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tNOX is both necessary and sufficient as a cellular target for the anticancer actions of capsaicin and the green tea catechin (-)-epigallocatechin-3-gallate

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Abstract. Capsaicin and the principal green tea catechin, (-)-epigallocatechin-3-gallate (EGCg), target tNOX, a tumor (cancer)-specific surface hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity (ECTO-NOX protein). Accordingly vector-forced over expression of tNOX in MCF-10A mammary epithelia or COS cells that lack tNOX or in COS cells that underexpress tNOX enhanced the susceptibility of growth and apoptosis to both EGCg and capsaicin. Additionally, the tNOX-transfected MCF-10A cells proliferated in Matrigel, a measure of invasiveness. In contrast, oligomeric antisense tNOX DNA abrogated growth inhibition by EGCg and capsaicin and reduced anchorage-dependent growth of HeLa (human cervical carcinoma) cells that naturally overexpress tNOX. The findings show cell surface expression of tNOX as both necessary and sufficient for the cellular anticancer activities attributed to both EGCg and capsaicin.

Keywords: ECTO-NOX, NADH oxidase, tNOX, cancer, capsaicin, (-)-epigallocatechin-3-gallate (EGCg)

1. Introduction

Our laboratories have described a cancer-specific hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity (ECTO-NOX protein) designated tNOX [6] (GenBank Accession No. AF207881). A defining characteristic of tNOX is that its activity is inhibited by capsaicin [13], antitumor sulfonylureas [14], adriamycin [19] and certain other natural products and drugs all with anticancer activity [20]. More recently, tNOX was postulated to represent the cellular target protein to explain the anticancer action of the principal green tea catechin, (-)-epigallocatechin-3-gallate (EGCg) [21].

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In this report, vector-forced expression and antisense were used to test the postulate that the tNOX target protein at the cell surface was the cellular target necessary and sufficient to explain the specific anticancer growth activity attributed both to EGCg [1,3,7,8,10] and to capsaicin [21]. The findings show gain (vector-forced expression in COS and MCF-10A cells) or loss (antisense with HeLa cells) of both drug and tumor growth response to EGCg and to capsaicin that correlated with cell surface tNOX expression.

2. Materials and methods

2.1. Materials

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO) unless otherwise specified. EGF (mouse, culture grade) was from Upstate Biotechnology, Inc. (Lake Placid, NY).

2.2. Growth of cells

COS-1 (ATCC CRL-1650) SV40 transformed monkey kidney fibroblast cells were cultured in Dulbecco's Modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 10% fetal bovine serum (heat-inactivated) plus 50 μg/L gentamycin sulfate (Sigma). Medium was renewed 1–2 times per week.

MCF-10A human mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium containing glutamine (292 mg/L), gentamycin sulfate (50 mg/L), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), EGF (20 ng/mL), and 5% horse serum.

HEK-293 cells (ATCC CRL 1573) adenovirus transformed human embryonal kidney were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

HeLa (ATCC CCL2) human cervical carcinoma cells were grown in 175 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/L gentamycin sulfate (Sigma). Cells were harvested by scraping and taken up in 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4, to a final cell concentration of 0.1 g wet weight (gww) per mL.

Medium was renewed every 2–3 days. Cell numbers were determined microscopically by counting the number of cells over defined areas consisting of a grid of 1 mm squares. Cell lines were obtained from the American Type Culture Collection (Rockville, MD).

2.3. Spectrophotometric assay of NADH oxidase

NADH oxidase activity was determined as the disappearance of NADH measured spectrophotometrically at 340 nm in a reaction mixture containing 25 mM Tris-methylethanesulfonate buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 μM NADH at 37°C with stirring. Activity was measured using a Hitachi U3210 or SLM Aminco DW2000 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity. EGCg or capsaicin was added at the final concentrations indicated at the beginning of the assay and was present during the assay period.

Proteins were estimated by the bicinchononic acid method [26] with bovine serum albumin (BSA) as standard.

2.4. tNOX overexpression in mammalian systems

tNOX protein was expressed in MCF-10A cells as a truncated form (beginning at M220, 46 kDa) [1]. tNOX cDNA was first amplified by PCR using primers 5′-TGGGAGTGTAAACAGCGTATG-3′ (forward) and 5′-TTTCTATGCTTGTCCAACACATAT -3′ (reverse). The PCR product was then amplified using primers 5′-AAACTTAAGCTTTGGGAGTGT3' (forward) and 5′- TTTCTATGCTTGTCCAA CACATAT-3′ (reverse) to construct a HindIII site. The DNA product was digested using HindIII and BamHI followed by purification (Invitrogen, Carlsbad, CA). A pcDNA3.1 mammalian expression vector of SV40 origin with a CMV enhancer-promoter and an ampicillin resistance gene (Invitrogen) was used.

