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Stress-induced down-regulation of tumor-associated NADH oxidase during apoptosis in transformed cells

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Abstract Tumor-associated NADH oxidase (tNOX) is a growth-related protein expressed in transformed cells. tNOX knockdown using RNA interference leads to a significant reduction in HeLa cell proliferation and migration, indicating an important role for tNOX in growth regulation and the cancer phenotype. Here, we show that tNOX is down-regulated during apoptosis in HCT116 cells. Treatment with diverse stresses induced a dose- and time-dependent decrease in tNOX expression that was concurrent with apoptosis. Moreover, shRNA-mediated tNOX knockdown rendered cells susceptible to apoptosis, whereas re-expression of tNOX partially recovered cell proliferation. Our results indicate that tNOX is suppressed during apoptosis and demonstrate that tNOX down-regulation sensitizes cells to stress-induced growth reduction, suggesting that tNOX is required for transformed cell growth.

Keywords: Apoptosis; Capsaicin; Down-regulation; EGCg; Tumor-associated NADH oxidase (tNOX); UVC

1. Introduction

Tumor-associated NADH oxidase (tNOX) belongs to a family of growth-related NADH (or hydroquinone) oxidases [1,2]. tNOX cDNA has been cloned [3] and functional motifs of tNOX have been identified using site-directed mutagenesis. These structural elements include a quinine-binding site, an adenine-nucleotide-binding site, and a CXXXC cysteine pair important for its activity [4]. tNOX was originally identified as a plasma membrane protein in rat hepatoma [5], and has since been identified in numerous cancer cell lines [6–8]. It has also been found in sera of cancer patients [9,10]. Accumulating evidence supports an important role for tNOX in regulating cancer cell growth [8,11,12]. Moreover, the survival of transformed cells is inhibited by antisera raised against bacterially expressed tNOX protein, a cytotoxic effect that is associated with apoptosis; in contrast, non-transformed cells are unaffected [13]. A key role for tNOX in regulating cell growth is further supported by the observation that the growth rate of MEF cells from tNOX-overexpressing transgenic mice is approximately twofold greater than that of wild-type cells [14].

A recent report has further demonstrated that suppression of tNOX using antisense oligonucleotides decreased HeLa cell colony formation, whereas tNOX overexpression increased the invasiveness of non-cancerous MCF-10A cells, suggesting that tNOX increased the ability of cancer cells to acquire an aggressive phenotype [15]. Additionally, tNOX knockdown using RNA interference led to a significant reduction in HeLa cell proliferation and migration [16]. Thus, it appears that the level of tNOX is critical for transformed cell growth. Here, we demonstrate that tNOX expression is down-regulated during apoptosis.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antisera to tNOX were generated as described previously [13]. The anti-PARP, anti-phosphorylated p53, and anti-rabbit IgG antibodies were from Cell Signaling Technology Inc. (Beverly, MA); the anti-actin antibody was from Chemicon International Inc. (Tamekula, CA); the anti-tubulin antibody was from Abcam Inc. (Cambridge, MA). The anti-cytochrome *c* antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The FITC-conjugated anti-mouse secondary antibody and Mitotracker were from Molecular Probes Inc. (Eugene, OR, USA). Z-IETD-FMK and Z-LEHD-FMK were purchased from Imgenex Inc. (San Diego, CA). The anti-p53 and anti-mouse IgG antibodies, and other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO).

2.2. Cell culture and transfection

HCT116 (human colon cancer) cells were grown in McCoy's 5A medium with 10% FBS. A549 (human alveolar basal epithelial) and HEK 293 (human embryonic kidney epithelial) cells were grown in DMEM with 10% FBS. All media contained 100 U/mL penicillin and 50 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air, and medium was replaced every 2–3 days. The tNOX-shRNA construct and transfection method have been previously described [16].

2.3. Measurement of cell viability

Cells were seeded into 96-well plates at 8×10^3 cells/well and permitted to adhere overnight at 37 °C. After incubation, cell viability was determined using the MTT assay [17].

2.4. Trypan blue exclusion assay

Cells were seeded at a density of 10^4 cells/dish and, after culturing for the indicated time periods, cell numbers were analyzed using the trypan blue exclusion assay as described previously [16].

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2.5. Apoptosis assay

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit as previously described [17]. Cells were also stained with propidium iodide (PI) to detect the sub-G1 population.

