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Research Article

Phosphorylation of serine-504 of tNOX (ENOX2) modulates cell proliferation and migration in cancer cells

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ABSTRACT

Tumor-associated NADH oxidase (tNOX; ENOX2) is a growth-related protein expressed in transformed cells. Consistent with this function, tNOX knockdown by RNA interference leads to a significant reduction in cell proliferation and migration in HeLa cells, whereas tNOX overexpression confers an aggressive phenotype. Here, for the first time, we report that tNOX is phosphorylated by protein kinase Cδ (PKCδ) both *in vitro* and *in vivo*. Replacement of serine-504 with alanine significantly reduces phosphorylation by PKCδ. Co-immunoprecipitation experiments reveal an interaction between tNOX and PKCδ. Moreover, whereas overexpression of wild-type tNOX in NIH3T3 cells increases cell proliferation and migration, overexpression of the S504A tNOX mutant leads to diminished cell proliferation and migration, reflecting reduced stability of the unphosphorylatable tNOX mutant protein. Collectively, these results suggest that phosphorylation of serine-504 by PKCδ modulates the biological function of tNOX.

Introduction

Previous studies have identified tumor-associated NADH oxidase (tNOX; ENOX2) as a member of a family of growth-related NADH (or hydroquinone) oxidases [1–4]. Unlike the NADH oxidase activity identified in normal rat liver plasma membranes (CNOX; ENOX1), which is responsive to growth factors and hormones, tNOX isolated from rat hepatoma cells is constitutively activated [1,4]. Further studies have revealed that tNOX is present in numerous cancer cell lines, including those derived from breast, cervix, colon, lung, and stomach cancers, as well as leukemias [5–10]; it is also detected in the sera of cancer patients [11–13]. tNOX cDNA has been cloned [14], and functional motifs of tNOX have been identified, including a quinone-binding site, an adenine-nucleotide-binding site, and a CXXXC cysteine pair that is important for tNOX activity [15].

Numerous anti-cancer drugs, including capsaicin [5,8], the major green tea catechin (-)-epigallocatechin-3-gallate (EGCg) [8,16], and phenoxodiol [17] have been shown to inhibit tNOX enzymatic activity in association with a reduction in cancer cell growth. Moreover, tNOX expression is suppressed during apoptosis and tNOX downregulation sensitizes cells to stress-induced growth reduction, suggesting that tNOX is required for transformed cell growth [8]. Similarly, tNOX-knockdown sensitizes TMC-1 cells to capsaicin-mediated oxidative stress and mitochondria-dependent apoptosis, leading to cell growth suppression [10]. Using small hairpin RNA (shRNA) to knockdown tNOX

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expression, we showed that reduced tNOX expression attenuates HeLa cell migration via membrane association of Rac protein [9]. In contrast, tNOX overexpression in non-cancerous MCF-10A cells results in the acquisition of invasivity, an aggressive characteristic of cancer cells, further supporting a key role for tNOX in cell migration [18]. These various lines of evidence suggest that tNOX acts as a critical regulator of physiological and pathological outcomes in responses to biological cues involved in redox signaling, cell proliferation, survival, and tumor progression. However, the molecular mechanisms by which tNOX function is regulated have not been thoroughly investigated. Here, we examined whether the phosphorylation status of tNOX affects its biological function in cell proliferation and migration, and found that protein kinase Cδ (PKCδ)-mediated phosphorylation of tNOX residue serine-504 plays a critical role in regulating the biological function of tNOX protein.

Materials and methods

Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). The anti-rabbit IgG antibody and PKCδ active enzyme were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-actin antibody was from Chemicon International, Inc. (Tamekula, CA). The anti-GST and PKCδ antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antisera to tNOX used in Western blot analyses were generated as described previously [7] and the tNOX protein band recognized by this antiserum was also identified by the commercially available anti-tNOX polyclonal antibody (Protein Tech Group, Inc. Chicago, IL) [9]. The anti-mouse IgG antibodies as well as other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA), unless otherwise specified.