2.5. COS-1 cells

COS-1 cells were seeded one day prior to transfection at 4×10^5 cells per 100-mm dish. A calcium phosphate transfection kit (Invitrogen) was used to transiently transfect COS cells according to the manufacturer's protocol using the same drug-resistance expression vector as for MCF-10A cells. tNOX expression was evaluated on the basis of enzymatic activity and Western blot analysis. For selection of stable transfectants, 0.5 mg/ml of G418 sulfate (Invitrogen) was added into the media twice a week as the antibiotic. A total of three colonies were selected, trypsinized individually and transferred individually to 24-well plates. Cells were harvested at 80% confluency. Transfections were confirmed by immunoblotting.

2.6. MCF-10A cells

MCF-10A cells were transiently transfected using calcium phosphate precipitation with a transfection kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Briefly, the cells were transfected with 30 μg plasmid (tNOX pcDNA3.1). After 24 h incubation, DMSO shock was performed to facilitate DNA uptake. Forty-eight hours after DMSO shock, cells were washed and replated in more diluted concentrations for further selection. After 15–20 days of G418 selection (150 μg/ml, Invitrogen, Life Technology), rapidly growing colonies were individually isolated and expanded serially to 24-well, 6-well and 60-mm dishes. The tNOX protein expression was examined by immunoblotting and the stably transfected cells were subsequently tested for tumorigenicity in immunocompromised mice.

2.7. HEK-293 cells

HEK-293 cells were transiently transfected with LipofectAMINE 2000 (Invitrogen, Life Technologies) according to the manufacturer's instructions. Briefly, 10 μg of plasmid (tNOX-pcDNA3.1/Zeo) was mixed with 25 μl of LipofectAMINE in 1 ml of MEM medium devoid of serum for 30 min and then added to cells that had attained 80–90% confluence in a 60-mm dish containing 5 ml of medium devoid of serum. After 6 h incubation, the medium was replaced with 10 ml of fresh culture medium containing serum. The transfected cells were placed in a CO_2 incubator at 37°C for 24 h and transferred at a 1:10 dilution into fresh growth medium for further selection. After 15–20 days of Zeocin selection (100 μg/ml, Invitrogen, Life Technology), rapidly growing colonies were individually isolated and expanded serially to 24-well, 6-well and 100-mm dishes. The tNOX protein expression was examined by immunoblotting and stably transfected cells were subsequently tested for tumorigenicity in immunocompromised mice.

2.8. Tumors in immunocompromized mice

To test for the ability of tNOX-transfected (non-cancer) cells to form tumors in immunocompromised mice, 6 week old, male mice (Strain Nuna, Harlan, Indianapolis) were injected subcutaneously with stably transfected MCF-10A or HEK-293 cells at two densities (2×10^6 or 6×10^6 cells per animal for the MCF-10A cells and 2×10^5 or 2×10^7 cells per animal for the HEK-293 cells). Animals were examined at intervals of about 3 days for palpable tumors. After 24 days, the animals were sacrificed and the tumors were excised and weighed.

2.9. Migration assay

To explore further the relationship between tNOX expression and the transformed phenotype, migration assays used Matrigel^{TM} (Collaborative Biomedical Products, Bedford, MA), a biologically active basement membrane model in an in vitro invasion assay. Matrigel was thawed at 4°C overnight and diluted in cold serum-free medium (1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium containing glutamine 292 mg/L, gentamycin sulfate 50 mg/L, insulin 10 μg/mL, hydrocortisone 0.5 μg/mL and EGF 20 ng/mL). Transfected MCF-10A cells were resuspended and seeded at 5×10^4 cells/ml of Matrigel-medium solution in 24-well plate. Cells were incubated at 37°C and inspected daily by light microscopy.

2.10. Oligomeric antisense transfection in HeLa cells

HeLa cells were transfected with either nonsense (5′-CGATGCTAGTGAGCC-3′) or antisense (5′-GGCTAGCATACGCTG-3′). Oligomeric nucleotides were synthesized by Genset Oligos (La Jolla, CA). Transfection used LipoTAXI mammalian transfection kits (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

2.11. Growth in soft agar

For soft agar cultures, transfected HeLa cells were suspended at 2.5×10^4 cells/mL in MEM medium plus 10% bovine calf serum with gentamycin sulfate containing 0.33% Noble agar (DIFCO Laboratories, Becton Dickson). Cell suspensions were seeded on top of 0.5% agar base in 10 cm culture dishes. Cells were cultured at 37°C for 2 weeks and 0.33% Noble agar in MEM with 10% FCS was added to provide additional nutrients and growth factors. Anchorage-independent colony formation was evaluated daily by light microscopy.

