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NOX5 is expressed at the plasma membrane and generates superoxide in response to protein kinase C activation

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Abstract

NOX5 is a ROS-generating NADPH oxidase which contains an N-terminal EF-hand region and can be activated by cytosolic Ca^{2+} elevations. However the C-terminal region of NOX5 also contains putative phosphorylation sites. In this study we used HEK cells stably expressing NOX5 to analyze the size and subcellular localization of the NOX5 protein, its mechanisms of activation, and the characteristics of the ROS released. We demonstrate that NOX5 can be activated both by the protein kinase C activating phorbol esther PMA and by the Ca^{2+} ionophore ionomycin. The PMA- but not the ionomycin-dependent activation can be inhibited by protein kinase C inhibitors. NOX5 activity is inhibited by submicromolar concentrations of diphenyl iodonium (DPI), but not by apocynin. Western blot analysis showed a lower (~70 kDa) than expected (82 kDa) molecular mass. Two arguments suggest that NOX5 is at least partially expressed on the plasma membrane: (i) the membrane-impermeant superoxide was readily detected by extracellular probes, and (ii) immunofluorescent labeling of NOX5 detected a fraction of the NOX5 protein at the plasma membrane. In summary, we demonstrate that NOX5 can be found intracellularly and at the cell surface. We also describe that it can be activated through protein kinase C, in addition to its Ca^{2+} activation.
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Keywords: NADPH oxidase; Inhibitors; Western blot; Immunofluorescence; Superoxide; Hydrogen peroxide

1. Introduction

Reactive oxygen species (ROS) have been implicated in a number of biological processes, such as differentiation, apoptosis, proliferation, changed transcription patterns, channel function among others (reviewed in [1,2]). The NADPH oxidase (NOX) family of enzymes is a major source of ROS production in cells, with the sole apparent purpose of generating ROS. The NOX family contains seven members (NOX1-5 and DUOX1 and 2) which show distinct tissue distribution and mechanisms of activation. The best studied NOX member is the phagocyte NADPH oxidase, NOX2, which is predominantly expressed in neutrophils and macrophages. Activation of NOX2 is responsible for the massive ROS production observed during the respiratory burst which is involved in the killing of ingested microorganisms. NOX2 itself is a membrane protein with six transmembrane domains, but its expression requires stabilization by another membrane protein, $p22^{phox}$. Activation of NOX2 requires translocation of cytoplasmic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$) and activators (Rac1 or Rac2) to the membrane-bound NOX2/$p22^{phox}$. NOX2 activation mechanisms include the phosphorylation of its cytoplasmic subunit $p47^{phox}$ [3]. The mechanisms of activation of NOX1 and NOX3 are not fully understood, but also depend

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on cytosolic regulatory subunits and may require phosphorylation at least under some circumstances [4–8].

In contrast to NOX1, 2 and 3, NOX4 and NOX5 do not appear to require cytosolic subunits for activation. NOX4 is associated with $p22^{phox}$ and seems to be constitutively active in most cell types, while NOX5, with 4 N-terminal EF-hand domains, can be activated by elevations of the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$ [9].

NOX5 is expressed in various fetal tissues, uterus, testis, spleen, lymph nodes and endothelial cells [10,11]. Interestingly, rodents appear to have selectively lost NOX5, while other mammals have one NOX5 gene, and plants express a multitude of NOX5-like genes [12]. In humans, there are at least five splice variants of NOX5: NOX5α is expressed in lymphoid tissues; the NOX5β form is found in testis: NOX5γ for which evidence for a presence in a cellular type has not been confirmed to date; NOX5δ, which is found in human microvascular endothelial cells (HMEC-1) [13]; and NOX5ε, also called NOX5S, which is a shorter form without EF-hands expressed mainly in embryonic tissues [10]. An increased expression of NOX5 has been observed in certain pathologies, such as hairy cell leukemia [14], melanoma cells [15], prostate cancer cells [16] and Barret's mucosa [17].

Herein, we used HEK293 cells stably expressing human NOX5β as a tool to study NOX5 activation and localization. We demonstrate that NOX5 can be activated by Ca^{2+} and by a pathway involving PKC and inhibited by DPI, but not by apocynin, the most widely used NOX inhibitors. We observed a perinuclear localization of NOX5 as well as cell surface expression, and a migration of NOX5 on SDS gels slightly lower than predicted by computer models.

2. Material and methods

2.1. Chemicals, enzymes and buffers

Dulbecco's modified Eagle's medium (DMEM), Hank's buffered salt solution (HBSS), fetal calf serum, neomycin (G418, geneticin) and Amplex Red were purchased from Invitrogen. pGEM-T vector was purchased from Promega. Streptavidin–horseradish peroxidase conjugate was purchased from Amersham. Protein kinase inhibitors were purchased as a kit from Calbiochem. Penicillin, streptomycin, phorbol myristate acetate (PMA), ionomycin, apocynin, luminol, cytochrome C and diphenylene iodonium chloride (DPI) were purchased from Sigma Aldrich.