Cell culture and transfection

HEK293 (human embryonic kidney epithelial) and NIH3T3 (mouse fibroblast) cells were grown in DMEM with 10% FBS, containing 100 units/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. Cells were transiently transfected with tNOX–GST or GST (control) using the jetPEI transfection reagent according to the manufacturer's protocol (Polyplus-transfection SA, Illkirch Cedex, France), as described previously [9].

Expression vector constructions and site-directed mutagenesis

The full-length and fragments of tNOX cDNA were cloned into pCMV–GST or pGEX-4T-1 GST expression vector with BamHI and SalI sites. Site-directed mutagenesis was performed on double-stranded plasmid by PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA, USA). The internal primers used for T402A mutant were 5′-GAA ATG ACA GAA ACA AAA GAA GCT GAG GAA TCA GCC-3′ (forward) and 5′-GGC TGA TTC CTC AGC TTC TTT TGT TTC TGT CAT TTC-3′ (reverse); for S504A mutant were 5′-GAA AAT CTT AAA GAA AAG GAA GCC TGT GCT TCT AGG CTG TGT GC-3′ (forward) and 5′-GCA CAC AGC CTA GAA GCA CAG GCT TCC TTT TCT TTA AGA TTT TC-3′ (reverse); for T543A mutant were 5′-CTA GTG GGG ATT ATC TCC GCA TTC CTT CAT GTT CAC CC-3′ (forward) and 5′-GGG TGA ACA TGA AGG AAT GCG GAG ATA ATC CCC ACT AG-3′ (reverse); and for S571A mutant were 5′-CTT GAT AAT AAG ATC TGC ACC GCC GAT GTG GAG TGT CTC ATG G-3′ (forward) and 5′-CCA TGA GAC ACT CCA CAT CGG CGG TGC AGA TCT TAT TAT CAA G-3′ (reverse).

Colony-formation assay

Approximately 200 cells were seeded onto a 6-cm dish and incubated in culture medium for 10 days to allow colony formation. After incubation, colonies were fixed in 1.25% glutaraldehyde at room temperature for 30 min, rinsed with distilled water and stained with a 0.05% methylene blue solution. The number of colonies was counted and recorded.

Continuous cell monitoring with the xCELLigence System

For continuous monitoring of changes in cell growth, approximately 1×10^4 cells/well were seeded onto E-plates and incubated for 30 min at room temperature, after which E-plates were placed onto the Real-Time Cell Analyzer (RTCA) station (xCELLigence System, Roche, Mannheim, Germany). Cells were grown overnight and impedance was measured every hour as previously described [19].

For continuous monitoring of cell invasion and migration (CIM), approximately 1×10^4 cells/well were seeded onto the top chamber of a CIM plate, which features microelectronic sensors integrated on the underside of the microporous polyethylene terephthalate (PET) membrane of a Boyden-like chamber. After incubating for 30 min at room temperature, CIM plates were placed onto the RTCA station (xCELLigence System). Cells' migration was continuously monitored throughout the experiments by measuring changes in the electrical impedance at the electrode/cell interface, as a population of cells migrated from the top to the bottom chamber.

Cell impedance is characterized by the cell index $(CI)=(Z_i-Z_0)$[Ohm]/15[Ohm], where Z_0 is the background resistance and Z_i is the resistance at an individual time point. A normalized cell index was determined as the cell index at a certain time point (CI_{ti}) divided by the cell index at the normalization time point (CI_{nml_time}).

Western blot analysis

Cell extracts were prepared in lysis buffer (20 mM Tris–HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM PMSF, 10 ng/mL leupeptin and 10 μg/mL aprotinin), and volumes of extract containing equal amounts of proteins (40 μg) were separated on SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked, washed, and probed with primary antibody. After washing to remove primary antibody, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The blots were washed again, and developed using enhanced chemiluminescence (ECL) reagents, according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

Boyden chamber assay

A Boyden chamber with filter inserts containing 8-μm pores (Neuro Probe, Inc., Gaithersburg, MD, USA) was used to measure cell migration. Cells (2.5×10^3) in DMEM containing 0.5% of FBS were placed in the upper chamber, and the lower chamber was filled with complete DMEM. After 24 h in culture, cells were fixed in methanol for 15 min and then stained with 10% Giemsa in phosphate-buffered saline (PBS) for 30 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. The number of cells on the bottom of filters (migrated cells) was counted and recorded. Data shown are representative of three independent experiments.

