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Acute inhibition of superoxide formation and Rac_1 activation by nitric oxide and iloprost in human vascular smooth muscle cells in response to the thromboxane A_2 analogue, U46619

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Abstract

Background—The over-production of superoxide ($O_2^{\cdot-}$) derived from NADPH oxidase (NOX) plays a central role in cardiovascular diseases. By contrast, nitric oxide (NO) and prostacyclin (PGI_2) are vasculoprotective. The effect of the NO donor, NONOate and iloprost on $O_2^{\cdot-}$ formation, $p47^{phox}$ and Rac_1 activation in human vascular smooth muscle cells (hVSMCs) was investigated.

Methods—hVSMCs were incubated with 10 nM thromboxane A_2 analogue, U46619 for 16 h, and then with apocynin (a NOX inhibitor), NONOate or iloprost for 1 h and $O_2^{\cdot-}$ measured spectrophotometrically. The role of cyclic AMP and cyclic GMP was examined by co-incubation of drugs with protein kinase (PK) A and G inhibitors listed above. Rac_1 was studied using pull-down assays.

Results—NONOate and iloprost inhibited $O_2^{\cdot-}$ formation, acutely, effects blocked by inhibition of PKG and PKA, respectively. Rac_1 and $p47^{phox}$ activation and translocation to the plasma membrane was completely inhibited by NONOate and iloprost, effects again reversed by co-incubation with PKG or PKA inhibitors.

Conclusions—NO and PGI_2 block the acute activity of NOX in hVSMCs via the cGMP–PKG axis (for NO) and by the cAMP–PKA axis (for iloprost) through inhibition of Rac_1 and $p47^{phox}$ translocation. These findings have implications in the pathophysiology and treatment of CVD.

1. Introduction

The intravascular over-production of superoxide ($O_2^{\cdot-}$) derived from NADPH oxidase (NOX) is involved in the pathogenesis of cardiovascular diseases that include diabetic angiopathy, sepsis, hypertension, atherogenesis, thrombosis, vein graft failure, restenosis and ischaemia reperfusion injury [1-4]. Among the vasculopathic effects of $O_2^{\cdot-}$ is the negation of the vasculoprotective actions of nitric oxide (NO) [5,6] and prostacyclin (PGI_2) [7]. Conversely, NO and PGI_2 block the expression of NOX subunits including $gp91^{phox}$ and $p47^{phox}$ induced by vasculopathic factors which include thromboxane A_2 (TXA_2), cytokines, hypoxia, isoprostanes and superoxide [5-11]. It appears, therefore, that NOX is in dynamic balance with endogenous NO and PGI_2 such that the over-expression of NOX and augmentation of $O_2^{\cdot-}$ formation negates the suppressive actions of NO and PGI_2 on NOX expression. Drugs that mimic or augment the action of PGI_2 and NO (NO-donors, PGI_2 mimetics and type-5 phosphodiestase inhibitors) also reduce the expression of NOX subunit

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proteins (e.g. $p47^{phox}$ and $gp91^{phox}$) and reduce $O_2^{\cdot-}$ formation in vascular cells and tissue [11]. It was suggested, therefore, that these drugs alter the balance in favour of inhibition of NOX proteins and $O_2^{\cdot-}$ formation which would, therefore, restore the vasculoprotection afforded by NO and PGI_2.

In these aforementioned studies, however, the acute effects of NO donors and PGI_2 mimetics on NOX activity were not studied. This is of importance as acute release of $O_2^{\cdot-}$ derived from NOX has been implicated in the aetiology of acute syndromes that include myocardial infarction, angina, acute respiratory distress syndrome and ischaemia reperfusion injury [1-5,12]. In vascular smooth muscle cells (VSMCs), acute activation of NOX involves the assembly of subunits of the whole enzyme complex, which is controlled by the activation and translocation of the Rho-like small GTPases, Rac_1 [13]. It is possible that PGI_2 and NO may block the acute activation of NOX through an inhibition of Rac_1 and translocation to the plasma membrane.

In order to test these proposal, the acute effects of NONOate and iloprost on $O_2^{\cdot-}$ formation in human VSMCs was studied. Human vascular smooth muscle cells (hVSMCs) derived from saphenous veins were incubated with 10 nM thromboxane A_2 analogue, U46619 (acutely and over the longer term) and the cells then incubated NONOate or iloprost and $O_2^{\cdot-}$ measured spectrophometrically. Cells were either incubated acutely with U46619 (i.e. for 1 h) or for 16 h before testing acute effects of NO or iloprost. Priming cells for 16 h with U46619 is of relevance, as NOX activity is low in healthy vasculature. However, cardiovascular disease is associated with an upregulation of NOX by vasculopathic factors, including TXA_2 [1-4]. The role of cyclic AMP and cyclic GMP was examined by co-incubation of drugs with protein kinase (PK) A and G inhibitors and effects on Rac_1 studied using Rac_1 pull-down assays. Effects on the translocation of $p47^{phox}$ were also studied as this is a key event in acute NOX activation.

2. Materials and methods

2.1. Drugs

9,11-Dideoxy-9α,11α-methanoepoxyprostaglandin $F_2\alpha$ (U46619), PKA inhibitor, 14–22 amide peptide, and PKG inhibitor, DT-3 peptide, were purchased from Calbiochem (Nottingham, UK). Iloprost was purchased from Schering (Berlin, Germany). Monoclonal antibody to Rac_1 was obtained from Upstate (NY, USA) and polyclonal antibody to $p47^{phox}$ was purchased from Santa Cruz (CA, USA). All the other drugs were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated.

