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Running title: Phosphorylation of NOX2 by PKA

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Abbreviations

cAMP	Cyclic adenosine monophosphate
CGD	chronic granulomatous disease
DFP	diisopropyl fluorophosphates
DPI	diphenyleneiodonium
FAD	Flavin adenine dinucleotide
fMLP	N-formyl-methionyl-leucyl-phenylalanine
INT	Iodonitrotetrazolium
MPO	myeloperoxidase
NBT	Nitro blue tetrazolium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
OPZ	opsonized zymosan
PKA	Protein kinase A
PKC	Protein kinase C
PMA	phorbol myristate acetate
PMN	Polymorphonuclear neutrophils
PMSF	Phenylmethylsulfonylfluoride
ROS	Reactive oxygen species

Abstract

Superoxide anion production by neutrophils is essential for host defense against microbes. Superoxide anion generates other reactive oxygen species (ROS) that are very toxic for microbes and host cells, therefore their excessive production could induce inflammatory reactions and tissue injury. Cyclic adenosine monophosphate (cAMP) elevating agents are considered to be physiological inhibitors of superoxide production by neutrophils but the mechanisms involved in this inhibitory effect are poorly understood. Superoxide is produced by the phagocyte NADPH oxidase, a complex enzyme composed of two membrane subunits, gp91^{phox} or NOX2 and p22^{phox}, and four cytosolic components p47^{phox}, p67^{phox}, p40^{phox}, and Rac2. Except Rac2, these proteins are known to be phosphorylated upon neutrophils stimulation. Here we show that forskolin, an activator of the adenylate cyclase-cAMP-PKA pathway, induced phosphorylation of gp91phox/NOX2 and inhibited fMLF-induced NADPH oxidase activation in human neutrophils. H89, a PKA inhibitor prevented the forskolin-induced phosphorylation of gp91phox and restored NADPH oxidase activation. Furthermore, PKA phosphorylated the recombinant gp91phox/NOX2-cytosolic C-terminal region *in vitro* only on a few specific peptides containing serine residues, as compared to PKC. Interestingly, phosphorylation of NOX2-Cterby PKA alone did not induce interaction with the cytosolic components p47^{phox}, p67^{phox} and Rac2, however it induced inhibition of PKC-induced interaction. Furthermore, PKA alone did not induce NOX2 electron transfer activity, however it inhibited PKC-induced activation. These results suggest that PKA phosphorylates NOX2 in human neutrophils, a process essential to limit ROS production and inflammation under physiological conditions. Our data identify cAMP-PKA-NOX2-axis as a critical gatekeeper of neutrophil ROS production.

Introduction

Polymorphonuclear neutrophils (PMN) are phagocytic cells representing 60-70% of blood leukocytes and are essential for innate immunity [1, 2]. They have a short half-life, lasting only for few hours to few days in the blood and tissue respectively; are highly motile and are the hallmark of acute inflammation as they are among the first circulating cells to migrate to the inflammatory site [3]. Upon activation, neutrophils release antimicrobial peptides, enzymes, superoxide anions and Neutrophil Extracellular Traps" [4-7]. Superoxide anion ($O_2^{-\bullet}$) generates other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) which is used by myeloperoxidase (MPO) to generate HOCl [8, 9]. These agents have a dual role as they are essential for pathogen killing but they can also induce tissue injury of the host. For instance the importance of ROS production in innate immunity is illustrated by chronic granulomatous disease (CGD), a severe primary immunodeficiency in which phagocytes do not produce ROS and patients rarely survived childhood due to infections [10, 11]. Inversely, the harmful role of ROS production in inflammatory disease is shown in several inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases and lung inflammation [12-17]. Thus, ROS production must be tightly regulated to avoid unwanted inflammatory reactions.

The enzyme responsible for superoxide anions production in phagocytes is called the phagocyte NADPH Oxidase or NOX2 [18, 19]. NADPH oxidase is a complex enzyme composed of two membrane proteins $gp91^{phox}$/NOX2 and $p22^{phox}$ (also called flavocytochrome b_{558}) and four cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2. In un-stimulated neutrophils, the complex is latent, with unassembled subunits located in the cytosol and the plasma membranes [8, 18]. Upon stimulation, the cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2 translocate to the plasma membrane and associate with flavocytochrome b_{558}.

Gp91^{phox} contains two hemes, a flavin and NADPH binding sites [18, 20]. The gp91^{phox} subunit is composed of N-terminal sequence, six putative trans-membrane segments, several loops and a long C-terminal domain (residues 289–570) containing the FAD and the NADPH binding sites [20, 21]. The activated enzyme transfers electrons from cytosolic NADPH to extracellular or phagosomal molecular oxygen through a transmembrane electron transport chain. The C-terminal flavin center of gp91^{phox} transfers electrons from the physiological electron donor, NADPH, to two membrane-imbedded hemes within the N-terminal domain of the cytochrome. The latter serves as the terminal electron donor to oxygen [18, 22]. Diaphorase activityis a reductase activity that measures the electron transfer activity from NADPH to FAD by using indicator dye such as INT or NBT which intercept the electron in stead of heme [22-25]. The cytochrome b_{558} is the central component that binds the subunits of the NADPH oxidase after stimulation allowing the transfer of electrons [20].

NADPH oxidase activation in neutrophils can be induced by different stimuli such as phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), and opsonized zymosan (OPZ) and bacteria [8]. NADPH oxidase activation is accompanied by the phosphorylation of p47phox, p67phox, p40phox as well as gp91phox/NOX2 and p22phox [19, 26]. Most of these phosphorylations are mediated by PKC and are believed to have a positive regulatory role [19]. Our team showed that NOX2 was phosphorylated in human neutrophils stimulated by PMA, fMLF and opsonized zymosan [26]. This phosphorylation occurs in the C-terminal domain of the protein (within residues 321–405 and 466–570) on serine and threonine residues and PKC was able to directly phosphorylate NOX2 and to stimulate interaction with the cytosolic proteins and diaphorase activity [26]. Cyclic adenosine monophosphate (cAMP)-elevating agents such as adrenaline, prostaglandin E2, forskolin and dibutyryl-cAMP are known to inhibit fMLF-induced superoxide production [27-30]. In general cAMP-elevating agents induce PKA activation and phosphorylation of several proteins. However, how PKA is able to inhibit ROS production

is not known. The aim of this study is to determine whether PKA is able to phosphorylate NOX2 and to investigate the consequences of this phosphorylation.

Materials and methods

Materials

Phenylmethylsulfonylfluoride (PMSF), diisopropyl fluorophosphate (DFP), iodonitrotetrazolium (INT), diphenyleneiodonium (DPI), FAD, PKA and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting reagents were purchased from Bio-Rad (Richmond, CA, USA). PKC was purchased from Calbiochem (San Diego, CA, USA). [γ-32P]-ATP, gamma-bind sepharose beads, pGEX-6p1, and gluthathione sepharose were purchased from GE Healthcare (Little Chalfont, UK). Monoclonal mouse anti-gp91^{phox} (clone 54.1) antibody was from Santa Cruz Biotechnology. Polyclonal rabbit anti-p47^{phox} and anti-p67^{phox} antibodies were previously described [31] anti-Rac1/2 antibody was from BD Bioscience (Palo Alto, CA, USA).