In vitro kinase assay

In vitro kinase assay was performed according to the procedure described previously with modification [20]. The active PKCδ was incubated with 5 μg of various fragments of recombinant GST–tNOX protein in a kinase reaction buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 and 1 mM NaF). The kinase assay was carried out in a total volume of 30 μL of a kinase reaction buffer containing 20 μM ATP, 2 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; GE healthcare) at 30 °C for 30 min. Phosphorylated GST–tNOX was resolved on 10% SDS-polyacrylamide gels followed by autoradiography.

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.

Results

tNOX is phosphorylated by PKCδ *in vitro*

Protein post-translational modifications play a key role in regulating an array of cellular processes, including cell proliferation, migration, and differentiation. Among these, protein phosphorylation is a reversible process that operates as a molecular switch, controlling protein functions and stability. However, the

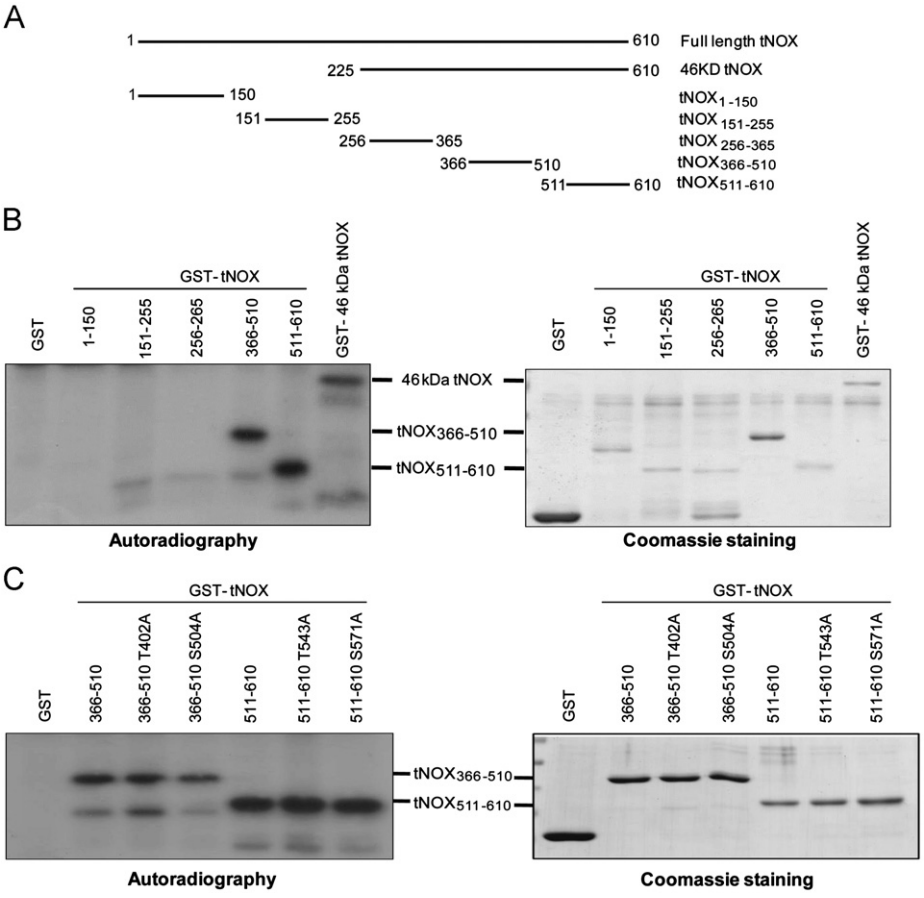

Fig. 1 – Phosphorylation of tNOX by PKCδ *in vitro*. (A) Schematic diagram depicting the different fragments of tNOX used for *in vitro* kinase assays. (B) Active PKCδ was mixed with 5 μg of GST–tNOX recombinant protein fragments in kinase reaction buffer. After incubating at 30 °C for 30 min, proteins were separated by SDS-PAGE on 10% gels, stained with Coomassie blue (right panel), and subjected to autoradiography (left panel). (C) T402 and S504 in $tNOX_{366–510}$, and T543 and S571 in $tNOX_{511–610}$ were substituted with unphosphorylatable alanine and used for *in vitro* kinase assays. Active PKCδ was mixed with 5 μg of GST–tNOX recombinant protein fragments in kinase reaction buffer. After incubating at 30 °C for 30 min, proteins were separated by SDS-PAGE on 10% gels, stained with Coomassie blue (right panel), and subjected to autoradiography (left panel). Representative images are shown.