2.12. Measurement of cell enlargement

Cells were grown for 24 h at 37°C on glass coverslips placed in 2.5 cm culture dishes with medium. Cell enlargement was evaluated using an Olympus Vanox-S Microscope, Model AHBS, using bright field and/or differential interference contrast optics. The microscope was coupled to a Hamamatsu CE400-07 video camera system with a Hamamatsu camera head (700 horizontal lines resolution) for viewing and data recording. As required, Hamamatsu Argus-10 and Argus-20 real time digital contrast and low light enhancement image processing were used. Increases in cell surface area were monitored for 90 or 120 min. In order to maintain the proper growth environment for the cells, a chamber was constructed to allow fresh medium to flow under the coverslip. Transparent tape and grease were applied along the

edges of the slide to secure the coverslip to avoid movement as medium flowed through the space. The coverslip was placed upside-down over the slide to avoid having to focus through the medium. The cells were regularly supplied with fresh medium. Temperature control was provided by a brass block with a viewing port that was placed over the microscope slide and through which water was circulated. Temperature at the slide surface was monitored by means of a thermocouple.

Using freeze frame technology, the tape was stopped at 1 min intervals. The cell perimeter was then digitally traced. Cell areas were calculated using the Hamamatsu Argus-10, equipped with the appropriate software. Each frame was measured three times. The measurements were then averaged and standard deviations were determined using Microsoft Excel.

2.13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was with the buffer system of Laemmli [9] and acrylamide slab gels. Proteins were denatured in sample buffer by boiling for 3 min and analyzed by SDS-PAGE (12% acrylamide). Standard marker proteins (Sigma markers, low molecular weight) were used. The resolved proteins were stained using 0.1% Coomassie Brilliant Blue R-250 or silver [2].

2.14. Western blotting

Proteins were resolved on SDS-PAGE (10% acrylamide) as described above and then were transferred by electroblotting onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After transfer, the proteins were stained with Ponceau S (Sigma, St Louis, MO). The molecular weight marker lane was cut off and the remaining membrane was destained using water and then incubated in a TBS-T (10 mM Tris-HCl, pH 8.0; 0.15 M NaCl, 0.05% Tween 20) solution containing 3% dry milk with shaking for 30 min at room temperature to block the unspecific antibody binding sites. The blocked membranes were incubated in 1:400 v/v primary antibody in TBS-T solution overnight with shaking at room temperature. The primary antibody was a polyclonal peptide antibody toward the adenine nucleotide binding site of human tNOX [23].

After washing four times with TBS-T solution for 15 min each, the nitrocellulose membrane was placed in secondary antibody in TBS-T solution (titer of 1:10,000) (goat anti-rabbit linked to alkaline phosphatase, Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The nitrocellulose membrane was washed with TBS-T solution four times, 15 min each and reacted with nitro blue tetrazolium/5-bromo-1-chloro-3-indolyl phosphate chromogenic alkaline phosphatase substrate followed by washing away the un-reacted substrate to stop the reaction.

3. Results

3.1. Vector-forced overexpression

Vector-forced overexpression experiments were carried out to test rigorously the hypothesis that the specific responses of cancer cells to EGCg and capsaicin as examples of tNOX inhibitors correlated with tNOX expression. When overexpressed in COS cells, Western blot analysis revealed both the 48 kDa tNOX expression product plus processed tNOX intermediates including the 34 kDa cell surface form (Fig. 1(A)). Results were similar with MCF-10A (Fig. 1(B)) and HEK 293 (Fig. 1(C)) cells. The transfected cells also exhibit increased cell surface tNOX activity as compared to wild type (Table 1, Fig. 2).

Table 1
Total cell surface NADH oxidase activities of cell lines transiently transfected with tNOX cDNA compared to vector alone and wild type cells and inhibition by 1 μM capsaicin. This concentration of capsaicin was sufficient to give complete inhibition of recombinant tNOX [6]. Averages ± standard deviations from 3 trials

Cell line	NADH oxidation, nmoles/min/10^6 cells ± standard deviation		
		No addition	+ 1 μM capsaicin
COS	Control	0.85 ± 0.05	0.78 ± 0.08
	Vector alone	0.80 ± 0.06	0.78 ± 0.075
	tNOX-transfected	1.05 ± 0.07*	0.80 ± 0.07
MCF-10A	Control	0.80 ± 0.05	0.80 ± 0.04
	Vector alone	0.80 ± 0.04	0.83 ± 0.04
	tNOX-transfected	1.00 ± 0.08*	0.80 ± 0.03
HEK-293	Control	0.80 ± 0.05	0.80 ± 0.05
	Vector alone	0.96 ± 0.09	0.84 ± 0.06
	tNOX-transfected	1.11 ± 0.12*	0.87 ± 0.03

Values marked by an asterisk were significantly different ($p < 0.02$).