Ethics statement and human neutrophils isolation

Neutrophils were isolated from healthy volunteers' venous blood with their signed informed consent. The collection and analyses of data were performed anonymously. All experiments were supported by the Inserm Institutional Review Board and ethics committee. Neutrophils were isolated from venous fresh blood of healthy donors, by dextran sedimentation, to remove red blood cells, followed by differential centrifugation through a Ficoll-Hypaque density gradient to remove mononuclear cells, and hypotonic lysis

to remove any remaining contaminating red blood cells [32]. Neutrophils in the pellet were washed and resuspended in phosphate-free media and counted [26]. Cell counting were determined by Turks' blue staining to evaluate the purity of neutrophils, and the viability by Trypan blue staining.

^{32}P labeling and stimulation

Containing 0.5 mCi of ^{32}P orthophosphoric acid/10^8 cells/ml for 60 min [26]. After centrifugation, the radiolabeled cells were resuspended in the same buffer containing 1 mM $CaCl_2$ and incubated at 37°C for 5 min. Following incubation, forskolin or fMLP were added for various lengths of time. The reactions were terminated by adding 10-fold excess of ice-cold phosphate-free loading buffer containing 25 mM NaF and 5 mM EDTA and centrifuged immediately (400 x g for 12 min at 4°C).

Neutrophil fractionation and gp91phox immunoprecipitation

Cells were lysed as previously described [26, 32]. Briefly, neutrophils were resuspended at 5 x 10^7 cells/ml in lysis buffer (8 mM HEPES pH 7.4, 2% Triton X-100, 25 mM NaF, 5.4 mM Na_3VO_4, 2 mM β-glycerophosphate, 1 mM Levamisole, 1 mM p-NPP, P8340 Protease Inhibitor Cocktail (1:1000 dilution), 8 mM NaCl, 80 mM KCl, 0.8 mM EDTA, 8 µg/ml Chymostatin, 0.8 mM PMSF, 0.08 mM DTT). After sonication on ice and mixing by rotation, the lysates were centrifuged at 114,000 x g for 30 min at 4°C. The supernatants were diluted twice in buffer containing 10 mM HEPES pH 7.4, 10 mM NaCl, 100 mM KCl, 1 mM EDTA, 10 µg/ml Chymostatin, 1 mM PMSF. Immunoprecipitation was performed by addition of anti-gp91^{phox} monoclonal antibody and protein A/G beads saturated in BSA and incubating for 2 h. Beads were then washed 4 times, and proteins were denaturated in Laemmli sample buffer by boiling at 100°C for 3 min [32].

Electrophoresis, immunoblotting, and autoradiography

Immunoprecipitated proteins were separated on SDS-polyacrylamide gels using standard techniques [32]. Proteins were transferred to nitrocellulose or PVDF, phosphorylation of $gp91^{phox}$ was monitored by autoradiography. $Gp91^{phox}$ was monitored by immunoblotting as described previously using anti-$gp91^{phox}$ monoclonal antibody (1:5000 dilution) and horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

Luminol-amplified chemiluminescence assay

Neutrophils ($5x10^5$) were suspended in HBSS (0.5 ml) and preincubated for 15 minutes at 37°C in the presence of luminol (10 μM), with or without forskolin or H89. Cells were then stimulated by fMLF (10^{-6} M). Luminol-amplified chemiluminescence was measured using a Berthold 953 apparatus for respectively 10 minutes at 37°C and light emission was expressed in counted photons per minute (c.p.m) [33].

Bacterial expression of the cytosolic $gp91^{phox}$/NOX2 (aa 291-570) domain, $p47^{phox}$, $p67^{phox}$, and Rac2

The truncated form of $gp91^{phox}$ (aa 291-570) was obtained by PCR using $gp91^{phox}$ cDNA in Blue-Script as template [34] (Zhen, 1993) and the following primers: forward primer (CGC GGA TCC TCT CAA CAG AAG GTG GT); reverse primer (CCG CTC GAG TTA GAA GTT TTC CTT GTT). PCR products were subcloned into the pGEX-6P1 vector (Pharmacia, Piscataway, NJ, USA) and sequenced to rule out unexpected mutations and to confirm the truncation. For expression, the pGEX-6P1 plasmid containing cDNA encoding glutathione *S*-transferase (GST)-NOX2 (291-570) was transformed into BL21 *Escherichia coli*, and the cells were cultured overnight in 100 ml of LB medium containing ampicillin (100 μg/ml) at 37°C. The overnight culture was then diluted 10-fold and allowed to grow until the optical

density at 600 nm reached 0.8. Expression of GST-NOX2 (291-570) was induced with isopropyl-β-D-thiogalactoside (20 μM) and FAD (20 μM) overnight at 16°C. Cells were pelleted by centrifuging at 2500 *g* for 25 min, lysed by sonication in lysis buffer (50 mM NaCl; 5 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.5; 1 mM DTT; 1% Triton X-100; and a mixture of protease inhibitors), and centrifuged at 10,000 *g* for 20 min at 4°C. This protocol gave 50% of soluble GST-NOX2 (291-570) and 50% of nonsoluble GST-NOX2 (291-570). The soluble GST-NOX2 (291-570) was affinity purified by incubation of the supernatant with glutathione-Sepharose 4B beads overnight at 4°C. Beads were then washed in lysis buffer, and the fusion protein was eluted with 15 mM glutathione; 50 mM Tris-HCl, pH 8; and 20 μM FAD. To separate NOX2 (291-570) from GST, the recombinant protein was treated with PreScission protease for 6 h at 4°C in 150 mM NaCl; 50 mM Tris-HCl, pH 7; 1 mM DTT; 1 mM EDTA; and 20 μM FAD and subjected to another round of glutathione-bead adsorption. NOX2 (291-570) migrates at 31 kDa as a monomeric protein in a non denaturating gel (data not shown). $P47^{phox}$, $p67^{phox}$, and Rac2 were expressed at 37°C, using the same protocol (pGEX plasmids as previously described [26].

In vitro phosphorylation of NOX2 (291-570)-domain and $p47^{phox}$

Phosphorylation by PKC was performed as described previously [26, 35]. Briefly, the reaction mixture contained 1 μg of recombinant protein, 25 ng of PKC in 40 mM HEPES, 10 mM $MgCl_2$, 1 mM DTT, 5 μg/ml diacylglycerol, 150 μg/ml phosphatidylserine, 3 mM $CaCl_2$, and 50 μM ATP (containing 1 μCi[γ-32P]-ATP) in a total volume of 50 μl. The reaction was incubated for 30 min at 30°C and terminated by addition of 1 Mm staurosporine. Phosphorylation by PKA was performed referring to the protocol recommended by sigma-aldrich: Briefly, Recombinant protein was incubated with [γ-32P]-ATP (2 μCi) in the presence of 200 ng of PKA in a total reaction volume of 40 μl at 30°C for 30 min. Reactions were

terminated at the indicated times by addition of 10 µl of 5X Laemmli sample buffer and denaturation at 100°C [32].