2.6. Western-blot analysis

Cell-extract preparation and Western-blot analyses were performed as reported previously [17].

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Primer sets used for PCR were as follows: tNOX sense, 5'-TGGCTGTGGGTCTACGAAT, tNOX antisense, 5'-GGAGTCTG-CCTGTGTCCTTC; β-actin sense, 5'-GATGATGATATCGCCG-CGCT, and β-actin antisense, 5'-TGGGTCATCTTCTCGCGGTT. Total RNA isolation and RT-PCR reactions were performed as described previously [17].

2.8. Confocal microscopy

Cells were grown overnight on coverslips, treated with 100 μM EGCg for 24 h, and incubated with Mitotracker (Molecular Probes Inc., Eugene, OR) for 10 min. Cells were then washed, fixed in 2% paraformaldehyde, washed with PBS, and permeabilized with 0.1% Triton X-100. Coverslips were stained with anti-cytochrome *c* antibody, and immunofluorescence images were obtained using a confocal microscope (TCS SP5, Leica Microsystems USA, Bannockburn, IL).

2.9. NADH oxidase assay

NADH oxidase assay was modified according to De Keulenaer et al. [18]. Briefly, cell extracts were prepared in lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM Na_3O_4, 1 mM PMSF, 1% aprotinin, 20 μg/ml leupetin, and 10% glycerol). The assay was performed in a 50 mM PBS (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, 1 mM KCN. Forty micrograms of protein and 100 μM NADH were added and incubated at 37 °C for 5 min. Chemiluminescence was measured by using a luminometer (Berthold, Sirius) for 10 s.

2.10. Statistics

All data are expressed as the means ± S.D. of at least three independent experiments. The differences between control and treatment groups were calculated by one-way ANOVA with a post hoc Dunnett's test to evaluate significance levels.

3. Results and discussion

3.1. Apoptosis induction in HCT116 cells

The presence of tNOX protein has been demonstrated in several lines of cancer cells where it is associated with cell growth [8,11–13]. Capsaicin, an important component of chili pepper, has been shown to induce apoptosis in various cancer cell lines [8,19,20]; however, with the exception of two reports using HT-29 cells [21,22], this has not been shown in colon cancer lines. EGCg, also a natural chemopreventative compound, has a well-established role in suppressing colon carcinogenesis via various pathways [23–25]. Using viability assays, we found that both EGCg and capsaicin induced significant cytotoxicity in HCT116 cells at concentrations of 100 μM and 250 μM, respectively (Fig. 1A and B); notably, these cells exhibit a higher tolerance to capsaicin. In this current study, the concentration of EGCg required to induce cytotoxicity is consistent with other reports [23,25,26]. We have previously shown that the cytotoxic effects of capsaicin and EGCg are associated with inhibition of tNOX activity and apoptosis in HeLa and BT-20 cells [8,11]; here, we verified their cytotoxic effects in HCT116 cells. At 24-h post-exposure, the percentage of apoptotic cells increased by 54.5% and 27.3% after treatment with 100 μM EGCg and 250 μM capsaicin, respectively. Exposure to DNA-damaging UVC radiation for 18 h also induced apoptosis in HCT116 cells (Fig. 1C). These results dem-