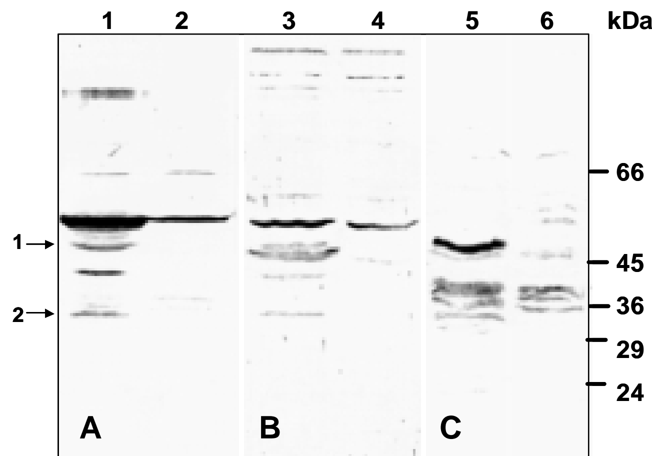

Fig. 1. Overexpression of tNOX. A. COS cells. B. MCF-A0A cells. C. HEK 293 cells. Lanes 1, 3, and 5 are tNOX transfected. Lanes 2, 4 and 6 are vector alone. Band 1 (arrow labeled 1) is the full length translation product at 48 kDa. Band 2 (arrow labeled 2) is the fully processed mature 34 kDa tNOX form. Detection used a peptide antibody to the semi-conserved adenine-binding region of tNOX C-terminus.

3.2. *Overexpression and cell enlargement*

Overexpression of tNOX enhanced enlargement of COS cells (Fig. 3). Cell diameter was increased on average by about 2-fold, yielding a 5-fold increase in cell volume.

The rate of cell enlargement was accelerated in the tNOX-transfected COS cells (Fig. 4). When measured at intervals of 1 min, cell enlargement rate oscillated with intervals of rapid enlargement separated by intervals of where little or no enlargement occurred. With the tNOX-transfected COS cells, rates of enlargement during both the rapid phases and the intervening slow phases were nearly doubled (Fig. 4).

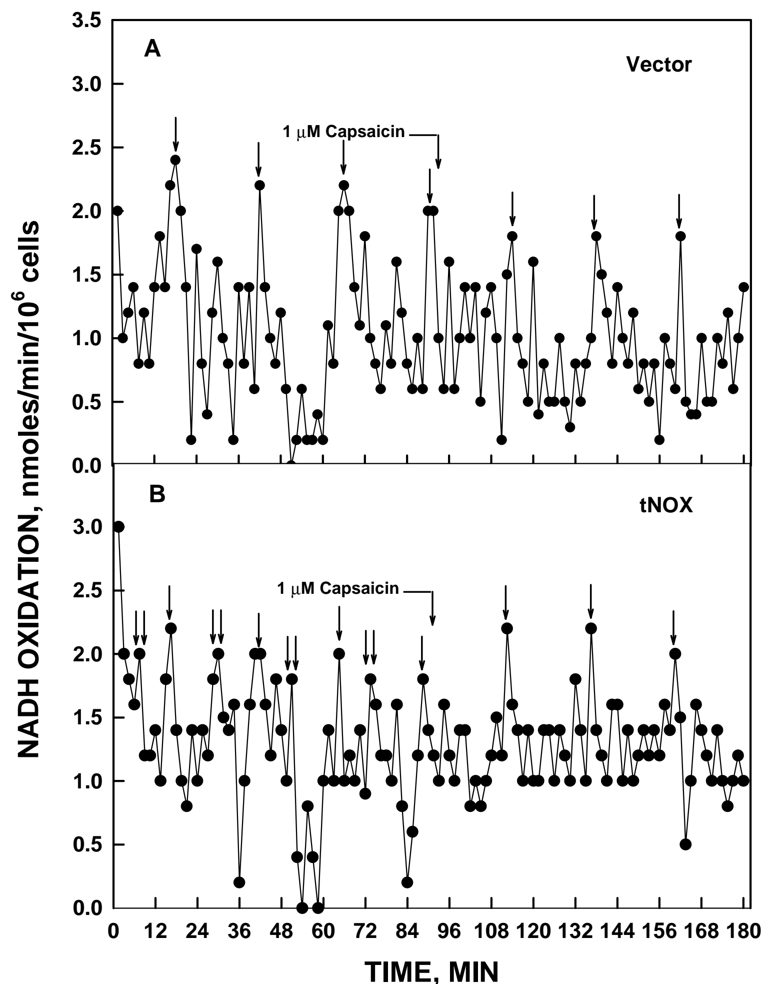

Fig. 2. NOX activity of tNOX transfected MCF-10A cells compared to cells transfected with vector alone. ECTO-NOX proteins are universally characterized by regular patterns of oscillations in enzymatic activity. Here rates of NADH oxidation based on decrease in A340 were determined over 1 min at intervals of 1.5 min over 180 min as described [24]. The definitive indicator of tNOX would be an oscillating activity with a period length of 22 min susceptible to drug inhibition. Capsaicin (1μM) was added at 90 min to inhibit tNOX. A. Vector alone. Arrows denote maxima at intervals of 24 min corresponding to the capsaicin-resistant constitutive CNOX. B. tNOX transfected. Single arrows denote maxima at intervals of 24 min corresponding to the capsaicin-resistant constitutive CNOX. Double arrows denote an activity resulting from transfection with maxima separated by 22 min. The activity resulting from transfection was inhibited completely by capsaicin to provide for its definitive identification as resulting from tNOX expression.