phosphorylation status of tNOX has not been studied previously. In this study, we employed phosphorylation site-prediction tools to reveal several possible PKC phosphorylation sites in tNOX, suggesting that phosphorylated status might modulate distinct biological functions of tNOX. To examine whether tNOX is phosphorylated *in vitro*, we cloned various fragments of tNOX into a pGEX-4T-1 expression vector to generate GST-fusion proteins (Fig. 1A). Recombinant GST-fusion proteins with different-length forms of tNOX were expressed and purified from *Escherichia coli* (Fig. 1B, lower panel), and then incubated with active PKCδ enzyme to test for *in vitro* kinase activity. We found that three fragments, tNOX-46kD, $tNOX_{366–510}$, and $tNOX_{511–610}$, were strongly phosphorylated by PKCδ (Fig. 1B, left panel). To identify phosphorylated residues, we substituted T402 and S504 in $tNOX_{366–510}$ and T543 and S571 in $tNOX_{511–610}$ with non-phosphorylatable alanine by site-directed mutagenesis, and then subjected recombinant mutant fusion proteins to *in vitro* kinase assays. Fig. 1C (left panel) shows that ^{32}P incorporation into S504A, but not T402A, T543A or S571A, was significantly decreased compared to its wild-type counterpart, indicating that serine-504 is robustly phosphorylated by PKCδ. However, the phosphorylation level in S504A was not completely reduced, suggesting other potential phosphorylation residues by PKC in the $tNOX_{511–610}$ fragment.

tNOX is phosphorylated by PKCδ *in vivo*

We next examined whether tNOX is phosphorylated by PKCδ *in vivo*. HEK293 cells were incubated with 160 nM phorbol myristate acetate (PMA), a potent PKC activator, for 1 h to activate endogenous PKC isoforms before cell extracts were harvested. Recombinant GST or GST-fused tNOX were incubated with extracts of PMA-treated cell followed by addition of an antibody against PKCδ to specifically immunoprecipitate PKCδ. Western blot analyses revealed that GST–tNOX, but not GST, was recovered in anti-PKCδ immune complexes (Fig. 2A), indicating that tNOX interacts with PKCδ. In addition, overexpressing GST–tNOX wild-type and GST–$tNOX^{S504A}$ in NIH3T3 cells were