2.2. Cell culture

HEK 293 cells, stably expressing NOX5, were generated by transfection of pcDNA3.1 with the full-length human NOX5β cDNA with a neomycin resistance gene followed by clone selection after neomycin culture for 10–14 days as described in [11]. All cells were cultured in DMEM, 4.5 g/l glucose, supplemented with 10% fetal calf serum and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in air with 5% CO_2. Selection pressure was maintained by continuous inclusion of neomycin (400 μg/ml) in transfected cell cultures.

2.3. Measurements of reactive oxygen species

Cells were trypsinized (1× Trypsin–EDTA), collected by centrifugation, and washed with PBS to remove medium. Cells were seeded into 96-well plates at a density of 20,000 cells in 200 μl testing buffer at 37 °C.

Extracellular hydrogen peroxide was measured by Amplex UltraRed with a testing buffer containing 25 μM Amplex UltraRed and 0.1 U/ml horseradish peroxidase in HBSS. Fluorescence was recorded with excitation and emission wavelengths of 550 nm and 600 nm respectively.

Extracellular superoxide was measured either by cytochrome *c* reduction, in a testing buffer containing 150 μM cytochrome c in HBSS with the absorbance at 550 nm recorded, or alternatively by a luminol assay, with a testing buffer containing 10 μM/ml luminol and 0.5 U/ml HRP with continuous recording of light emission.

For each detection method, one measurement per minute was recorded for 40–60 min in either a Victor3 Wallac or a BMG Fluostar microplate reader. When indicated, ionomycin (1 μM) or PMA (100 nM), protein kinase inhibitors, DPI (0.01–10 μM) or apocynin (2–63 μM) was added just before measurement.

2.4. Western blots

Cells were lysed on ice in 1% Triton X-100 in 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 50 mM Tris–HCl, pH 7.4, supplemented with a protease inhibitor cocktail (CompleteTM mini, Boehringer-Mannheim) and sonicated. Protein concentration was determined by the BioRad protein assay. Samples of equal protein quantity were separated on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVD) membranes for Western blotting according to standard techniques, and probed with rabbit anti NOX5 antibodies 1/1000 as previously described [9]. Antibody-binding was visualized by the addition of HRP-labeled rabbit anti-goat IgG (1/20 000) and enhanced chemiluminescence reagents (ECL reagent, Amersham Biosciences). Molecular weights were determined by interpolation of the standard curve generated using the BioRad Precision Plus Dual Stained Color Standard.

2.5. In vitro translation

Full-length NOX5β was subcloned into pGEM-T vector containing a T7 promoter and added to a reticulocyte mix provided by TNT® Quick Coupled Transcription/Translation System (Promega). Transcription and translation were performed according to the manufacturer's instructions with the insertion of chemiluminescent labeled amino acids using a Transcend™ chemiluminescent tRNA (Promega). The reaction was performed in a 50 μl volume using 1 μg of NOX5 plasmid. The translated protein was separated by electrophoresis on SDS PAGE gel and revealed by chemiluminescence (not shown)

or transferred to a PVD membrane and revealed with a NOX5 antibody.

2.6. Confocal microscopy

Cells were plated 24 h prior to experiments on glass slides mounted with plastic chambers (8-well slides). The media was replaced by 250 µl HBSS containing 20 µM Alexa 488-labelled Concanavalin A (ConA). After 15 min incubation at 4 °C, medium was taken off and replaced by ice-cold paraformaldehyde (4%) in HBSS. The cells were permeabilized and non-specific staining blocked by incubation in 10% goat serum and 0.1% Triton X-100 in PBS for 20 min. Anti-NOX5 antibody, diluted 1/1000 in blocking solution, was added and the cells were then incubated for 60 min at room temperature. Following washes, a secondary Alexa 555-goat anti rabbit antibody (Molecular Probes) was added (1/2000). DAPI (5 µM) was added in the secondary antibody mix to counterstain the nuclei. After 30 min, the cells were washed carefully, mounted on cover-slips and immunofluorescence images were acquired using a laser scanning confocal microscope (LSM 510 Meta Carl Zeiss AG, Switzerland) with a plan-Apochromat 63×1.4 NA oil immersion objective.

3. Results

3.1. Prediction of phosphorylation on NOX5

The NetPhos 2.0 Server was used for prediction of serine-threonine phosphorylation of NOX5 [18]. Eleven serine residues were considered to have a probability higher than 95% of being phosphorylated (Fig. 1a). S111, S114 are located in the specific intracellular NOX5 N terminal region between EF hands domains III and IV. S346 lies in the intracellular loop between transmembrane domains IV and V. S486, S490, S492, S498, S501, S502 and S505 are located in a serine rich region in NOX5 intracellular C-terminal with no identity with any other members of the NOX family. S574 and S659 are located within the NADPH binding sites. Only S346 aligns with a serine residue in NOX4, and S505 aligns with NOX4 and DUOX2 (Fig. 1b).