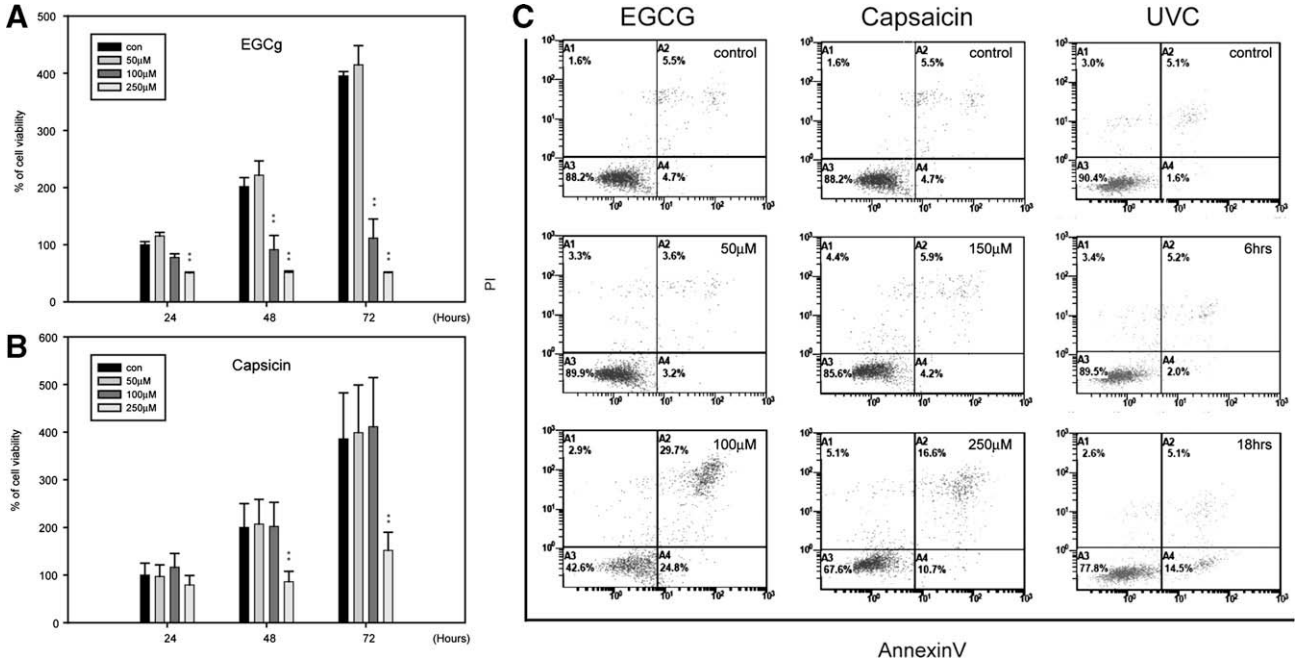

Fig. 1. Diverse stresses decrease cell viability and induce apoptosis in HCT116 cells. (A and B) Cells were treated with EGCg (A) or capsaicin (B) and viability was measured using the MTT assay. $^{**}P < 0.01$. (C) Cells were treated with EGCg or capsaicin for 24 h, or were UVC-irradiated (40 J/m^2), and the distribution of necrotic (A1 in Fig 1C quadrants), late apoptotic (A2), early apoptotic (A4), and viable (A3) cells was analyzed by double-staining with PI (necrotic) and FITC-annexin V (apoptotic).