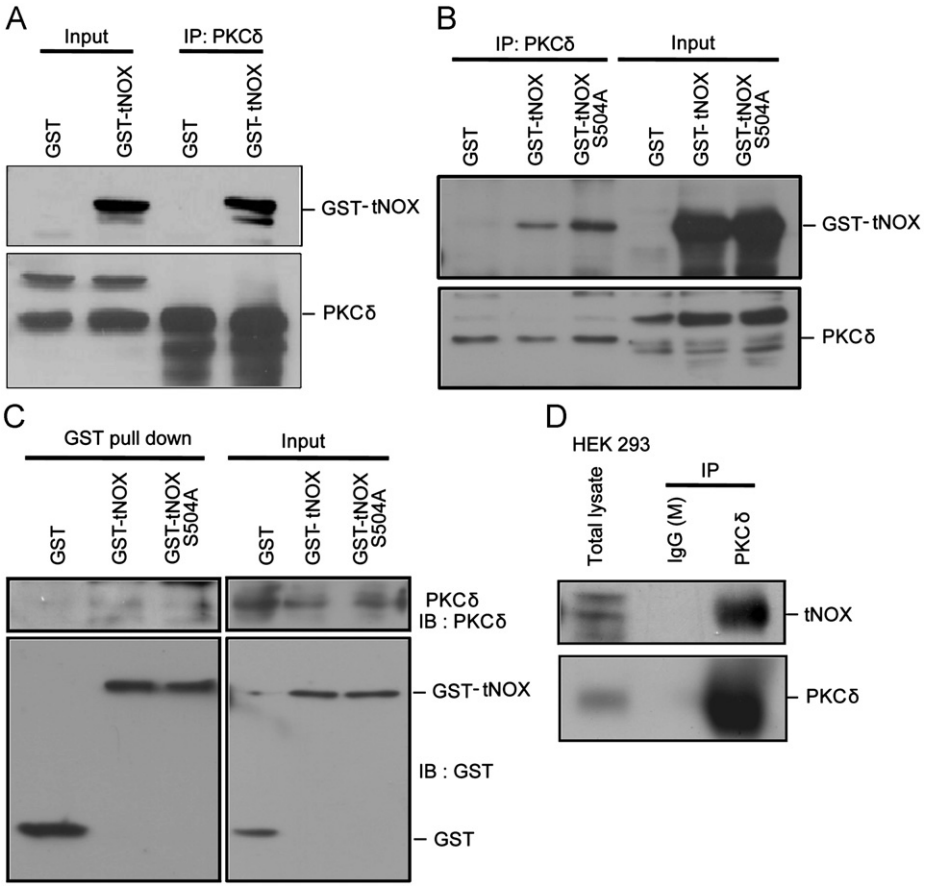

Fig. 2 – Interaction between tNOX and PKCδ *in vivo*. Cell extracts were prepared and incubated with an antibody against PKCδ to specifically immunoprecipitate PKCδ, followed by Western blot analysis. Total cell lysates were also separated by SDS-PAGE. The presence of PKCδ and GST–tNOX in immunoprecipitates were detected with anti- PKCδ and anti-tNOX antibodies, respectively (A) and (B). (A) HEK293 cells were transfected with GST or GST–tNOX and used for immunoprecipitation. (B) NIH3T3 cells were transfected with GST, GST–tNOX, or GST–$tNOX^{S504}$ and used for immunoprecipitation. (C) NIH3T3 cells were transfected with GST, GST–tNOX wt, or GST–$tNOX^{S504}$ and cell extracts were prepared and subjected to GST pull-down assays. The presence of PKCδ and GST–tNOX in GST pull-down complexes was detected with anti- PKCδ and anti-GST antibodies, respectively. (D) HEK293 cell extracts were prepared and incubated with an antibody against PKCδ to specifically immunoprecipitate PKCδ, followed by Western blot analysis. The presence of endogenous tNOX and PKCδ in immunoprecipitates was detected with anti-tNOX and anti- PKCδ antibodies, respectively. Representative images are shown.

utilized for the PKCδ immunoprecipitation experiments and our results showed that both GST–tNOX wild type and GST–tNOX^{S504A} were also recovered in anti-PKCδ immune complexes (Fig. 2B). Alternatively, the interaction between tNOX and PKCδ was confirmed by GST pull-down experiments (Fig. 2C). Furthermore, Western blot analyses verified that endogenous tNOX protein was retrieved in anti-PKCδ immune complexes (Fig. 2D).