Phosphoamino acid analysis

Phosphorylated NOX2 (291-570) was separated by SDS-PAGE, transferred to PVDF, excised, and then subjected to hydrolysis with 6N HCl for 90 min at 110°C [26, 32]. The supernatant was dried in a speed-vac and lyophilized. Phosphoamino acids were collected and mixed with or without 2 µg of standard markers (phosphoserine, phosphothreonine, phosphotyrosine) and separated by thin-layer chromatography. Samples were then applied to a cellulose plate and separated by electrophoresis at 1100 V for 45 min at 4°C in a buffer consisting of water/acetic acid/pyridine (189:10:1), pH 3.5. Standard phosphoamino acids were visualized by spraying with 0.2% ninhydrin, and phosphorylated amino acids were detected by autoradiography.

Two-dimensional tryptic phosphopeptide mapping

The nitrocellulose area containing ^{32}P-labeled gp91^{phox}/NOX2 was incubated for 30 min at 37°C with polyvinylpyrrolidone and incubated overnight with trypsin (50 µg/ml) in carbonate buffer. Released peptides were washed 3 times, redissolved in electrophoresis buffer, and applied to one corner of a cellulose thin-layer plate. After electrophoresis (1000 V for 20 min), ascending chromatography was performed in isobutyric acid buffer [26]. The plates were autoradiographed at −75°C.

GST pull-down assay

Here 80 pmol of GST-Rac2, GST-p67^{phox}, phosphorylated GST-p47^{phox}, and GST alone were incubated in the presence of 5 pmol of phosphorylated or not phosphorylated recombinant NOX2 (291-570) and

glutathione-Sepharose beads in interaction buffer (20 mM Hepes, pH 7.5; 1% Nonidet P-40; 50 mM NaCl; and 1 mM EGTA) for 1h [26]. After washing, the complex was eluted with 10 mM glutathione and analyzed by SDS-PAGE and Western blot using protein-specific antibodies.

Diaphorase activity assay

INT-reductase activity was measured according to the protocols of Han *et al*. [24] and Nisimoto *et al*. [25, 26]. The NOX2 (291-570) flavoprotein domain was preincubated with 20 μM FAD for 16 h at 4°C. Activity was measured in 500 μl of assay buffer (0.3% Triton X-100, 50 mM NaCl, 4 mM $MgCl_2$, and 20 μM FAD in 50 mM phosphate buffer, pH 7.3) containing 0.2 mM INT and 1 μg of phosphorylated or unphosphorylated NOX2 (291-570) in the absence or presence of phosphorylated $p47^{phox}$, $p67^{phox}$, and Rac2. The reactions were started by addition of 0.2 mM NADPH at 37°C, and absorbance at 500 nm was monitored. The reaction was performed with or without DPI (20 μM) to check the specificity. The rate of INT reduction was quantified using an extinction coefficient of 11 mM^{-1} cm^{-1}. After addition of NADPH, the INT reductase activity was measured for 6 min, as the absorbance change at 500 nm was almost linear throughout this interval.

Statistical analysis

All results are expressed as means ± SED. Data were analyzed with GraphPad Prism 7 software (GraphPad Software, San Diego, CA). Differences between groups were analyzed by One-way ANOVA test with Tukey's Multiple Comparison post-test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ values were considered as significant.

Results

Forskolin induces gp91^{phox}/NOX2 phosphorylation and inhibits fMLF-induced superoxide production in human neutrophils

To evaluate the effect of cyclic-AMP elevating agents on the phosphorylation of gp91^{phox}/NOX2 in human neutrophils, cells were labeled with ^{32}P and treated with forskolin, an adenylate cyclase activator and thus a cyclic-AMP increasing agent [36]. Gp91^{phox}/NOX2 was immunoprecipitated from lysates using a specific monoclonal antibody, and the samples were analyzed by SDS-PAGE, immunoblotting and autoradiography. Our results show that, while gp91^{phox}/NOX2 was weakly phosphorylated in resting cells, its phosphorylation was clearly induced in forskolin-treated neutrophils in a concentration dependent manner (Fig. 1A). Furthermore, corresponding Western blot analysis confirmed that this phosphoprotein was gp91^{phox}/NOX2 and showed that the same amount of protein was present for each condition. Gp91^{phox}/NOX2 is heavily glycosylated, and its mature form migrates as a broad band in SDS-PAGE with an average size of 91 kDa because of variable glycosylation [26, 37]. Indeed, this broad band in SDS-PAGE is a characteristic signature of gp91^{phox}/NOX2. Likewise, the phosphorylated protein and corresponding blot both demonstrate a similar broad band, supporting the conclusion that the phosphorylated protein corresponds to gp91^{phox}/NOX2. To re-evaluate the effect of forskolin on neutrophil ROS production, purified human neutrophils were treated with different concentration of forskolin in the absence or presence of fMLF and ROS production was measured using luminol-amplified chemiluminescence. Results show that forskolin alone did not induce ROS production (Fig. 1B), however it was able to inhibit fMLF-induced ROS production by neutrophils as shown in Fig. 1C. These results show that forskolin induced gp91phox phosphorylation without inducing NADPH oxidase activation, however it inhibited fMLF-induced NADPH oxidase activation.