3.3. Overexpression and drug response

COS cells are malignantly transformed by the SV40T antigen and express a low level of drug-responsive NOX (tNOX) activity as illustrated both for EGCg (Fig. 5(A)) and for capsaicin (Fig. 5(B)). However, the tNOX transfected COS cells were more responsive to these and other tNOX-inhibitory drugs (Table 2). Generally, the dose necessary to inhibit proliferation by 50% was reduced by one log as a result of tNOX overexpression. Response to the cancer inactive sulfonylurea LY181985, which differs from the active LY181984 by a single chlorine, was unaffected by tNOX overexpression as was the response to

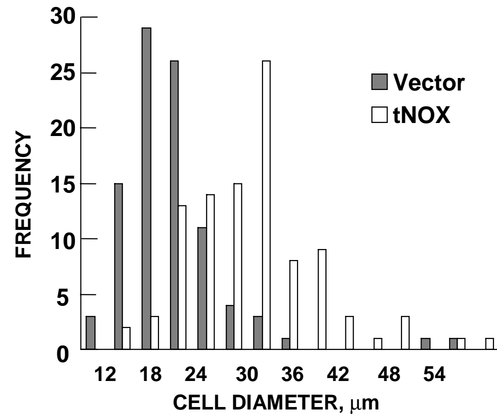

Fig. 3. Longitudinal diameters of tNOX transfected COS cells (open bars) measured directly from light microscope (40X) images were approximately two times larger than those of untransfected COS cells (solid bars).

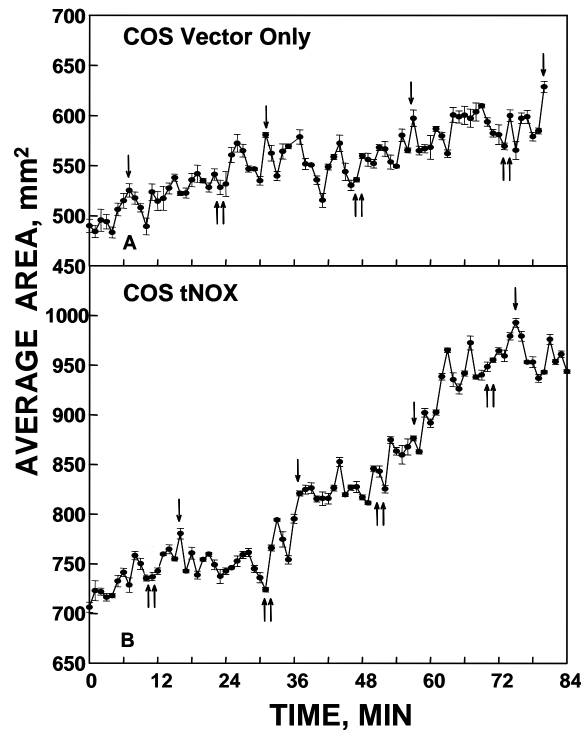

Fig. 4. Increase in area (enlargement growth) of COS cells transfected with the vector alone (A) or with tNOX cDNA (B) as determined by image-enhanced light microscopy [25]. The growth rates fluctuated with a complex pattern of periodicity but were approximately 2-fold greater with the tNOX transfected cells. In A, single arrows separated by intervals of 24 min indicate periods of rapid enlargement alternating with resting periods (double arrows). In B, the single arrows indicating periods of rapid enlargement and intervening rest periods (double arrows) are separated by intervals of 22 min.

tamoxifen and methotrexate, neither of which affected tNOX activity.

The transfection experiments were extended to MCF-10A mammary epithelial cells which lack tNOX at their cell surface and were refractory to growth inhibition by tNOX inhibitors [13,21]. Expression