tNOX^{S504A} decreases tNOX stability and cell proliferation

The impact of serine-504 phosphorylation on tNOX biological function was further investigated in NIH3T3 cells transfected with wild-type or S504A mutant forms of GST-fused tNOX. In the first set of experiments, cells were treated 48 h after transfection with the protein synthesis inhibitor cycloheximide (CHX; 50 μg/ml), and changes in tNOX protein levels over time (3–9 h) were examined by Western blotting. As shown in Fig. 3A, S504A substitution (tNOX^{S504A}) led to a decrease in tNOX protein stability compared to the wild-type tNOX that was evident as early as 3 h after the addition of CHX. Given in previous reports that reduced tNOX expression decreases cell proliferation and migration and restores stress-induced growth inhibition [8–10], we next examined the effect of overexpressing tNOX^{S504A} protein on cell proliferation and migration. Fig. 3B shows that GST–tNOX proteins, whether wild-type or tNOX^{S504A} mutant, were overexpressed in NIH3T3 cells (Fig. 3B). Next, the xCELLigence System, a novel real-time cell monitoring system that measures electrical impedance and displays results as cell index (*CI*) values [21,22] was used to continuously monitor the effect of tNOX overexpression on the growth of NIH3T3 cells. In this application of the xCELLigence System, the presence of cells on the top of the electrodes produces an elevation in electrode impedance whose magnitude is determined by the cell number and the degree of cell adhesion. Using this approach, we found that wild-type tNOX significantly enhanced cell proliferation compared to the vector control (Fig. 3C). Interestingly, the tNOX^{S504A} mutant had no impact on cell proliferation, suggesting that phosphorylation of serine-504 is necessary for tNOX effects on cell proliferation.

tNOX^{S504A} attenuates cell migration

tNOX deficiency results in decreased cell migration [9], and tNOX overexpression increases the ability of non-cancerous cells to acquire invasiveness [18]. Accordingly, we next determined