Figure 1

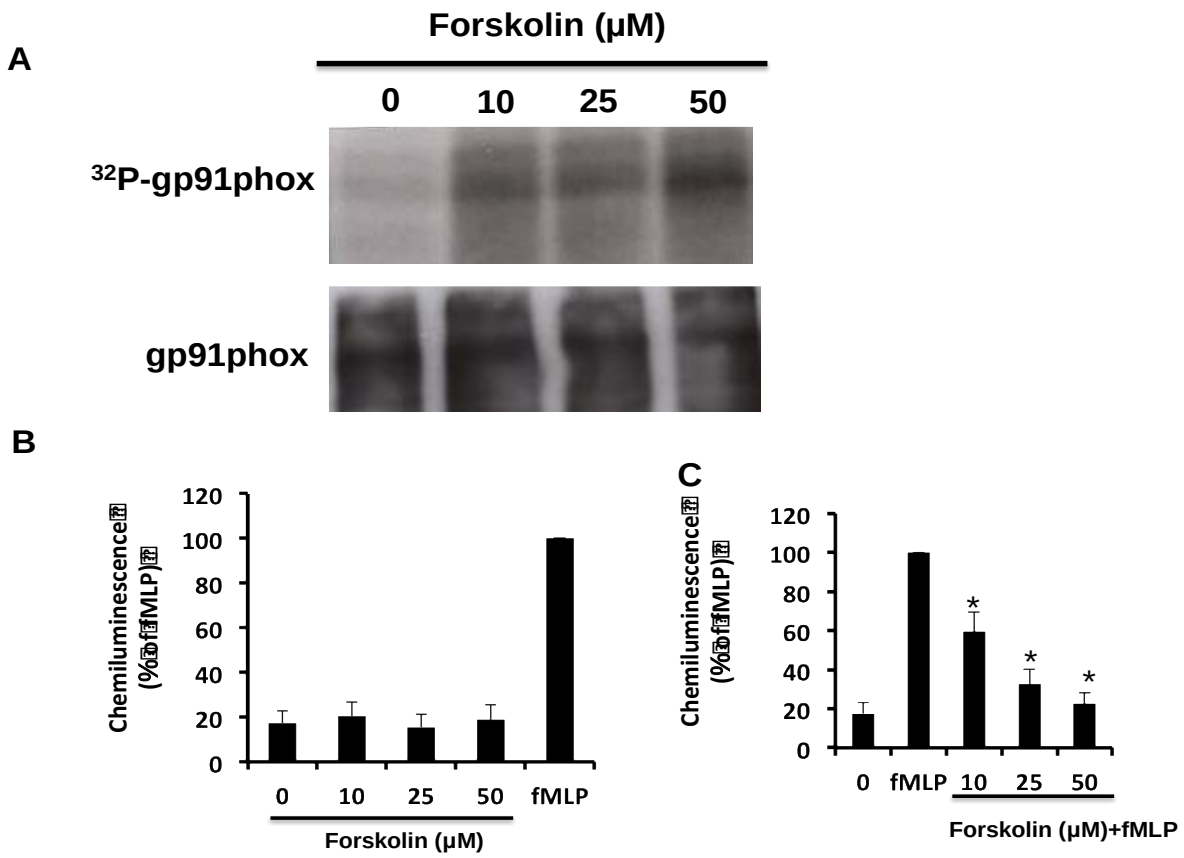

Fig. 1 Effect of forskolin on gp91^{phox}/NOX2 phosphorylation and ROS production in human neutrophils. (A)^{32}Pi-labeled neutrophils (5 x 10^7 cells/ml) were incubated in the absence (Resting) or presence of different concentration of forskolin. Cell lysates were prepared and gp91phox was immunoprecipitated with monoclonal anti-gp91^{phox}. Proteins were subjected to SDS-PAGE, blotted on nitrocellulose, and detected by autoradiography and immunoblotting with anti-gp91^{phox}.The data are representative of 3 experiments. (B) Neutrophils (1 x 10^6 cells/ml of HBSS) were incubated in the absence or presence of different concentration of forskolin alone or fMLP(1 μM) and luminol-amplified chemiluminescence was measured.(C) Neutrophils (1 x 10^6 cells/ml of HBSS) were incubated in the absence or presence of different concentration of forskolin, stimulated by fMLP(1 μM) and luminol-amplified chemiluminescence was measured.Values are expressed as means ± SED of 3 independent experiments, *: $p<0.05$, **: $p<0.01$ as compared to the control (fMLP alone).

H89, a PKA inhibitor decreased forskolin-induced gp91^{phox}/NOX2 phosphorylation and prevented forskolin-induced inhibition of NADPH oxidase activation

Forskolin is known to activate adenylate cyclase and to induce cAMP production in cells. As cAMP is a PKA direct activator, we evaluated whether this kinase played a role in gp91^{phox}/NOX2 phosphorylation. To investigate this possibility, we evaluated the effect of H89, a selective PKA inhibitor [38], on gp91^{phox}/NOX2 phosphorylation in forskolin treated human neutrophils. As shown in Figure 2A, H89 dramatically inhibited gp91^{phox}/NOX2 phosphorylation induced by forskolin in neutrophils. Interestingly H89 was able to restore ROS production in forskolin-treated neutrophils (Fig. 2B). These results suggest that the phosphorylation of gp91phox/NOX2 by PKA could inhibit NADPH oxidase activation in human neutrophils.

Figure 2

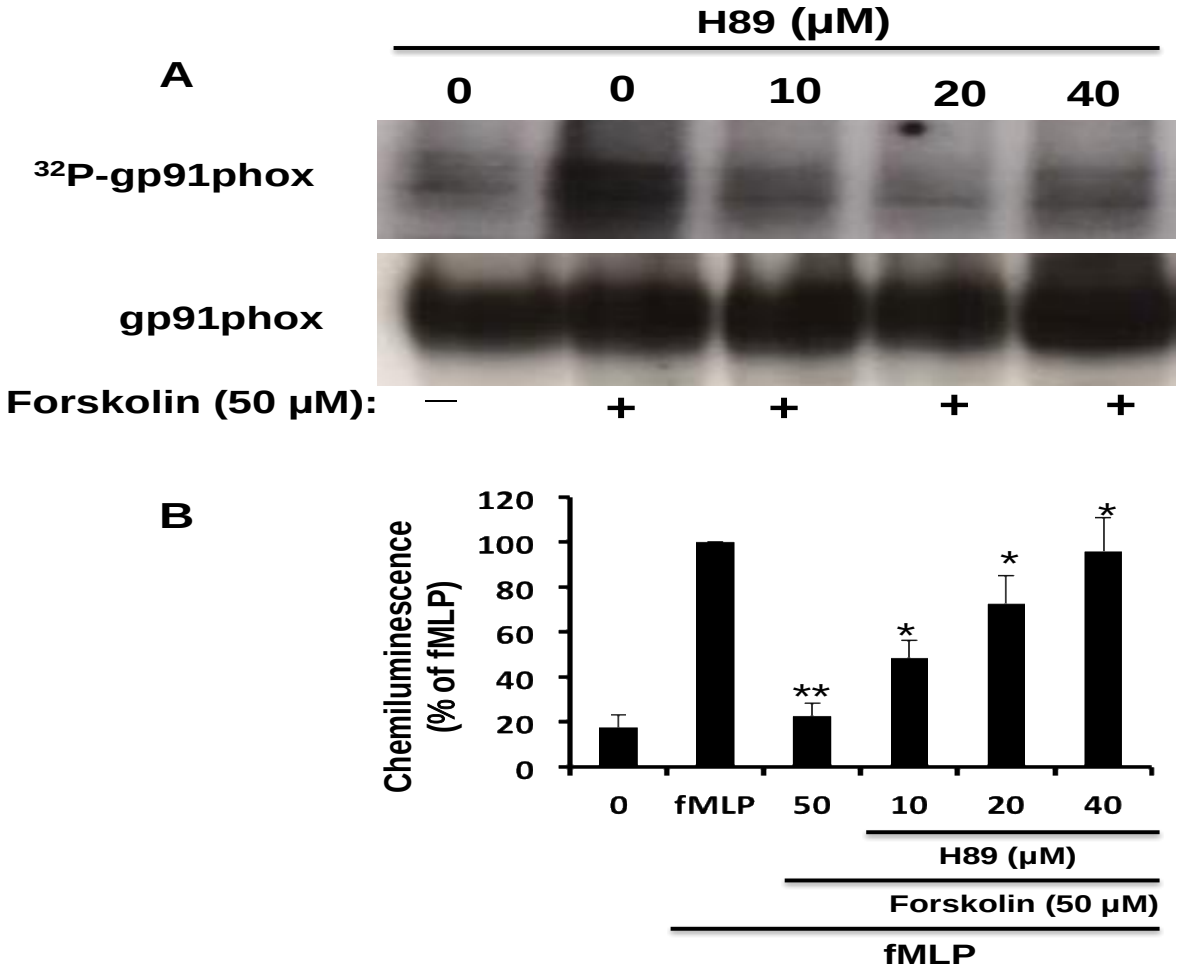

Fig. 2 Effect of H89 on forskolin-induced gp91^{phox}/NOX2 phosphorylation and forskolin-inhibitory effect on ROS production by human neutrophils. (A)^{32}Pi-labeled neutrophils (5 x 10^7 cells/ml) were incubated in the absence or presence of different concentration of H89 for 30 min then forskolin was added. Cell lysates were prepared and gp91phox was immunoprecipitated with monoclonal anti-gp91^{phox} as described in materials and methods. Proteins were subjected to SDS-PAGE, blotted on nitrocellulose, and detected by autoradiography (^{32}P) and immunoblotting with anti-gp91^{phox} (NOX2). (B) Neutrophils (1 x 10^6 cells/ml of HBSS) were incubated in the absence or presence of different concentrations of H89 for 30 min, forskolinwas added for 15 min and luminol-amplified chemiluminescence was measured before and after the addition of fMLF (1 μM). Values are expressed as means ± SED of 3 independent experiments, *: $p<0.05$ as compared to the control (fMLP alone).