3.2. PMA stimulates NOX5-dependent superoxide production

We therefore investigated the effect of the phorbol esther PMA, which binds to and activates protein kinase C, on stably NOX5 expressing HEK293 cells and compared it to the effect of the Ca^{2+} ionophore ionomycin. Neither PMA nor ionomycin induced ROS generation in untransfected HEK293 cells (data not shown). In the absence of a stimulus, NOX5-expressing cells produced very low amounts of ROS. PMA stimulation activated ROS generation in NOX5 expressing cells as efficiently as ionomycin. This ROS generation could be detected by cytochrome *c* and by luminol-enhanced chemiluminescence (Fig. 1a–d), which detect extracellular superoxide, as well as by Amplex Red, which detects extracellular hydrogen peroxide (Fig. 2e,f). Thus, we demonstrate that NOX5 can be activated by PMA, in addition to its well-known Ca^{2+}-mediated activation by ionomycin. We also demonstrate that extracellular superoxide can be detected. As the negatively charged superoxide ion does not penetrate through the phospholipid bilayer of biological membranes, this demonstrates that at least a fraction of the NOX5 is localized at the plasma membrane, where it can transfer electrons from the intra- to the extra-cellular space.

3.3. Effect of different protein kinase inhibitors on PMA- and ionomycin-induced activation of NOX5

We next investigated the identity of the protein kinases involved in the PMA activation of NOX5. For that purpose, a panel of cell permeant serine-threonine kinase inhibitors was tested on the ROS generation induced by either PMA or ionomycin. Prior to stimulation, cells were incubated for 5 min with inhibitors; bisindolylmaleimide I (BMI) (a specific PKC inhibitor); staurosporine (a broad range kinase inhibitor); KN92 (an inhibitor of calmodulin dependent protein kinase); ML-7 (a selective inhibitor of myosin light chain kinase); H-89 (an inhibitor of cAMP dependent protein kinase A); and PKG-I (an inhibitor of cGMP dependent protein kinase). Both PKC inhibitors BMI and staurosporine blocked the ROS generation induced by PMA, while the other inhibitors had no effect (Fig. 3a). This effect was specific for the PMA-activated pathway as neither inhibited ionomycin-induced NOX5 activity (Fig. 3b). PMA itself did not induce an elevation in $[Ca^{2+}]_c$ (data not shown). Taken together these results strongly suggest that Ca^{2+}-induced NOX5 activity is independent of PKC and that PMA-stimulated NOX5 activity is mediated by PKC.

3.4. NOX5 is inhibited by submicromolar DPI concentrations

DPI and apocynin are inhibitors of NOX2 and, to varying extent, of other NOX enzymes. DPI appears to broadly inhibit NOX enzymes, but is relatively non-specific and inhibits the mitochondrial respiratory chain with a half maximal inhibitory concentration (IC50) between 5 and 10 µM. In contrast, apocynin has a more restricted activity, as it seems to inhibit subunit assembly of NOX2 and might even require metabolism by a peroxidase (for review see [1]). The IC50 for DPI was in the nanomolar range for both Ca^{2+}-induced NOX5 activity (137 nM) and PKC-mediated NOX5 activity (343 nM) (Fig. 4a). In contrast, even at a concentration of 63 µM, apocynin had very little effect on NOX5 activity (Fig. 4b). Thus, we conclude that DPI inhibits NOX5 (similar to other NOX enzymes) in the submicromolar range, i.e. at concentration where it does not inhibit the mitochondrial respiratory chain [19,20]. In contrast, apocynin is not a NOX5 inhibitor.