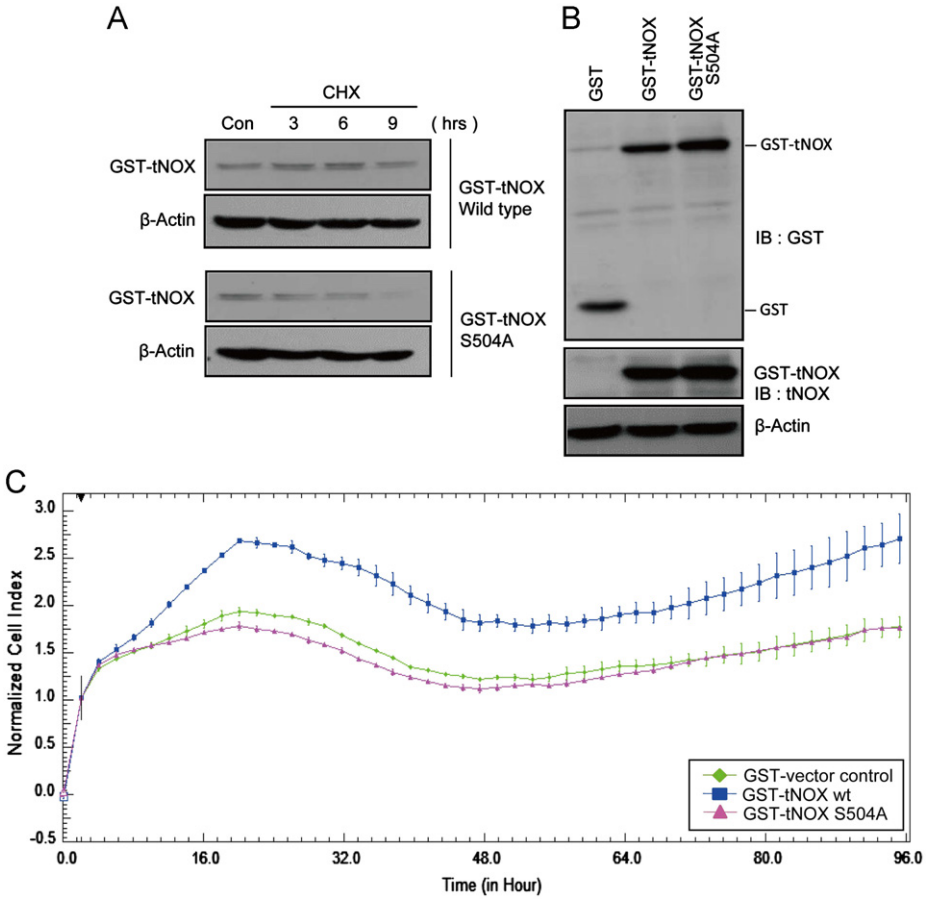

Fig. 3 – Stability of wild-type and mutant GST–tNOX proteins and effects of their overexpression on cell growth. NIH3T3 cells were transfected with GST, GST–tNOX wt, or GST–tNOX^{S504A}. (A) Cells expressing either GST–tNOX wt or GST–tNOX S504A were pretreated with 50 μg/ml CHX followed by analysis of tNOX expression. β-actin was used as an internal control to monitor for equal loading. (B) Overexpression of GST–tNOX wt and GST–tNOX S504A fusion proteins was analyzed using anti-GST and anti-tNOX antibodies. β-actin was used as an internal control to monitor for equal loading. Representative images are shown. (C) Dynamic monitoring of cell proliferation using impedance technology, as described in Materials and methods. Shown are normalized cell index values measured over 96 h.

whether tNOX^{S504A} affects cell migration in NIH3T3 cells. Boyden chamber assays showed that overexpression of wild-type GST–tNOX significantly enhanced cell migration compared to cells transfected with the GST–vector control, whereas tNOX^{S504A} had little effect on cell migration (Fig. 4A). We further confirmed these results by continuously monitoring the effect of tNOX overexpression on cell migratory behavior, using the xCELLigence System. Using this approach, we similarly found that overexpression of wild-type GST–tNOX, but not tNOX^{S504A}, enhanced cell migration compared to that observed in cells transfected with GST–vector control (Fig. 4B). On the basis of our data presented here, we conclude that phosphorylation of tNOX on serine-504 by PKCδ modulates tNOX biological functions in cell proliferation and migration by increasing protein stability.

Discussion

Signal transduction pathways are important in converting extracellular stimuli into a wide range of cellular responses; notably, these pathways are often deregulated in human cancers. Among these, signaling pathways involving members of the PKC family are important in various cellular processes, including cell proliferation, migration, and tumorigenesis [23,24,25]. The function of PKC in cancer is complicated owing to its multiple different isoforms, which have unique impacts in cellular pathways involved in transformation. PKCδ classified as a member of the new/novel PKC (nPKC) subgroup, has been reported to act primarily as a pro-apoptotic kinase [26]. However, accumulating evidence also suggests that PKCδ positively regulates cancer cell survival. For example, PKCδ knockdown using antisense oligonucleotides has been demonstrated to inhibit the survival of breast cancer cells [27]. Similar findings were reported by Clark et al. [28], who suggested that PKCδ promotes survival and enhances chemotherapeutic resistance of non-small cell lung cancer cells. In the present study, we provide evidence in support of the idea that tNOX, a tumor-associated NADH oxidase related to cancer cell growth, is a substrate for PKCδ phosphorylation, which enhances tNOX protein stability and leads to increased cell proliferation.

Recent evidence also suggests that PKCδ acts as a positive regulator of cell motility through phosphorylation of adducin [29]. Furthermore, PKCδ contributes to prostaglandin E2-enhanced motility of oral cancer cells as well as epidermal