The gp91^{phox}/NOX2 cytosolic flavoprotein domain is phosphorylated by PKA *in vitro* on serine residues located on selective peptides

To investigate whether PKA can directly phosphorylate gp91^{phox}/NOX2, we first analyzed gp91^{phox}/NOX2 cytosolic sequences for potential PKA-phosphorylation sites using the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) and protein Scan program for PKA sites (NCI, Bethesda, MD, USA). Gp91^{phox}/NOX2 is an integral membrane protein that is predicted to have a cytosolic N terminus, 2 cytosolic intracellular loops, and a cytosolic C terminus containing a flavoprotein domain [20, 21]. Scanning these cytosolic regions for potential phosphorylation sites indicated none in the N-terminal sequence. In contrast, the cytosolic flavoprotein domain has 33 potential phosphorylation sites (Supplemental table 1). Thus, we evaluated whether PKA could phosphorylate the NOX2 cytosolic flavoprotein domain [NOX2 (291-570)]. As shown in Figure 3A, purified recombinant GST-NOX2 (291-570) fusion protein was phosphorylated *in vitro* by PKA in a time-dependent manner, whereas control GST alone was not phosphorylated by PKA (data not shown). Furthermore, phosphoamino acid analysis of PKA-phosphorylated NOX2 (291-570) showed that this protein was phosphorylated on serine residues (Fig. 3B), supporting a role for PKA-mediated phosphorylation. These results suggest that gp91phox/NOX2 is phosphorylated by PKA in its carboxy-terminal fragment on serines located in the sequence aa 291-570.

Figure 3

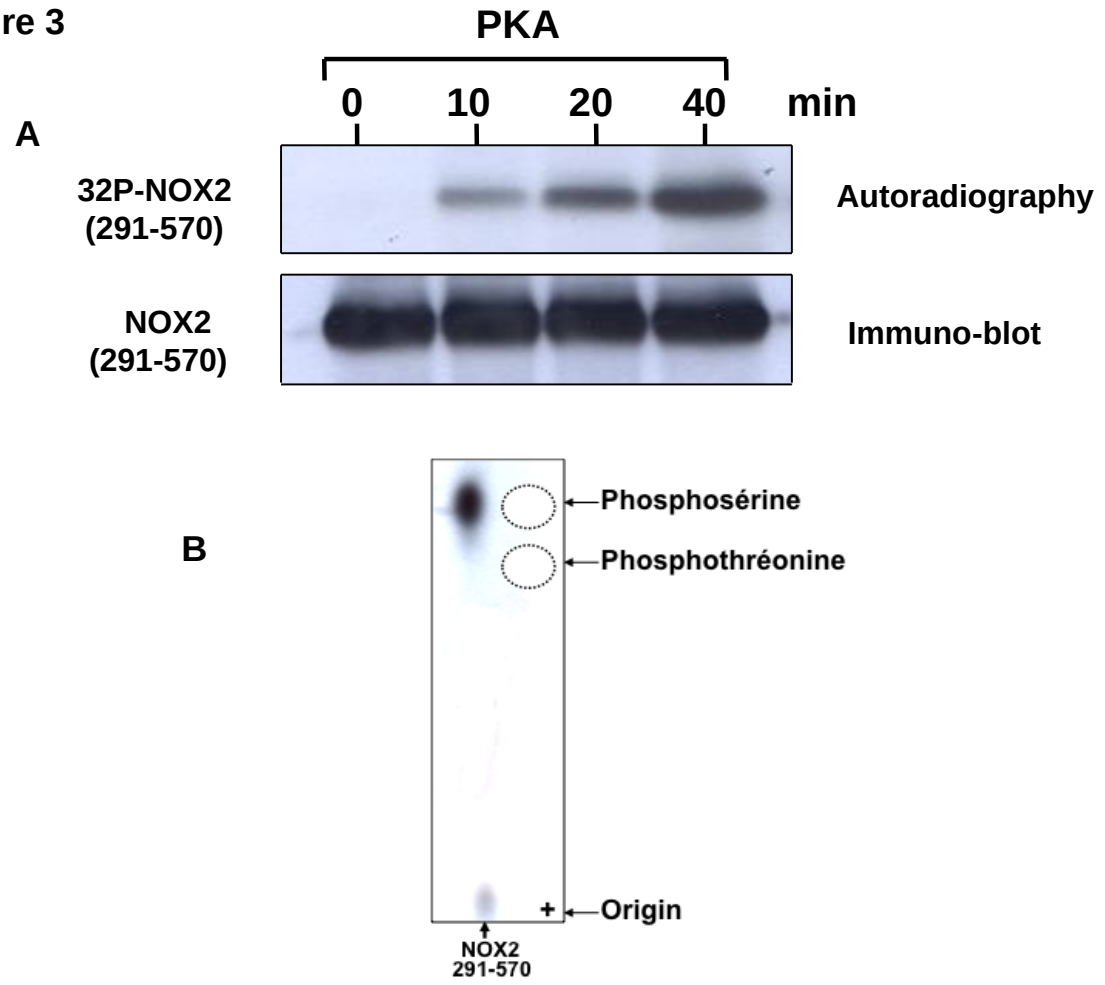

Fig. 3 Phosphorylation of recombinant gp91phox/NOX2 cytosolic flavoprotein domain by purified PKA *in vitro* and phosphoamino acid analysis. (A) 2µg of recombinant NOX2 (291-570) protein was incubated with [γ-32P]-ATP (2 µCi) in the presence of 200 ng of PKA in a total reaction volume of 40 µl at 30°C, reactions were terminated at the indicated times by addition of 10 µl of Laemmli 5X sample buffer and denaturation at 100°C. Proteins were then separated by SDS-PAGE and analyzed by autoradiography and immunoblotting with the anti-NOX2 antibodies. (B) Phosphorylated NOX2 was separated by SDS-PAGE, tranferred to PVDF, excised, and subjected to acid hydrolysis, as described. Phosphoamino acids were collected and mixed with or without standard markers (phosphoserine, phosphothreonine, phosphotyrosine) and separated by thin layer electrophoresis. Standard phosphoamino acids were visualized by 0.2% ninhydrin, and phosphorylated amino acids were detected by autoradiography. The data are representative of 4 experiments.

In our previous study, we showed that PKC phosphorylated gp91phox/NOX2 sites on several peptides [26]. We first compared the intensity of PKC- and PKA-induced NOX2 phosphorylation. Results show that PKA phosphorylates NOX2 less than PKC (Fig. 4A). To check whether the phosphorylation of the gp91phox/NOX2-cytosolic tail *in vitro* by PKA or PKC occurred on the same or on different peptides, we analyzed phosphorylated protein by 2-dimensional tryptic phosphopeptide mapping. Interestingly, phosphopeptide mapping analysis of the recombinant gp91phox/NOX2-cytosolic tail phosphorylated *in vitro* by PKA showed that this kinase phosphorylated NOX2 (291-570) on only two major peptides (b and c) while PKC phosphorylated NOX2 on more peptides (Fig. 4B).