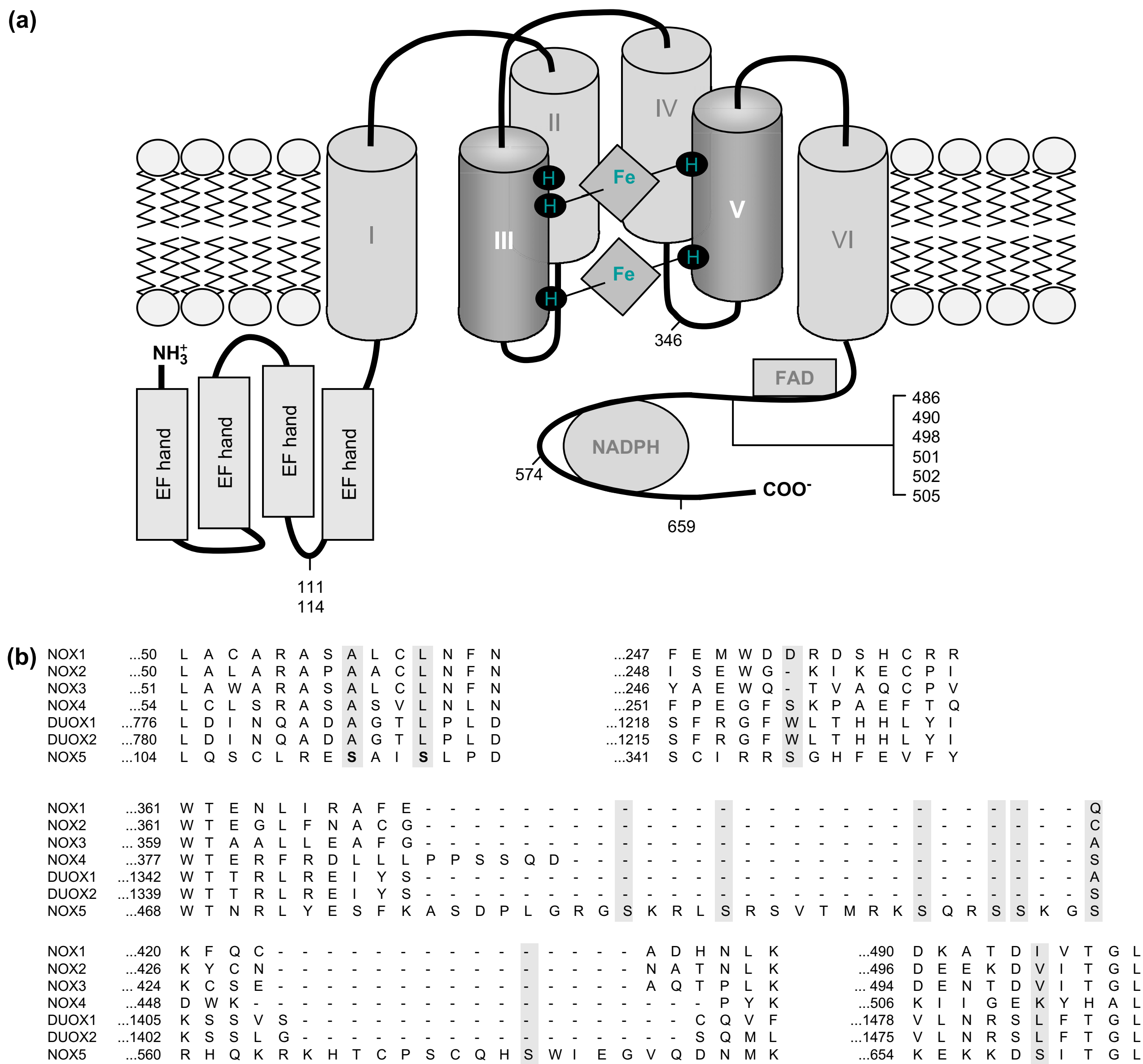

Fig. 1. Predicted phosphorylation sites on NOX5. (a) Schematic representation of NOX5 with predicted phosphorylation sites for serine-threonine kinases. Using NetPhos 2.0 Server, it was calculated that 11 serine residues had a probability >95% of being phosphorylated. The predicted phosphorylation sites are noted on the schematic representation of NOX5. (b) Alignment of NOX1 (DQ314883), NOX2 (NM_000397) NOX3 (NM_015718), NOX4 (NM_016931), NOX5β (AF325189), DUOX1 (AAI14939) and DUOX2 (EAW77288) showing the regions encompassing the predicted phosphorylation sites of NOX5. With the exceptions of S346 and S505, all other serines predicted to be phosphorylated are unique to NOX5.

3.5. Apparent molecular weight of NOX5 on SDS gel electrophoresis

The predicted molecular mass of NOX5 protein is 82 kDa. However, an anti NOX5 antibody consistently recognized a band of approximately 70 kDa (and a smaller band of approximately 42 kDa) in NOX5 expressing cells (Fig. 5).

A band of ∼70 kDa was also detected by the anti-NOX5 antibody when NOX5 was transcribed/translated in vitro. The same band of 70 kDa appeared in the in vitro translation experiment using labeled amino acids (data not shown). The ∼42 kDa band could potentially correspond to a cleavage product. It might be a homodimer of the cleaved off EF hand regions (monomeric MW ∼18 kDa) or a single fragment cleaved at around amino acid 400.

3.6. Determination of cellular localization of NOX5 by immunofluorescence

Confocal images of HEK cells stained with ConA (green) and anti-NOX5 (red) demonstrate that ConA selectively binds to plasma membrane sugars while NOX5 was detected only in cells expressing NOX5 (Fig. 6). A strong perinuclear staining and a more discrete, but clearly discernable staining of the plasma membrane is observed. The perinuclear staining may correspond to the endoplasmic reticulum (ER), however as