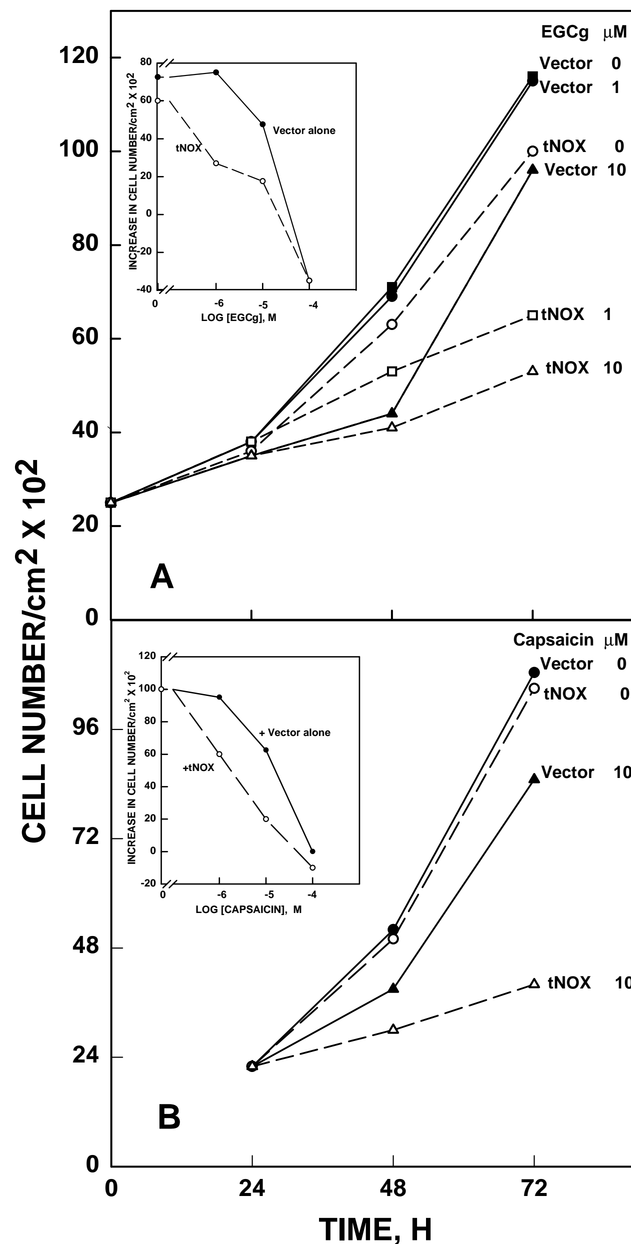

Fig. 5. Response of untransfected (vector alone) and transfected (tNOX) COS cells to the tNOX inhibitor EGCg (A) and to capsaicin (B). The insets show the dose response. Sensitivity of the COS cells to both EGCg and to capsaicin was increased by approximately one log order by transfection.

of tNOX was verified by Western blot (Fig. 1(B)) Stable MCF-10A transfectants were sensitive to both capsaicin and EGCg (Table 2). Furthermore, tNOX transfectants of MCF-10A cells formed colonies after one week incubation in Matrigel and the colonies rapidly invaded the Matrigel (Fig. 6). The tNOX-transfected MCF-10A cells then increased in size over a second week of incubation (Fig. 6(C,D)). With both the wild type MCF-10A cells and the cells transfected with vector alone, many fewer colonies were

Table 2
Inhibition by tNOX-targeted drugs plus LY181985, tamoxifen and methotrexate on growth of COS cells (inhibition of the increase in cell number) as a result of stable transfection with tNOX cDNA. Values were estimated from triplicate dose response determinations as shown for Fig. 5

Drug1	IC_{50} (μM)	
	Nontransfected	tNOX transfected
EGCg	10	0.1
Capsaicin	15	2.3
Adriamycin	0.3	0.04
LY181984 (active)	20	3.0
LY181985 (inactive)	>100	>100
Tamoxifen	16	8
Methotrexate	1	1

1EGCg (-)-epigallocatechin-3-gallate; Capsaicin, 8-methyl-N-vanillyl-6-noneamide; LY181984, N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl)urea; LY181985, N-(4-methylphenylsulfonyl)-N'-(4-phenyl)urea.

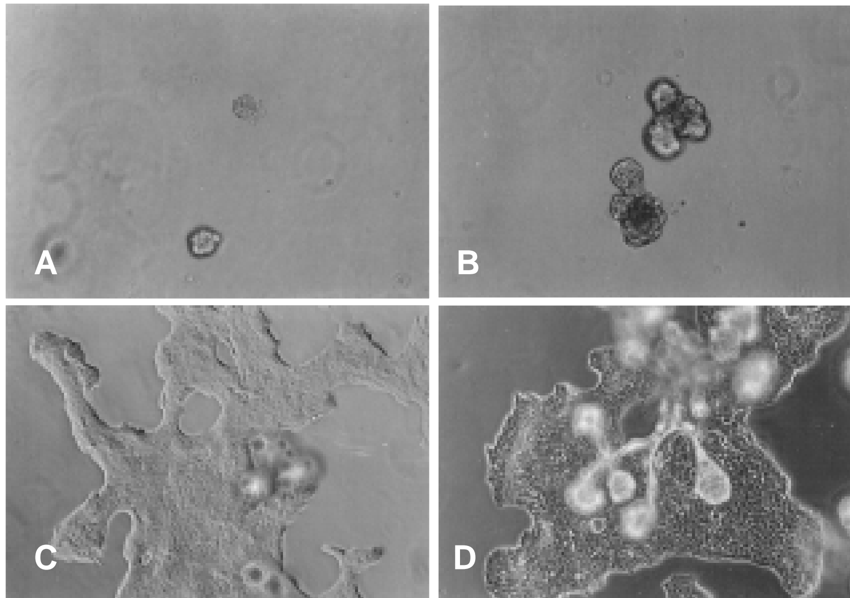

Fig. 6. Effect of tNOX on invasion ability of MCF-10A cells (non-cancer human mammary epithelia) transfected with tNOX evaluated by growth in Matrigel. A. Wild-type MCF-10A cells. B. Empty pcDNA 3.1-transfected MCF-10A cells. Neither are able to grow in the Matrigel. C and D. Two different clones of MCF-10A cells stably transfected with tNOX pcDNA 3.1. The cells exhibited a transformed phenotype and formed extensive colonies in the Matrigel.

formed and the colonies failed to expand in size (Fig. 6(A,B)).