Figure 4

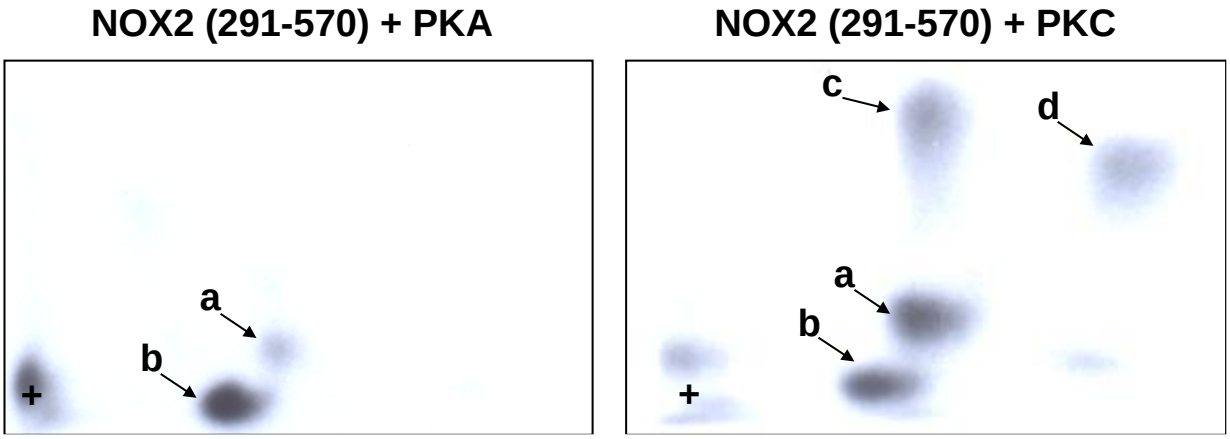

Fig. 4 Comparaison of two-dimensional tryptic phosphopeptide mapping of recombinant gp91phox/NOX2 (291-570) phosphorylated *in vitro*by PKA and PKC. Recombinant NOX2 (291-570) was incubated with [γ-32P]-ATP in the presence of PKA or PKC, reactions were terminated at the indicated times, and proteins were separated by SDS-PAGE and analyzed by autoradiography. ^{32}P-NOX2 (291-570) were recovered from nitrocellulose sheets and submitted to trypsin digestion. Peptides were subjected to first-dimension electrophoresis and second-dimension thin layer chromatography. Phosphorylated peptides were detected by autoradiography. Data are representative of 3 experiments.

Phosphorylation of the gp91^{phox}/NOX2 cytosolic flavoprotein domain by PKA antagonizes PKC-induced interaction with p67^{phox}, p47^{phox} and Rac2

Phosphorylation of NOX2(291-570) by PKC has been shown to induce a clear increase of its interaction with the cytosolic components Rac2, p67phox, and p47phox [26]. Thus, we studied the consequences of PKA-mediated phosphorylation of recombinant NOX2 (291-570) on these interactions. Results showed that non-phosphorylated NOX2 (291-570) weakly interacted with phosphorylated p47phox and Rac2 (Fig. 5). As expected, phosphorylation of NOX2 (291-570) by PKC induced a clear increase in these interactions, which suggests that phosphorylation of gp91phox/NOX2 may potentiate the assembly of NADPH oxidase. Interestingly, phosphorylation of NOX2 (291-570) by PKA induced a clear decrease in PKC-induced interactions. The same result was obtained with p67phox and control experiments with GST alone showed no interaction with NOX2 (Fig. 6). These results show that phosphorylation of NOX2 (291-570) by PKA induced inhibition of its interaction with the cytosolic subunits suggesting that NOX2 phosphorylation by PKA could play a negative role in the assembly of NOX2 complex in phagocytes.

Figure 5

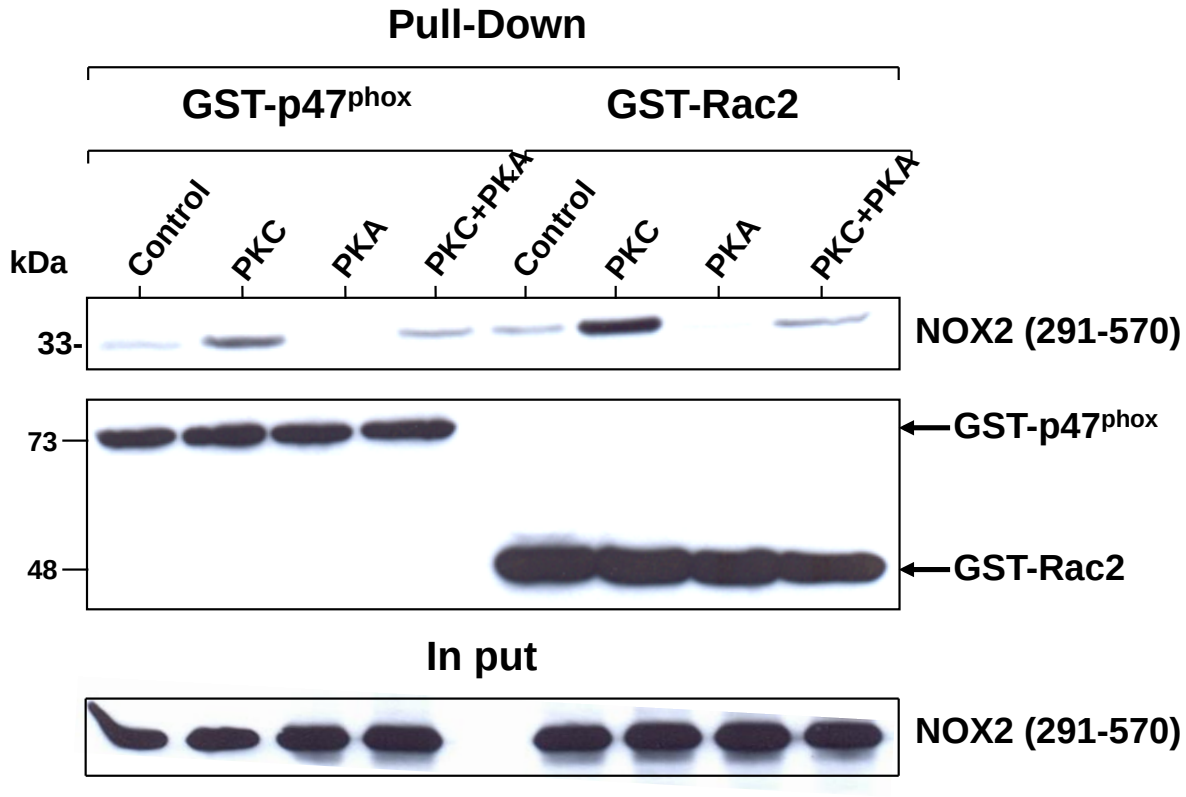

Fig. 5 Effect of PKA-induced phosphorylation of gp91phox/NOX2-cytosolic flavoprotein domain on PKC-induced interaction with p47phox and Rac2. Recombinant NOX2 (291-570) unphosphorylated or phosphorylated by PKC or PKA or PKA+PKC was incubated in the presence of GST-p47phox (0.4 μM) or GST-Rac2 (0.4 μM) (Panel B), for 1h at room temperature in a total volume of 500 μl. The glutathione Sepharose 4B beads were therefore added for 90 min. After washing and elution with reduced glutathione, SDS-PAGE and western-blot analysis were performed. The data are representative of 3 experiments.