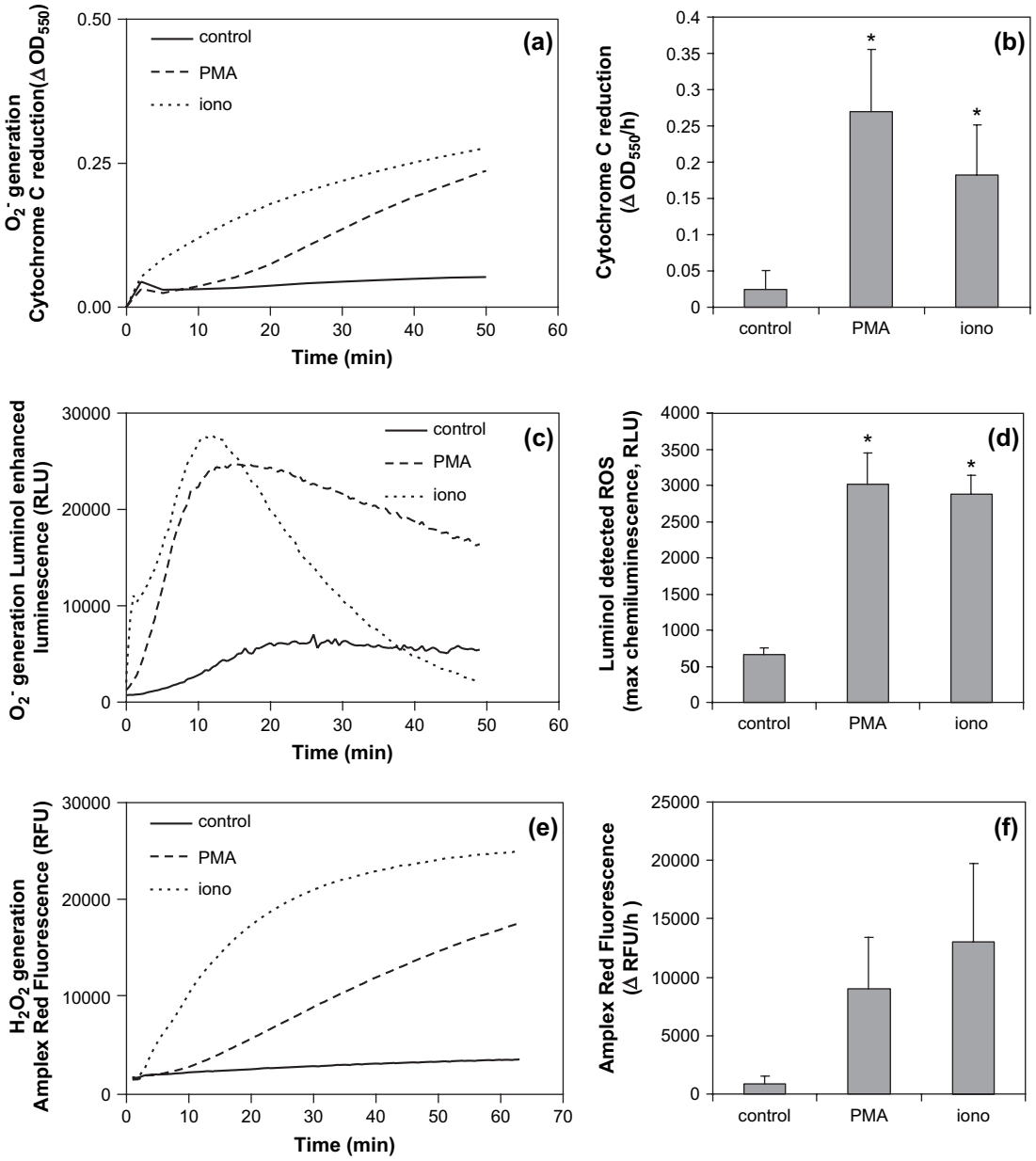

Fig. 2. ROS generation from NOX5 expressing HEK cells. ROS production was measured following stimulation with PMA (0.1 μM) or ionomycin (1 μM). Superoxide was detected as cytochrome *c* reduction (a,b) and Luminol enhanced luminescence (c,d). Hydrogen peroxide was detected as Amplex Red fluorescence (e,f). (a,c,e) show representative traces. (b,f) show the mean rate of ROS production ± SEM and (d) shows the mean maximal amplitude ± SEM for 3–8 experiments. Significant differences from control determined by one-way ANOVA followed by Dunnett's post-hoc analysis are indicated with an asterisk.

these are NOX5 overexpressing cells it is difficult to distinguish between ER retention due to NOX5 overexpression and a true ER localization. In contrast, the localization to the plasma membrane most likely corresponds to a physiological site of action of NOX5.

Interestingly, whereas the membranes in cell–cell contact show more intense staining with ConA, NOX5 labeling looks stronger at surfaces without cell–cell contact.

4. Discussion

After the initial description of several EF-hand containing NOX5 isoforms [11], it appeared that from a biochemical point of view, NOX5 was the most straightforward of the NOX enzymes. With no need for subunits and phosphorylation steps, expression of the NOX5 protein and $[Ca^{2+}]_c$ elevations appeared sufficient for activity [9]. The present study demonstrates that NOX5 is more complex than this, and can be activated by phosphorylation by protein kinase C, likely through phosphorylation of serine residues. This activation process has no equivalent in the NOX family and is likely to occur on regions of this enzyme which are distinct from other NOX enzymes.

