRNAs.

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fected with NRF2 siRNAs compared with control siRNA (Fig. 3A). Additionally, sulforaphane did not affect u-PA mRNA and PAI-1 mRNA expression levels in the cells (Fig. 3B). These results indicate that NRF2 pathway activation selectively suppresses t-PA expression. Taken together, our data suggest that the transcription factor NRF2 may play a role in down-regulating endothelial t-PA synthesis and fibrinolytic activity.

It has been reported that the cAMP pathway is involved in the suppression of endothelial t-PA synthesis (Francis and Neely, 1989). Meanwhile, our previous experiments showed that cAMP, which down-regulates t-PA synthesis, was not involved in endothelin-1’s inhibitory effect on endothelial t-PA synthesis in human umbilical vein endothelial cells (Kaji et al., 1992a). Recently, we also reported that copper diethyldithiocarbamate down-regulates endothelial t-PA synthesis in human coronary endothelial cells but the cAMP pathway is not involved in the inhibitory effect (Fujie et al., 2017). Because copper diethyldithiocarbamate is an activator of NRF2 in vascular endothelial cells (Fujie et al., 2016), the compound likely activates the NRF2 pathway and then inhibits the t-PA synthesis in human coronary endothelial cells and EA.hy926 cells. Additionally, we previously demon-

![Image of Fig. 2](image_url)
Stratified that lead, a toxic heavy metal, disturbs endothelial fibrinolytic activity by down-regulating t-PA synthesis (Kaji et al., 1992b). Because the NRF2-KEAP1 system plays a role in protection against lead toxicity in vascular endothelial cells (Shinkai and Kaji, 2012), it is possible that lead inhibits endothelial t-PA synthesis (Kaji et al., 1992b) through NRF2 pathway activation. Thus, our data may contribute to the elucidation of the toxic mechanisms of fibrinolytic systems induced by lead and cadmium known to activate the NRF2 pathway.

In conclusion, the transcription factor NRF2 may be a selective mediator for regulating endothelial t-PA production. A previous report has shown that NRF2 knockdown in mouse embryonic fibroblast stimulates the PAI-1 protein synthesis (Zagotta et al., 2013), but there is no report that the NRF2 pathway is involved in the synthesis of t-PA and PAI-1 in vascular cells. Our finding is significant because, to our knowledge, this is the first report that the NRF2 pathway may be involved in fibrinolytic activity in the vascular system. However, because the mechanism by which NRF2 pathway down-regulates endothelial t-PA synthesis is unclear, further studies should be performed to clarify this.

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Conflict of interest---The authors declare that there is no conflict of interest.

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