Figure 6

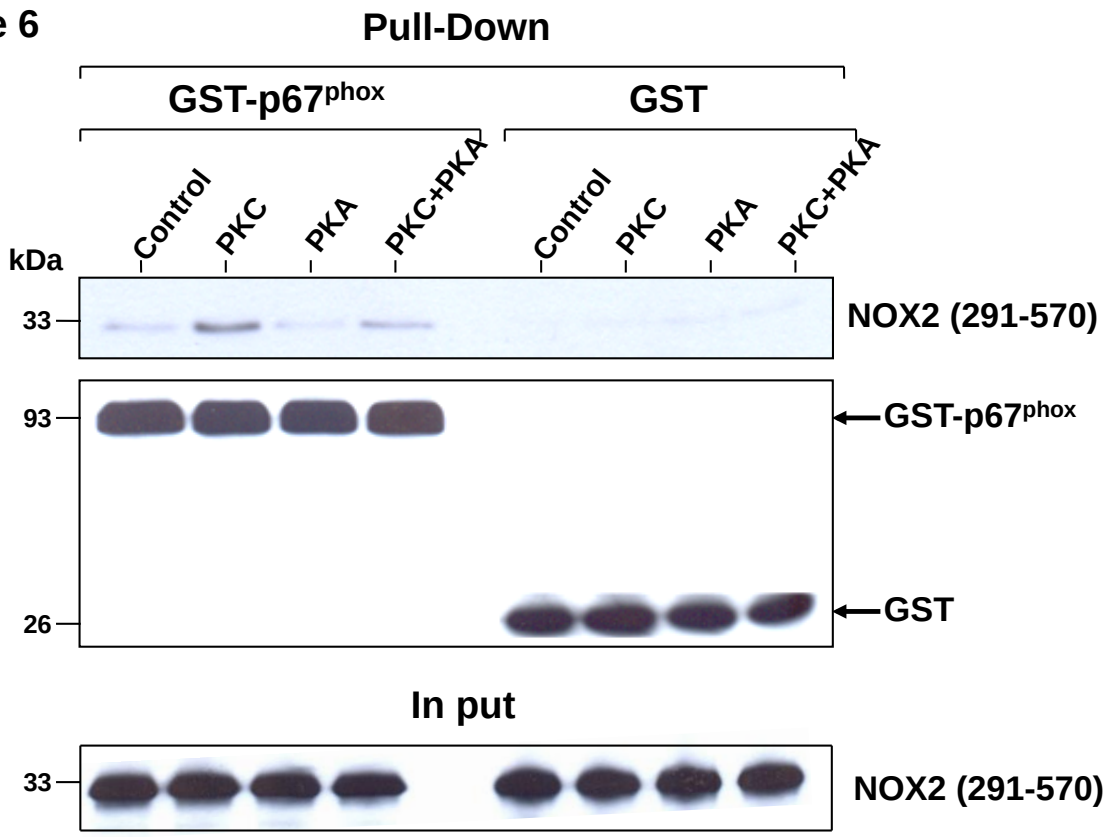

Fig. 6 Effect of PKA-induced phosphorylation of gp91phox/NOX2-cytosolic flavoprotein domain on PKC-induced interaction with p67phox and GST control. Recombinant NOX2 (291-570) unphosphorylated or phosphorylated by PKC or PKA or PKA+PKC was incubated in the presence of GST-p67phox (0.4 μM) or GST (0.4 μM) for 1h at room temperature in a total volume of 500 μl. The glutathione Sepharose 4B beads were therefore added for 90 min. After washing and elution with reduced glutathione, SDS-PAGE and western-blot analysis were performed. The data are representative of 3 experiments.

PKA-mediated phosphorylation of the gp91^{phox}/NOX2 cytosolic flavoprotein domain inhibits its diaphorase activity

Gp91^{phox}/NOX2 was shown to have an intrinsic INT-reductase activity, which is also known as diaphorase activity [23, 25], and the cytosolic flavoprotein domain (amino acids 291 to 570) was shown to be responsible for this activity [24, 25]. PKC phosphorylation of this domain was able to increase its intrinsic enzymatic diaphorase activity [26, 39]. In the presence of PKC, FAD and NADPH, phosphorylation of

NOX2 (291-570) by PKC induced an increase in diaphorase activitythat can be inhibited by DPI (Fig.7). Phosphorylation of NOX2 (291-570) by PKA alone did not induce diaphorase activity, however phosphorylation by PKA before the addition of PKC significantly inhibited this diaphorase activity. Taken together, these results suggest that phosphorylation of gp91^{phox}/NOX2 by PKA may negatively regulate its catalytic activity in neutrophils.

Figure 7

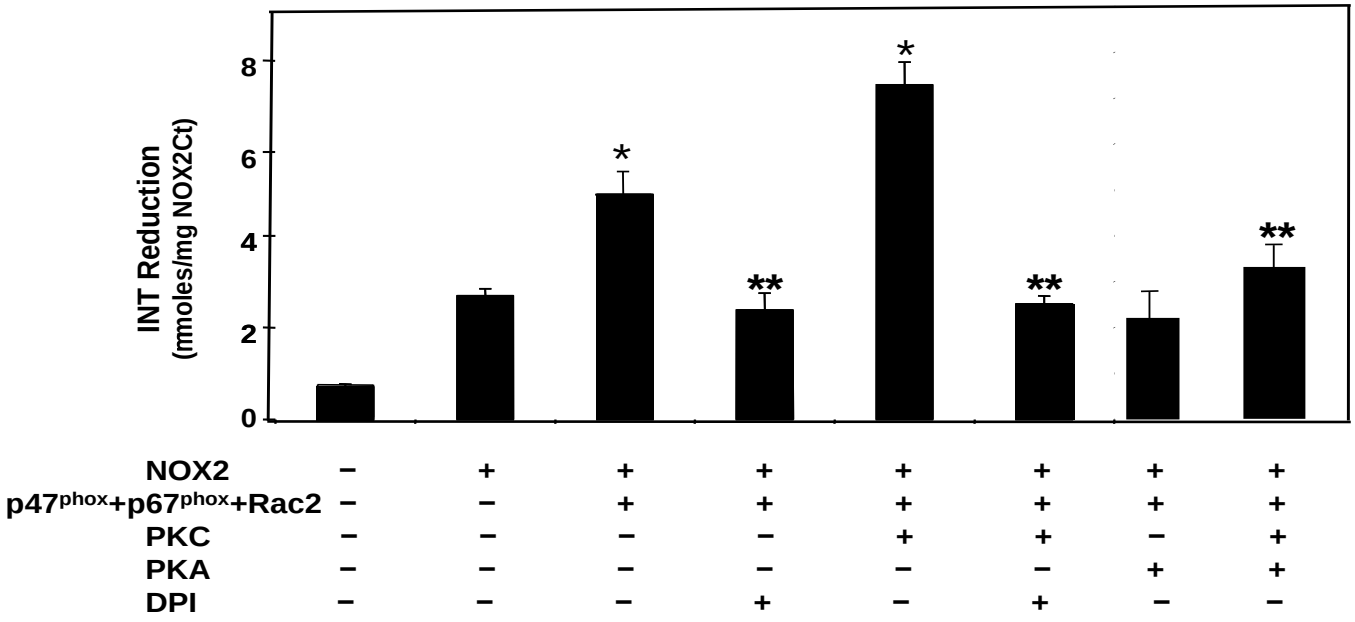

Fig. 7 Effect of PKA-induced phosphorylation of gp91^{phox}/NOX2-cytosolic flavoprotein domain on INT-reductase activity in the absence or presence of PKC. Recombinant NOX2(291-570) alone or in the presence of p47phox, p67phox and Rac2 was incubated with or without PKC, and INT-reductase activity was measured. Activity was determined in a 500 µl volume of assay buffer containing 0.2 mM INT and 1 µg of the phosphorylated or non-phosphorylated NOX2(291-570) which was preincubated with (+) or without (-) DPI (20 µM) for 15 min. The reaction was initiated by addition of 0.2 mM NADPH, and the rate of INT reduction was monitored by measuring absorbance at 500 nm. The data are representative of 6 experiments. Statistically significant differences (* $p<0.05$) are indicated.