The results described here are of interest with respect to the potential physiological role of the enzyme. Indeed, while it is premature to conclude the main physiological function of

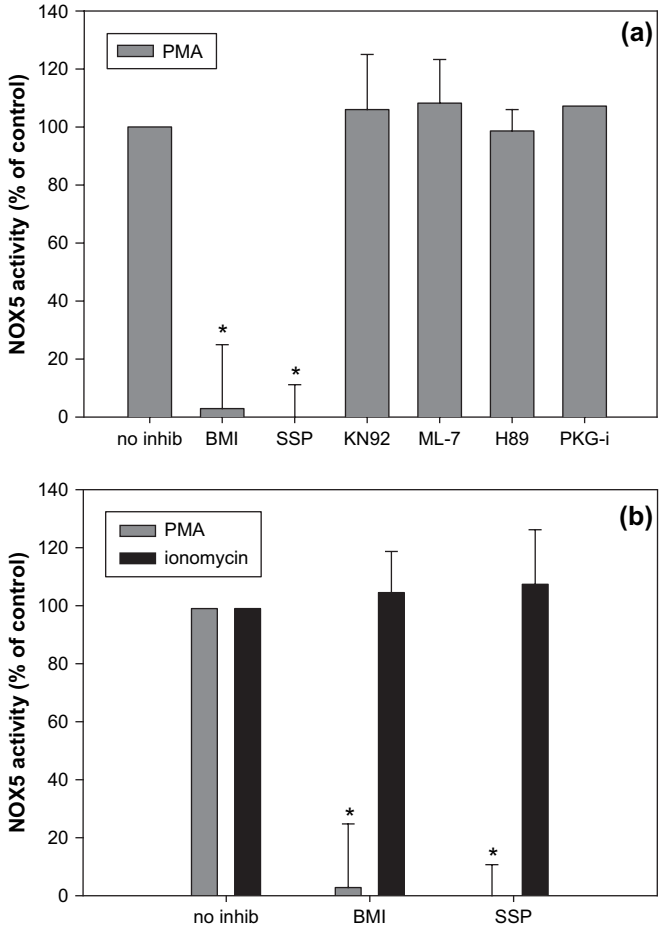

Fig. 3. PKC inhibitors block PMA induced NOX5 activation. Amplex Red was used to assess PMA (0.1 μM) or ionomycin (1 μM) induced NOX5 activity in the presence of serine-threonine kinase inhibitors. (a) The PKC inhibitor PKC-bisindolylmaleimide I (BMI) (1 μM) and the broad range inhibitor staurosporine (SSP) (100 nM) inhibited PMA induced NOX5 activity, while the Cam kinase II inhibitor KN92 (400 nM), the MLCK inhibitor ML-7 (300 nM), the PKA inhibitor H-89 (100 nM), and the protein kinase G inhibitor PKG-i (100 uM) had no effect. The rate of ROS production is presented as the percent of control, which is the rate of ROS production in the absence of inhibitors. Asterisks indicate a significant difference from control determined by one-way ANOVA followed by Dunnett's post-hoc analysis. (b) BMI and SSP block PMA induced but not ionomycin induced NOX5 activity. Asterisks indicate a significant difference between PMA and ionomycin treated groups, as determined by Student's *t*-test. Results are shown as mean ± SEM from 3 to 5 experiments.

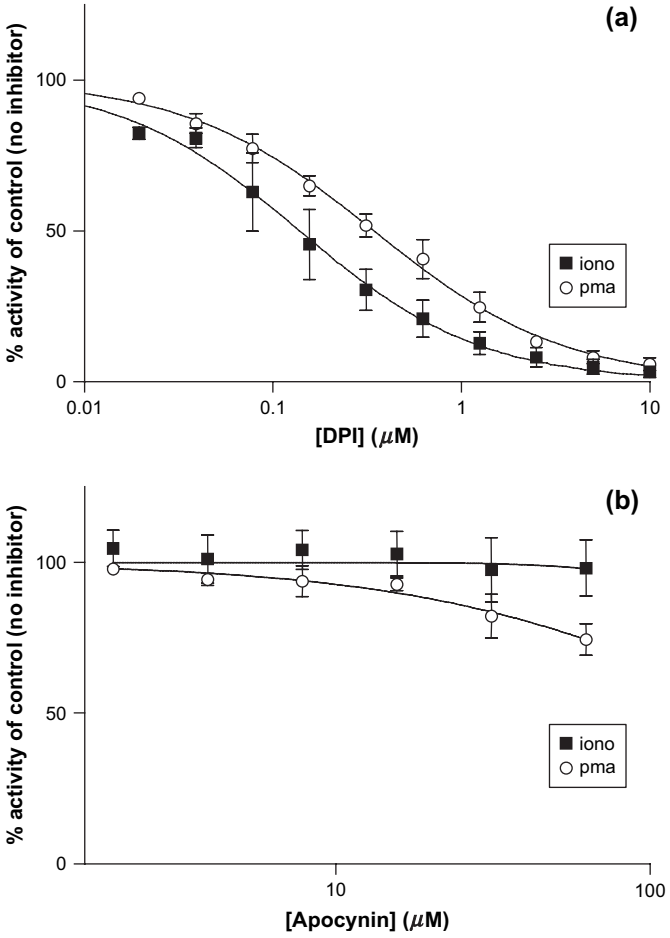

Fig. 4. The effect of DPI and apocynin on NOX5 activity. NOX5 activity was measured with Amplex Red. Cells were stimulated with PMA (0.1 μM) or ionomycin (1 μM). The effect of DPI or apocynin on NOX5 activity is expressed as the percent of the signal generated after 20 min in the absence of inhibitor. The inhibition curve was fitted with a four-parameter Hill curve, and the IC50 value determined. The figure shows mean values ± SEM for three experiments.