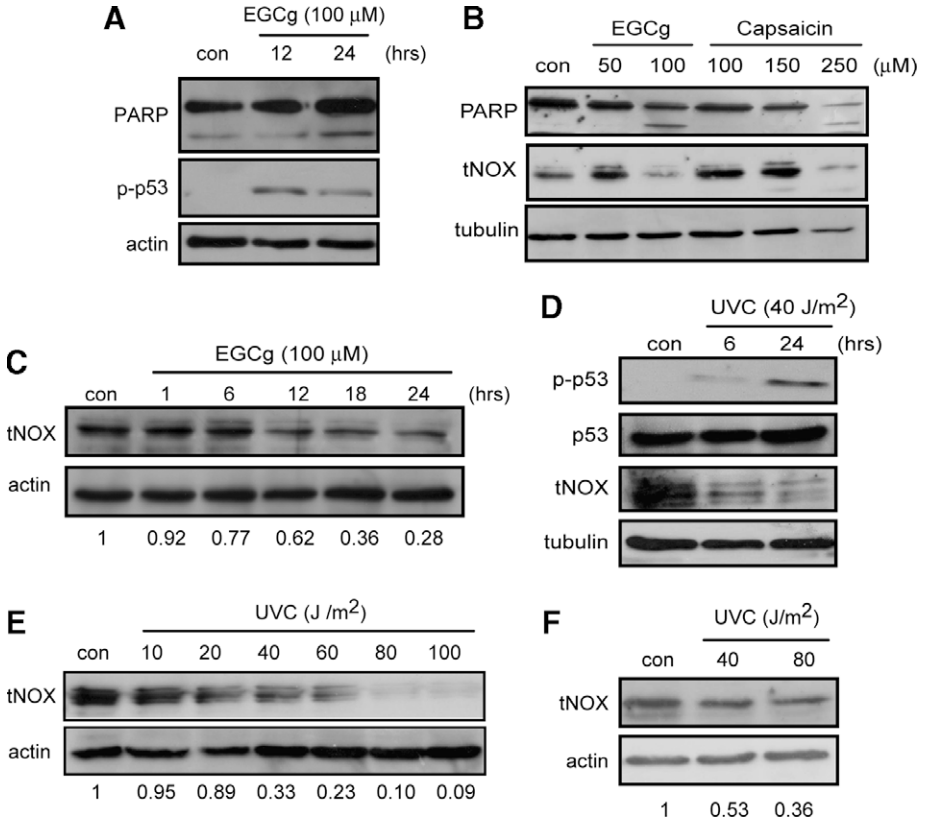

Fig. 2. Effects of apoptotic stresses on protein expression. (A) HCT116 cells treated with EGCg (100 μM) for 0–24 h. (B) HCT116 cells treated with EGCg (0–100 μM) or capsaicin (100–250 μM) for 24 h. (C) HCT116 cells treated with EGCg (100 μM) for 0–24 h. (D) HCT116 cells exposed to UVC (40 J/m^2) for 0–24 h. (E) HCT116 cells exposed to UVC (0–100 J/m^2) for 18 h. (F) A549 cells exposed to UVC (0–80 J/m^2) for 18 h. Values were determined by densitometric analysis and normalized to controls.

onstrate that diverse stress stimuli induced a concentration- and time-dependent increase in cytotoxicity and apoptosis.

3.2. Apoptosis is associated with suppressed tNOX level

Analyzing the mechanism of EGCg-induced apoptosis further, we found that PARP cleavage and p53 phosphorylation increased over time in HCT116 cells treated with EGCg (Fig. 2A). Using an antiserum that recognizes multiple tNOX forms [13], we showed that the increase in PARP cleavage occurred concomitantly with a decrease in tNOX levels (Fig. 2B); moreover, this EGCg-induced reduction in tNOX occurred in a time-dependent manner (Fig. 2C). At lower EGCg concentrations, an increase in tNOX was observed (Fig. 2B).

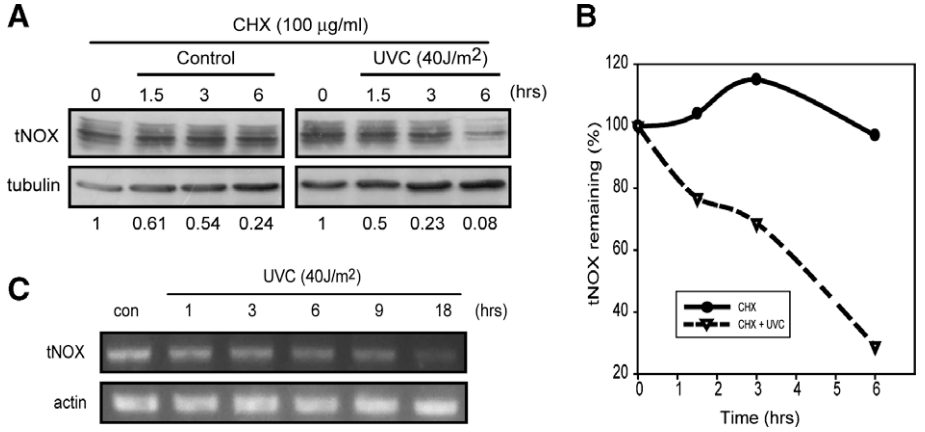

Fig. 3. UVC irradiation decreases tNOX expression in HCT116 cells. (A) Cells were pretreated with 100 μg/mL CHX for 30 minutes and exposed to UVC (40 J/m^2) for 0–6 h, followed by analysis of tNOX expression. The tNOX values have been normalized to tubulin values. (B) tNOX protein half-life was determined by densitometric analysis based on equal amount of total proteins of each sample. (C) Cells were exposed to 40 J/m^2 UVC for 0–18 h and tNOX mRNA levels were assessed by semi-quantitative RT-PCR.