Discussion

Regulation of the phagocyte NADPH oxidase is mediated by several pathways such as the phosphorylation of p47phox, p67phox, p40phox, p22phox and gp91phox. Although the role of the phosphorylation of p47phox is extensively studied, less is known about the phosphorylation of the other subunits and particularly gp91phox. In this study we clearly show that PKA phosphorylates gp91phox/NOX2 in neutrophils and show that the cytosolic fragment NOX2(291-570) is phosphorylated *in vitro* by PKA. Unlike PKC, phosphoamino acid analysis of PKA-phosphorylated NOX2 (291-570) showed that this protein was phosphorylated only on serine residues. In addition, tryptic phosphopeptide mapping of recombinant gp91phox/NOX2 (291-570) phosphorylated *in vitro* by PKA shows that this kinase targets the phosphorylation of two peptides on NOX2 (291-570) which can be phosphorylatedin intact neutrophils and by PKC *in vitro*. Our results also show that phosphorylation of NOX2 (291-570) by PKA induced inhibition of its PKC-induced interaction with the cytosolic partners (Rac2, p67phox and p47phox) and inhibits its diaphorase activity.

The hydrophilic C-terminal domain of gp91phox/NOX2 contains the FAD-binding and consensus NADPH-binding sequences [20, 40, 41], and it has been proposed that gp91phox/NOX2 is the catalytic core of NADPH oxidase because it contains all the required factors [40-42]. The NADPH oxidase appears to catalyze superoxide $O_2^{-\bullet}$ production *via* a 2-step electron transfer process. The first step, catalyzed by the flavin center, is called diaphorase activity [22–25], and it has been shown previously that the cytosolic tail of gp91phox/NOX2, which contains the flavoprotein domain, also exhibits NADPH diaphorase activity [24, 25]. Currently, little is known how this electron transfer process is induced and regulated. We have previously shown that the phosphorylation of the cytosolic tail of gp91phox/NOX2 increased diaphorase activity [26]. Here, we further show that the phosphorylation *in vitro* of NOX2 (291-570) by PKA induces

a decrease of diaphorase activity of the cytosolic protein tail. These data suggest that phosphorylation by PKA (in contrast to PKC) could induce a conformational change in the gp91phox/NADPH, which may reduce electron transfer or binding of alignment components of the chain, such as NADPH or FAD. In addition, the phosphorylation of the cytosolic fragment of NOX2 by PKA inhibited its interaction with p67phox, p47phox, and Rac2 and suggesting that this phosphorylation also modulates the assembly of the complex. Taken together, these results suggest that phosphorylation of gp91phox/NOX2 by PKA down regulate the assembly of the complex and its catalytic activity.

In contrast to PMA, physiological agonists such as fMLF, C5a and PAF, induce a transient and short activation of superoxide production by neutrophils. The inactivation is preceded by an increase of cAMP level in cells [27] and may be due to either phosphorylation or dephosphorylation of several targets upstream of the NADPH oxidase complex or the belonging to the enzyme. In addition, inhibitors of PKA potentiated superoxide production by neutrophils [43]. Studies from the literature showed that cAMP elevation in neutrophils inhibited PLD [44-47] and PLA2 activation [30, 48]. It was also shown that Rap1A, a small G-protein associated with the cytochrome b558, is a phosphorylated by PKA and this phosphorylation could induce inhibition of neutrophil functions such as ROS production [49].

Regarding the NADPH oxidase targets of the cAMP-PKA pathway, p47phox was suggested as a possible candidate as it is a good substrate of PKA [31, 50]. In this study we show that NOX2 is a PKA substrate and show that kinase inhibits its electron transfer and interaction with cytosolic partners p47phox, p67phox and Rac2. This result suggests that cAMP enhancers (activators) may have anti-inflammatory effects. In addition to the cAMP-PKA pathway, ataxia telangiectasia-mutated kinase (ATM kinase) was shown to phosphorylate gp91phox and to inhibit NADPH oxidase activation in neutrophil-like cells [51]. The Btk Kinase was reported to negatively regulate ROS production by neutrophils probably by controlling the PI3Kinase-Rac2 pathway [52]. These three kinases (PKA, ATM Kinase and Btk) are themself controlled

by specific phosphatases, PP2A [53], Wip1 [54] and SHP-1/SHP-2 [55] respectively, ensuring a tight regulation of the NOX inhibitory mechanisms. PKA, ATM Kinase and Btk could play a role in limiting ROS production by neutrophils and constitute a family of gatekeepers of neutrophil ROS production.

Regarding the regulation of the other NOXs by phosphorylation and the possible involvement of PKA, NOX5 and DUOX1/2 have been described to be directly phosphorylated by different protein kinases [56-59]. NOX5 is phosphorylated by PKCα, PKCε, PKCδ, ERK, CAM-kinase II [56, 57] and the tyrosine kinase c-Abl [58]. Interestingly, only phosphorylation by PKCε and PKCδ inhibited PMA-induced NOX5 activity [56, 57]. The phosphorylation of NOX5 by PKA and its effect are not known, PKA could be involved in this process in physiological conditions. DUOX1 but not DUOX2 was phosphorylated by PKA and its activity is stimulated by forskolin via protein kinase A-mediated Duox1 phosphorylation on serine 955 and Duox2 was phosphorylated by PKC and PMA stimulated its activity via PKC [59]. At our knowledge, NOX1 is not known to be directly phosphorylated by PKA, however PKA phosphorylated its regulatory component NOXA1 and inhibited its activity [60, 61].

In conclusion, our results show that NOX2 (291-570) is phosphorylated by PKA *in vitro* and this phosphorylation plays an antagonistic role to that of PKC by inhibiting activation of NADPH oxidase. This work suggests that phosphorylation of gp91phox/NOX2 by PKA participates in the regulation of electron transfer and the disassembly of cytosolic proteins of NOX2. NOX2 phosphorylation is a novel mechanism which can regulate ROS generation by neutrophils at inflammatory sites and could constitute a novel target for new anti-inflammatory agents.

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Author contributions

HR designed and performed the experiments. HM, HH, MES, RAD and TB performed the experiments. MAGP, PMCD and JEB designed the experiments and analyzed the data. All authors contributed to writing the manuscript.

Conflicts of Interest

The authors declare no competing conflict of interest.

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