NOX5, there are suggestions that it might be involved in the regulation of cell proliferation [13,16], the regulation of protein tyrosine phosphatase activity [14], and the esophagus sphincter tone [21]. The switch in paradigm for NOX5 from a simple Ca^{2+} regulated enzyme to an enzyme which can also be activated by protein kinase C adds an additional dimension to the possible mechanisms regulating the above-described functions. The protein kinase C activation of NOX5 is also particularly interesting with respect to NOX5ε, a short isoform devoid of Ca^{2+}-binding EF hand regions. Indeed, so far, nothing is known about the activation mechanisms of this isoform. Could it also be activated through protein kinase C, either alone or in conjunction with other second messenger systems? Further experiments will be necessary to address this point.

NOX5 activity, as for other NOX isoforms, is very sensitive to DPI inhibition with IC50s in the nanomolar range for both Ca^{2+} and PMA stimulation. Apocynin, on the other hand had only a minor inhibitory effect at 63 μM. This lack of effect of apocynin is not surprising as: (i) apocynin mechanism was described to affect the activation of NOX2 by inhibiting the translocation of two essential cytosolic subunits, $p47^{phox}$ and $p67^{phox}$, thereby inhibiting the assembly of the NADPH oxidase [22]; (ii) apocynin requires oxidation by a myeloperoxidase for activity [23–25], which is absent in HEK293 cells. This explains why apocynin is effective at low concentrations (IC50 = 10 μM) in opsonized zymosan stimulated neutrophils where a substantial amount of myeloperoxidase is released [26], while inhibition of ROS production by apocynin occurs at 600 μM in vascular endothelial cells which are devoid of myeloperoxidase [27]. At these concentrations the effect of

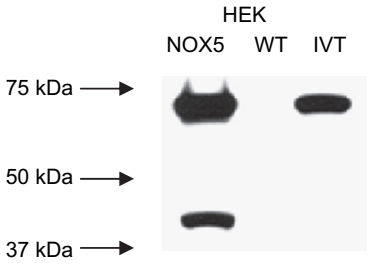

Fig. 5. NOX5 is detected as a protein of ~70 kDa despite a predicted mass of 82 kDa. Lysates (20 μg) from NOX5 HEK cells and WT HEK cells and in vitro generated NOX5 (5 μL) were analyzed by Western blot using the anti-NOX5 antibody. The molecular weight was estimated by interpolation of the standard curve generated using the BioRad Precision Plus Dual Stained Color Standard. This picture is a representative of four experiments.

apocynin probably represents a ROS scavenging effect rather than a direct NOX inhibition.

One of the bottlenecks in NOX research resides in the difficulty of developing good antibodies. Here, we demonstrate that a rabbit polyclonal antibody raised against 18 kDa of the NOX5 N-terminal is highly specific in Western blot experiments using in vitro translated protein and transfected cells, and also in immunocytochemistry experiments using transfected cells. On SDS PAGE, a 70 kDa band was detected. The calculated NOX5 molecular mass is 82 kDa, but the apparent molecular mass on SDS PAGE is consistent with another report where a polyclonal antibody directed against a region in the C-terminal was used [13]. The smaller band of 43 kDa observed with the NOX5 antibody most likely