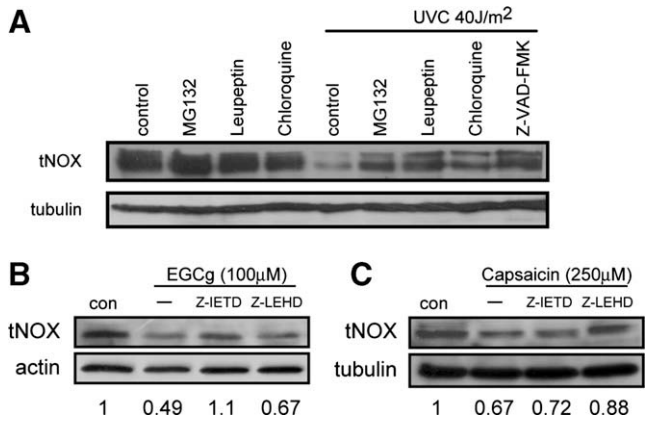

Fig. 4. Pretreatment with diverse protease inhibitors suppresses tNOX down-regulation in HCT116 cells. (A) Cells were pretreated with 1 μM MG132, 100 μM leupeptin, 100 μM chloroquine, or 20 μM Z-VAD-FMK for 30 min, and exposed or not to UVC (40 J/m^2) for 18 h. (B and C) Cells were pretreated with 15 μM Z-IETD or Z-LEHD for 30 min and exposed to 100 μM EGCg (B) or 250 μM capsaicin (C) for 24 h.

Similarly, UVC (40 J/m^2) irradiation led to p53 activation and a time- and dose-dependent decline in tNOX levels (Fig. 2D and E). To extend these observations to another cancer cell line, we exposed A549 cells to UVC irradiation. In these cells, UVC treatment also induced tNOX down-regulation (Fig. 2F), indicating that this response is not specific to a single cancer cell line. Many pathways have been reported to couple UV irradiation to apoptosis, including several that involve protein regulation [27–29]. In this first-reported use of UV radiation to assess tNOX expression, we found that tNOX was significantly down-regulated by DNA-damaging stress.

3.3. The half-life of tNOX is decreased after UVC exposure

To verify that UVC-mediated tNOX down-regulation occurred at the protein level, we pretreated cells with cycloheximide (CHX) for 30 min and then evaluated tNOX levels. tNOX levels progressively decreased in CHX/UVC-treated cells to an extent greater than seen after CHX treatment alone (Fig. 3A). A densitometric analysis based on equal amount of total protein of each sample confirmed that tNOX deteriorated more rapidly in the CHX/UVC-treated cells (Fig. 3B), suggesting that UVC exposure decreased the half-life of tNOX. Additionally, a reduction in the tNOX RNA level was observed after UVC exposure (Fig. 3C); this was also observed in EGCg-treated cells. UVC radiation regulates many genes by influencing the activities of transcription factors [30] and by affecting mRNA stabilization [31,32]. It is not yet clear, however, how tNOX protein is regulated at the transcriptional