3.4. Inhibition of tNOX expression by antisense oligonucleotides to tNOX

In HeLa cells transfected with the tNOX antisense oligonucleotide, tNOX expression was substantially reduced (Fig. 7). Growth inhibition in response to both capsaicin and EGCg was reduced or eliminated

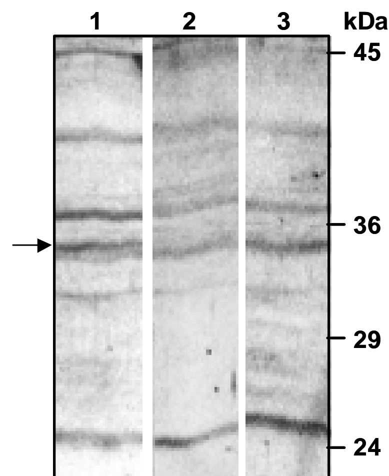

Fig. 7. Western blot analysis of antisense transfected of HeLa cells using a peptide antibody to the adenine nucleotide binding region of the tNOX C-terminus. Lane 1, nonsense transfected. Lane 2, antisense transfected. Lane 3, untransfected HeLa cells. The arrow indicates the 34 kDa processed plasma membrane form of tNOX as the upper band of an apparent doublet. The band at ca. 24 kDa serves as a protein loading control. The most striking effect of the antisense was the reduction in the upper band of the doublet at 34 kDa. Quantitation relative to the ca. 24 kDa band together with measurements of drug-responsive and periodic NADH oxidase activity were consistent with at least a 30–50% overall reduction in the processed tNOX 34 kDa drug-responsive tNOX protein as a result of transfection with antisense.

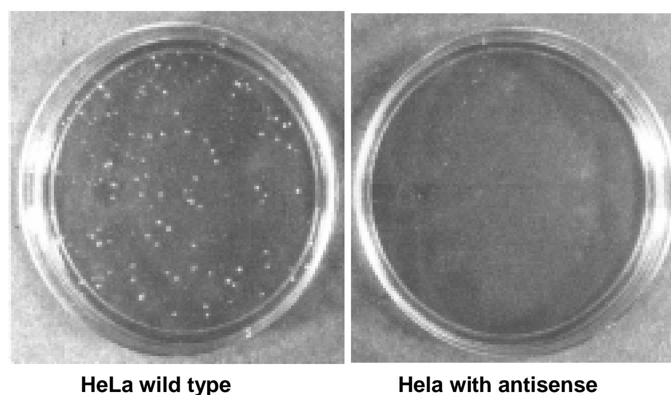

Fig. 8. Transfection of HeLa cells with tNOX antisense results in a reduced ability to form colonies on soft agar (B) compared to wild type (A).

by antisense tNOX (Table 3). With HeLa cells, the IC50 for growth inhibition was approximately 10 μM for both EGCg [21] and for capsaicin [13]. With subcultures transiently transfected with tNOX antisense oligonucleotides, the IC_{50} for both EGCg and for capsaicin was increased to about 100 μM. Additionally, HeLa cells transfected with antisense demonstrated a reduced tNOX activity along with a reduced ability to grow on soft agar (Fig. 8).

3.5. Tumors in immunocompromized mice

To determine if tNOX overexpression was sufficient to result in tumorigenic transformation, stably transfected MCF-10A cells (Fig. 1(B)) were implanted in immunocompromised mice. Animals injected

Table 3
HeLa cells transfected with tNOX antisense were no longer inhibited by capsaicin or EGCg. Determinations were in duplicate as for Table 2 ± mean average deviations

Transfection	% Inhibition after 72 h	
	100 μM Capsaicin	10 μM EGCg
None	76 ± 4	40 ± 8
Nonsense	60 ± 6	25 ± 6
Antisense	5 ± 2*	0 ± 0*

Values within each column marked by an asterisk were significantly different ($p < 0.002$).

with cells transfected with vector alone were tumor free after 20 days as were most of the animals receiving the transfected MCF-10A cells. Only one animal out of eight developed a small tumor in the two experiments. With stably transfected HEK-293 cells (Fig. 1(C)), tumor incidence was unaffected by transfection. At least one animal in all three treatment groups (parental, vector and tNOX) developed a tumor. For animals developing tumors, average tumor weights were not significantly different among animals after 24 days.

4. Discussion

A defining characteristic of the tNOX protein, a tumor- (cancer-) specific and growth-related hydroquinone oxidase with protein disulfide thiol interchange activity of the cell surface, is its inhibition by a series of quinone site inhibitors or putative quinone site inhibitors including EGCg, capsaicin, adriamycin and antitumor sulfonylureas, all of which have anticancer activity [20]. They inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting the constitutive NOX or growth of non-cancer cells [13,14,21].