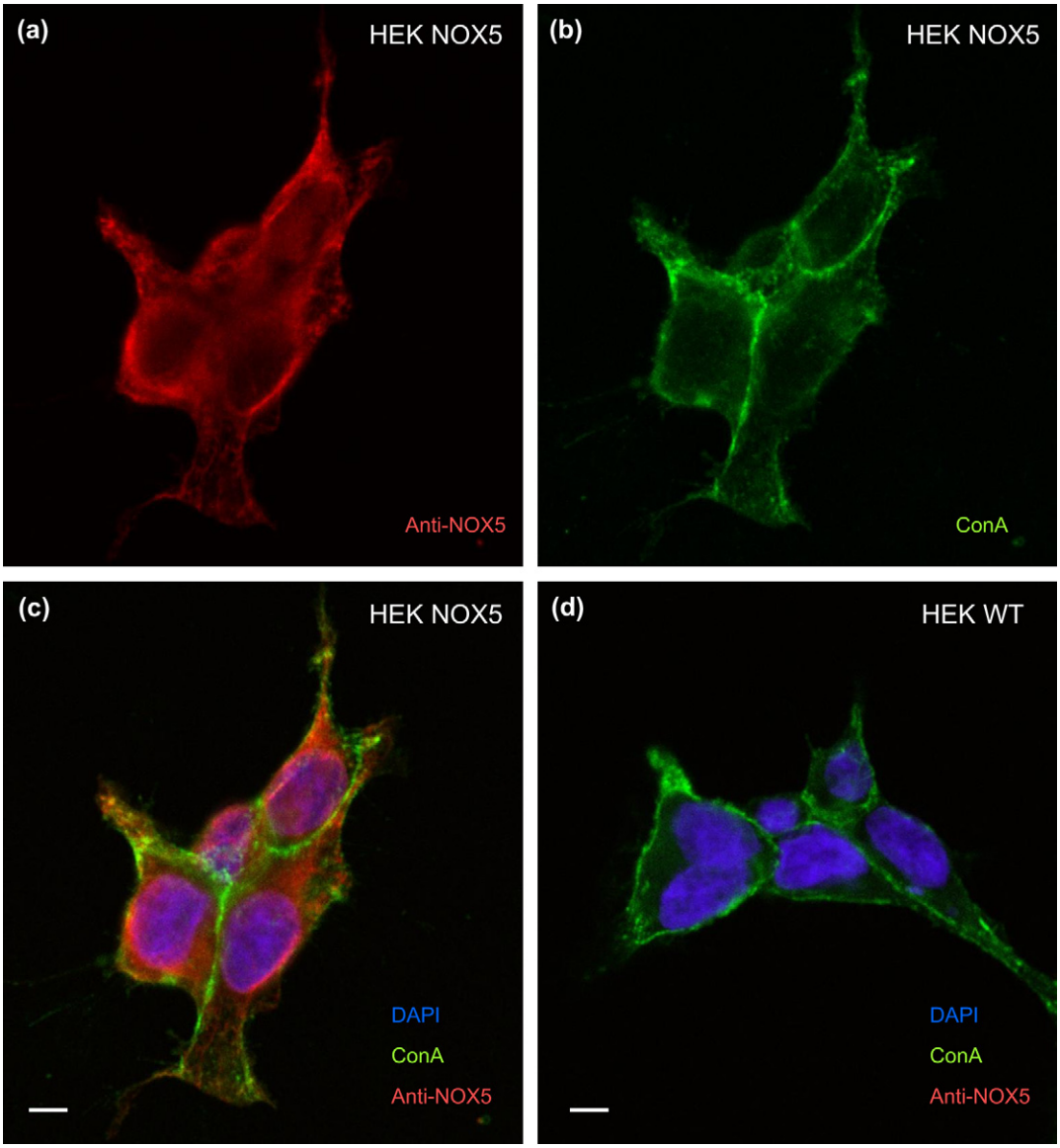

Fig. 6. The subcellular localization of NOX5. NOX5 transfected and untransfected HEK cells were incubated with ConA-Alexa 488 (20 μM) for 15 min before fixation, permeabilization and labeling with anti-NOX5 antibody, followed by incubation with a secondary anti-rabbit-alexa 555 antibody and DAPI (5 μM). Panels (a)–(c) show NOX5 HEK cells, and panel (d) shows WT HEK cells. Confocal images show (a) anti-NOX5; (b) ConA. (c),(d) Overlay of ConA and NOX5 DAPI was used to stain all nuclei. Scale bar = 5 μm.

represents a cleaved fragment. Discrepancies between calculated and apparent molecular masses have been observed for other members of the NOX family: the de-glycosolated form of NOX2 runs at 55 kDa, rather than the predicted 65 kDa, and NOX1 runs at 55–60 kDa rather than the predicted 65 kDa. The reason for this discrepancy is not known, but it has been proposed that this phenomenon may result from the basic nature of these proteins (p$I = 9.0$ for NOX1; p$I = 8.9$ for NOX2; and p$I = 9.0$ for NOX5) [28].

Both superoxide and hydrogen peroxide produced following NOX5 activation can be detected outside of the cell. Since superoxide does not readily cross membranes, it is expected that NOX5 must be found at the plasma membrane. Indeed, immunostaining confirmed the presence of NOX5 at the plasma membrane, but most of the staining can be seen in the perinuclear region. In addition, the immunostaining cannot distinguish between the full size protein and a cleaved fragment appearing at approximately 43 kDa on SDS PAGE, which may accumulate in the ER or in lysosomes. Even if subcellular localization in over-expressing cells must be taken with caution, this observation is consistent with what was observed in human endothelial cells constitutively expressing NOX5 [13] and in mouse fibroblast transduced with NOX5 (Bedard, unpublished data).

While we were in the process of writing this manuscript, an e-publication of an in-press article appeared [29]. Observations shown in this study corroborate several aspects of our study. In particular, PMA-enhancement of NOX5-sensitivity to Ca^{2+} was observed. The use of PKC inhibitors and site directed mutagenesis demonstrated that T494 and S498 in the NOX5 C-terminal described above are responsible for a PMA enhancement through protein kinase C.

In conclusion, the present study demonstrates that NOX5 can be activated by PMA through a direct PKC involvement. Moreover, differential sensitivity of NOX5 to the NOX inhibitors was described, apocynin being inactive and DPI showing efficiency in the submicromolar range. Finally, the cellular localization of NOX5 was intracellular and at the plasma membrane.

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