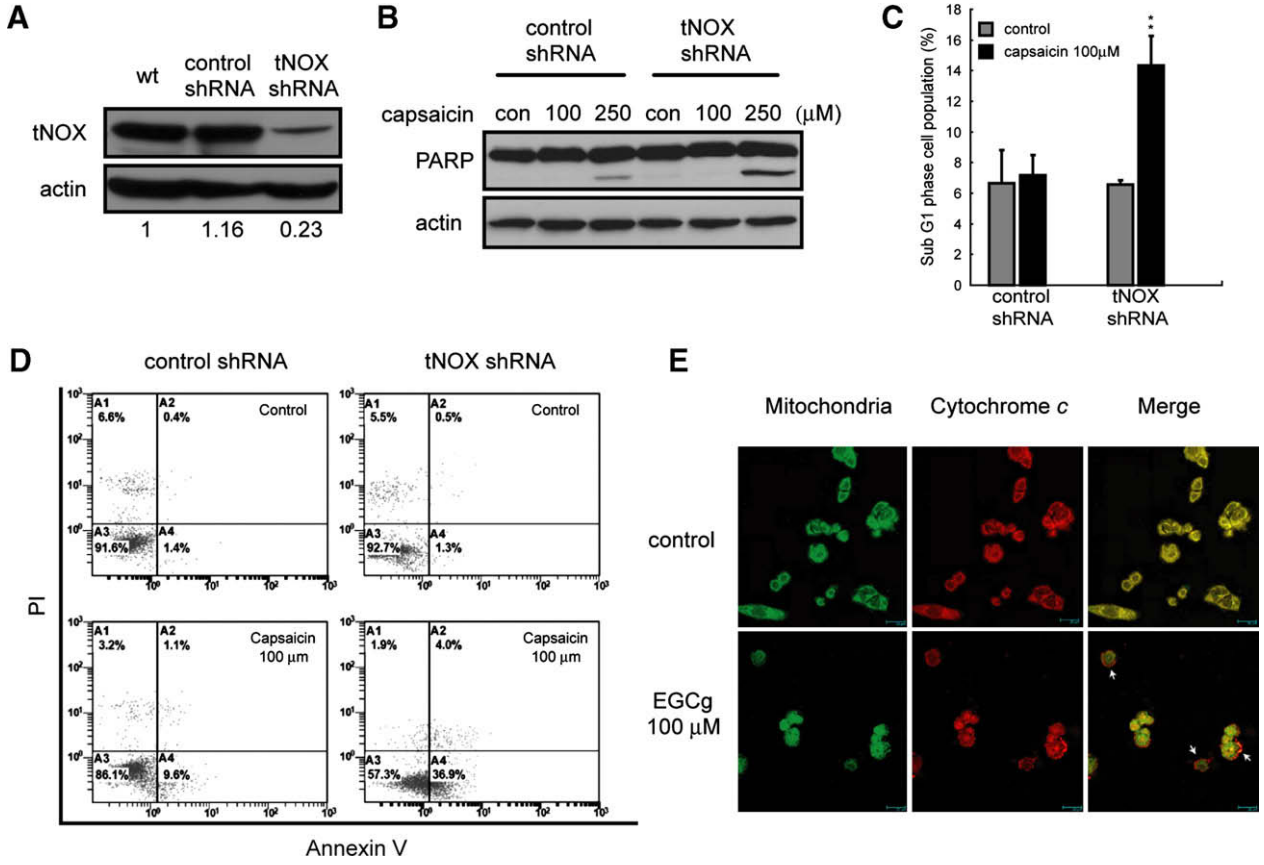

Fig. 5. Effect of tNOX knockdown on protein expression and apoptosis. (A) tNOX protein was analyzed in wild-type, control-shRNA (scrambled), and tNOX-shRNA HEK293 cells. (B) HEK293 cells were treated with capsaicin (0–250 μM) for 24 h and analyzed. (C) HEK293 cells were incubated with or without 100 μM capsaicin for 24 h and the sub-G1 population was analyzed. There is a significant difference in capsaicin-treated tNOX-shRNA cells compared with their controls. $^{**}P < 0.01$. (D) Apoptosis was determined by flow-cytometry analysis in HEK293 cells incubated with or without 100 μM capsaicin. (E) Laser confocal microscopy analyses of Mitotracker-stained mitochondria and cytochrome *c* subcellular localization in control and EGCg-treated transient transfected tNOX-shRNA HCT116 cells.