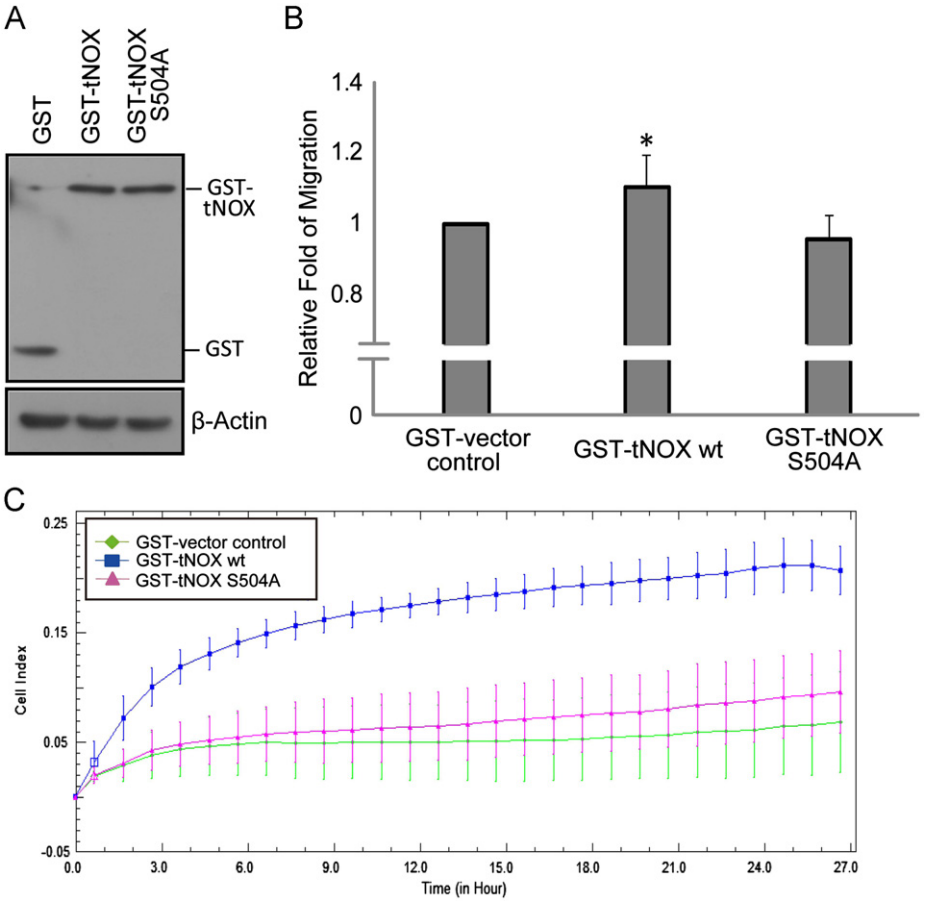

Fig. 4 – Effect of overexpressed wild-type and mutant GST–tNOX proteins on cell migration. NIH3T3 cells were transfected with GST, GST–tNOX wt, or GST–tNOX S504A and incubated for 2 d. (A) Overexpression of GST–tNOX wt and GST–tNOX S504A fusion proteins was analyzed using GST antibody. β-actin was used as an internal control to monitor for equal loading. Representative images are shown. (B) Cell migration was measured using a Boyden chamber system. Values (means ± SEs) are from at least three independent experiments. Cell migration was significantly increased in cells expressing GST–tNOX wild type compared with control ($^{*}p < 0.05$). (C) Dynamic monitoring of cell migration using impedance technology, as described in Materials and methods. Shown are normalized cell index values measured over 27 h.

growth factor-mediated cell migration of fibroblast cells [30,31], verifying a role of PKCδ in promoting cell migration. We have previously shown that cells depleted of tNOX exhibit attenuated cell migration ability [9], whereas non-cancerous cells over-expressing tNOX acquires invasiveness [18]. The fact that phosphorylation of tNOX residue serine-504 by PKCδ increases tNOX protein stability and leads to enhanced cell migration—a conclusion from this study—further supports the cell migration-promoting effects of PKCδ.

Interestingly, Chen et al. have proposed that reactive oxygen species (ROS) function as an upstream signal to activate PKCδ, which subsequently, triggers additional ROS generation through the NADPH oxidase complex, inducing cell scattering [32]. The superoxide-generating NADPH oxidase complex comprises a membrane-bound catalytic subunit and a number of cytosolic regulatory subunits that respond to growth factors; accordingly, it is involved in numerous intracellular signaling pathways [33]. PKC is involved in phosphorylating the cytosolic regulatory subunits $p47^{phox}$ subunit of NADPH oxidase, which leads to the generation of superoxide important in intracellular signaling [34]. Previously, we showed that capsaicin induces divergent effects on the growth of gastric cancer cells that parallel its effects on tNOX expression, and demonstrated that forced tNOX down-regulation sensitizes TMC-1 cells to capsaicin-induced oxidative stress and mitochondria-dependent apoptosis, leading to cell growth suppression [10]. Collectively, these observations suggest a possibility that tNOX functions as a modulator of intracellular ROS generation. In this study, we propose that phosphorylation of tNOX residue serine-504 by PKCδ might lead to modulation of ROS, thereby providing a positive feedback loop for ROS/tNOX/PKCδ signal transduction, which is an important participant in a variety of cellular processes, especially those involved in cancer cell proliferation and migration.

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