As detailed in published reports [22,24,25], NOX activities correlate with the enlargement phase of cell growth. When inhibited, the cells divide normally and DNA and protein synthesis are not inhibited but the cells fail to enlarge [24]. The resultant small cells, unable to enlarge, fail to divide and, after a few days, begin to undergo apoptotic cell death [12,19,21,24].

The presence of the tNOX protein has been demonstrated in a small sampling of human tumor tissues (mammary, prostate, neuroblastoma, colon xenografts). However, serum analyses suggest a much broader association with human cancer. tNOX proteins are ectoproteins reversibly bound at the outer leaflet of the plasma membrane [11]. As is characteristic of other examples of ectoproteins (sialyl and galactosyl transferases, dipeptidylamino peptidase IV, etc), the tNOX proteins are shed. They appear in soluble form in conditioned media of cultured cells [16] and in patient sera [17,18]. The serum form from cancer patients exhibits the same degree of drug responsiveness as does the membrane-associated form [17,18]. When assayed for drug-responsive tNOX activities, activity is seen in sera of a variety of human cancer patients, including patients with leukemias, lymphomas or solid tumors (breast, colon, prostate, lung, pancreas, ovarian, liver). With sera from more than 500 cancer patients, the majority (ca 490) were found to exhibit the drug-responsive activity [17, 18, results unpublished]. In contrast, no drug-responsive NOX activities were found with sera of healthy volunteers. We postulate that the serum presence of the antitumor sulfonylurea-responsive NADH oxidase represents an origin due to shedding from the patient's cancer. As such, the antitumor-responsive tNOX activity represents the first reported cell surface change universally associated with most, if not all, forms of human cancer.

The significance of tNOX rests principally in its potential as a cell surface alteration specifically and universally associated with human cancer. Specificity (absence from normal cells and tissues) is indicated from lack of a drug-responsive NOX activity in normal tissues, lack of a serum form of tNOX in healthy volunteers and patients with disorders other than cancer and indications from tNOX-specific antisera [4]. Findings to date with both cultured cells and sera offer no evidence for a tNOX cross-reactive protein other than with human cancer cells or sera of cancer patients.

To test rigorously the hypothesis that tNOX is related to the tNOX-specific drug response, a series of overexpression experiments were carried out. When overexpressed in COS cells (Fig. 1(A)), the tNOX cDNA resulted in larger cells (up to 5-fold increase in volume) (Fig. 3) and an accelerated rate of cell enlargement (Fig. 4). COS cells are a cancer line and express a low level of drug-responsive NOX (tNOX) activity (Table 1). However, the COS cells overexpressing the tNOX protein exhibit one to two log orders of increased drug sensitivity to the entire spectrum of anti-NOX quinone site directed anticancer drugs (Table 2; Fig. 5).

The transfection experiments were extended to MCF-10A mammary epithelia which lack tNOX at their cell surface and are refractory to growth inhibition by NOX inhibitors [13,21]. We were able to obtain stable tNOX transfectants with this cell line (Fig. 1(B)) and induce, for example, an increased rate of enlargement and growth on soft agar as well as sensitivity to EGCg and capsaicin. Additionally, the tNOX-transfected MCF-10A cells acquired a potentially invasive phenotype as evidenced by an ability to infiltrate through Matrigel not shown with wild type or sham-transfected MCF-10A cells (Fig. 6). As a positive control, comparisons were with transfectants with tNOX cDNA mutated in the drug binding pocket (M396A) in which NOX activity was unaffected but where drug-responsiveness was absent [25].

These findings demonstrate that tNOX expression is sufficient to result in an inhibitory cell growth-response to EGCg as well as other tNOX inhibitors. The necessity of tNOX for a growth response to low doses of EGCg or capsaicin was demonstrated using oligomeric antisense to tNOX where loss of cell surface tNOX from normally responsive HeLa cells resulted in both loss of growth inhibition (Table 3) and a reduced capacity to form colonies on soft agar (Fig. 8). This response is unrelated to transfection per se [15]. For example, rat kidney cells which lack a drug-responsive ECTO-NOX activity at their surface were unaffected by the sulfonylurea LY181984, a potent tNOX inhibitor, when stably transfected with K-ras. In contrast stable H-ras transfectants of the same cell line exhibited both a sulfonylurea-inhibited ECTO-NOX activity and growth response.

tNOX, however, cannot be formally regarded as an oncogene. Its expression alone as a result of transfection is insufficient to result in tumorigenic transformation. This was evidenced by an inability of stably transfected MCF-10A or HEK-293 cells to exhibit an increased incidence or size of tumors when implanted in immunocompromised mice.

The findings support the concept that tNOX, by facilitating unregulated cell enlargement, serves an essential role in cancer etiology. When combined with oncogenic loss of checkpoint control, tNOX expression would insure uninterrupted traverse through the cell cycle as required by the unregulated cell proliferation that typifies cancer.

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