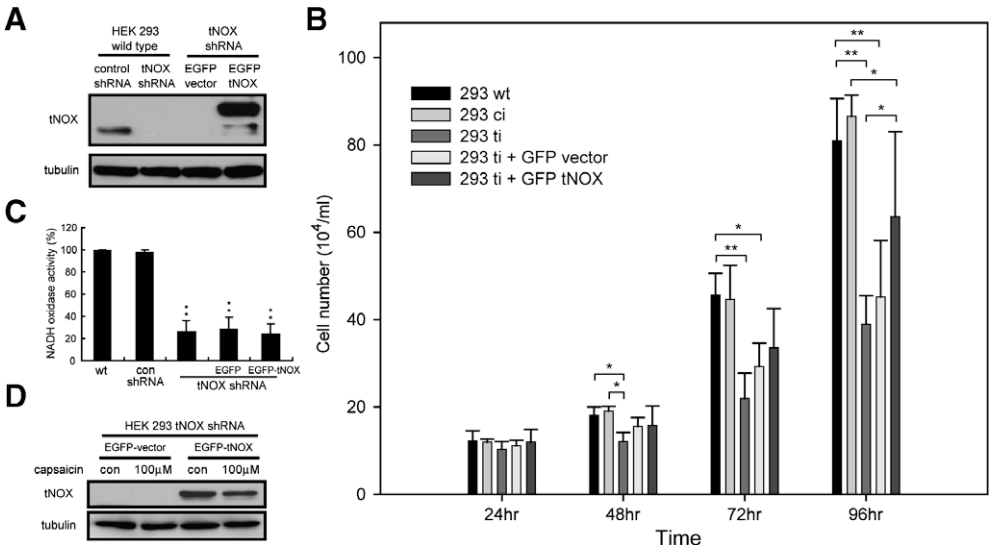

Fig. 6. Effects of EGFP-tNOX re-expression in tNOX-shRNA HEK293 cells. (A) tNOX protein was analyzed in control-shRNA, tNOX-shRNA, EGFP-vector, and EGFP-tNOX HEK293 cells. (B) Cells were seeded in quadruplicate and the number of viable cells was determined using the trypan blue exclusion assay. $^{*}P < 0.05$, $^{**}P < 0.01$ for difference in cell number between groups. (C) NADH oxidase activity in HEK homogenates. Data are expressed as percentage and presented as means ± S.D. There is a significant difference in tNOX-shRNA groups compared with wild-types or control shRNA. $^{**}P < 0.01$. (D) HEK293 cells were treated with or without capsaicin for 24 h and analyzed.

level, although UVC irradiation apparently induced a reduction in tNOX RNA transcription.

3.4. tNOX down-regulation is protease-dependent and important for apoptosis

UV irradiation mediates the degradation of numerous proteins, including survivin [27], myc [33], and p21 [34]. To determine whether protein degradation might contribute to tNOX down-regulation, we tested various protease inhibitors and showed that they rescued UVC-induced tNOX down-regulation (Fig. 4A). In addition, pretreatment with the caspase-8 inhibitor Z-IETD or the caspase-9 inhibitor Z-LEHD partially recovered tNOX degradation (Fig. 4B and C). He and colleagues [35] had reported that EGFR is cleaved at the consensus sequence DXXD by caspase during apoptosis; furthermore, the potential consensus cleavage sequence D^{193}KKD has been identified in tNOX protein [3]. Whether this site is actually cleaved by caspases requires further investigation.

Using shRNA-mediated silencing of tNOX in HEK293 cells (Fig. 5A), we showed that tNOX knockdown enhanced capsaicin-mediated apoptosis, as indicated by increased PARP cleavage (Fig. 5B), an elevated sub-G1 population (Fig. 5C), and augmented annexin V-staining (Fig. 5D), indicating that the loss of tNOX sensitized transformed cells to apoptosis. Additional evidence for apoptosis in tNOX-knockdown cells was provided by confocal microscopy, which showed that cytochrome *c* was released from mitochondria into the cytosol by treatment with 100 μM EGCg, whereas cytochrome *c* remained in the mitochondria in untreated cells (Fig. 5E). Re-expression of EGFP-tNOX in tNOX-knockdown cells partially recovered cell proliferation (Fig. 6A and B). NADH oxidase activity in tNOX-shRNA cells was notably decreased compared to wild-types or control shRNA, however, the activity in re-expression of EGFP-tNOX cells was not significantly different from EGFP control (Fig. 6C). Additionally, capsaicin treatment of these cells caused a decrease in the levels of the exogenous protein (Fig. 6D). The reason for conducting tNOX-shRNA experiments in HEK293 system was due to low transfection efficiency and the failure of generation of stable lines in HCT116 cells. Capsaicin and EGCg have been shown to preferentially inhibit tNOX activity [8,11], and our results here suggest that this inhibitory effect is caused by down-regulation of tNOX. However, EGFP-tNOX failed to protect against apoptosis, possibly reflecting a less-than-optimal functional configuration of the fusion protein as suggested in oxidase activity assay. Alternatively, because exogenous tNOX requires a prolonged expression interval (>96 h) to exert a significant biological effect as suggested by our proliferation data (Fig. 6B), the absence of an anti-apoptotic effect may simply reflect limitations inherent in the transient transfection system used. It is also possible that other function of tNOX protein, besides NADH oxidase, might be involved in regulating cell proliferation. tNOX is known to have a role in transformed cell growth [8,11,12], although this may possibly be due to regulation of proliferation rather than inhibition of apoptosis [15,16].

In conclusion, we report the novel finding that tNOX, which is required for transformed cell growth, is suppressed during apoptosis. The down-regulation of tNOX results in decreased cell proliferation and renders cells sensitive to apoptosis, leading to decreased growth.

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