2.2. Culture and incubation of vascular smooth muscle cells

Saphenous veins were obtained from patients undergoing coronary artery bypass graft surgery CABG for which ethical approval and patient consent had been obtained. Veins were placed in medium RPMI 1640 (Gibco BRL; Paisley, Scotland) containing 2% amphotericin (Gibco BRL and 0.4% heparin; Sigma Chemical Co.). hVSMC were then grown in Dulbecco’s minimum essential medium—Glutamax without sodium pyruvate (DMEM; Gibco BRL), containing 100 units/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 10% foetal calf serum (Gibco BRL). After passage 4, hVSMCs were seeded in a 6-well or 24-well plates at a density of 6×10^4 cells per well and cultured for 2 days in DMEM/fetal calf serum. Cells were then rendered quiescent for 3 days in serum-free medium (Gibco BRL) before commencing experiment. Under these conditions, there was no loss of cell numbers over this time course. Cells were incubated with thromboxane A_2 analogue, U46619 (100 nM) for either 1 or 16 h in the continual presence of either iloprost (100 ng/ml) or Deta-NONOate (10 μM) or NADPH oxidase inhibitor, apocynin (10 μM).

Acute effects of iloprost and Deta-NONOate were studied by exposing cells to these compounds for 1 h following overnight incubation with U46619. To assess the role of PKA and PKG in mediating the effects of iloprost and Deta-NONOate, in some experiments, cells were pre-incubated for 30 min with the heat-stable and cell-permeable inhibitors of PKA, 14-22 amide peptide sequence [14], or PKG, DT-3 peptide [15], before the addition of iloprost or Deta-NONOate. These are highly specific inhibitors of PKA and PKG [14,15]. Following incubation, cells were rinsed in PBS and superoxide release was measured by ferricytochrome *c* assay and protein expression was measured by the Western analysis and Rac_1 by pull-down assay.

2.3. Measurement of superoxide

The measurement of superoxide formation and release by cultured cells was performed by detection of ferricytochrome *c* reduction, as previously described [6]. We opted for this method rather than the lucigenin method as even though more sensitive lucigenin itself undergoes auto-oxidation and thereby act as a source of superoxide generation. We also found that cytochrome *c* was adequate as sensitive in detecting superoxide release in our system. Following incubation, cells were washed three times with phosphate buffered saline (PBS) and equilibrated in DMEM without phenol red for 10 min at 37 °C in a 95% air–5% CO_2 incubator (Heraeus, Hera Cell, Kandro Laboratory Products, Germany). Twenty micromolar horseradish cytochrome *c* with or without 500 U/ml copper–zinc SOD was added to the cells and incubated at 37 °C in a 95% air–5% CO_2 incubator for 1 h. The final volume of the reaction mixture was 0.5 ml per well. After 1 h, the reaction medium was removed and maximum rate of reduction of cytochrome *c* was determined at 550 nM on a temperature-controlled Anthos Lucy 1 spectrometer (Lab-tech International, Ringmer, East Sussex, UK) and converted to micromoles of superoxide, using $\Delta E_{550\ \mathrm{nM}}$ = 21.1 mM/cm/min as the extinction coefficient for (reduced–oxidised) cytochrome *c*. The reduction of cytochrome *c* that was inhibitable with SOD reflected actual formation release. Cells were rinsed in PBS, lysed with 0.1% (v/v) Triton X-100 and total protein content measured using BCA-protein assay kit.

2.4. Western blotting

For Western analysis of NOX1, HVSMC were washed and lysed with Tris buffer (100 mM, pH 6.8) containing 10% glycerol and 1% sodium dodecyl sulphate (SDS) [16-19]. Extracts were boiled at a 1:1 ratio with the loading buffer containing Tris (125 mM, pH 6.8), 4% (w/v) SDS, 10% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol and 2 mg/ml bromophenol blue. Total cell lysates of equal protein (50 μg) were loaded onto 10% Tris–glycine sodium dodecyl sulphate gels and separated by electrophoresis. After transfer to nitrocellulose, the blots were primed overnight with the primary antibodies to Rac_1 (1:1000) and $p47^{phox}$ (1:500). The blots were then incubated with either goat anti-mouse (1:3000; Dako, UK; for Rac_1) or goat anti-rabbit (1:100,000; for $p47^{phox}$) conjugated to horseradish peroxidase for 1 h and developed by enhanced chemiluminescence (Amersham International, UK). Rainbow markers (10–250 kDa; Amersham International) were used for molecular weight determination.

2.5. Rac_1-activation and translocation assays

In order to investigate the involvement of Rac_1 in U46619-stimulated $O_2^{\cdot -}$ generating system and the effect of iloprost and NONOate on Rac_1 activation, hVSMCs were treated for 1 h with a Rho–GTPase inhibitor, *Clostridium difficile* toxin B, following a 16-h incubation with U46619. Activation of Rac_1 was analysed by GST-PAK-CRIB pull down assays. Briefly, cells were washed in cold PBS and lysed in 800 μl of lysis buffer: 50 mM Tris–HCl, pH 7.2, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM AEBSF,

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10 μg/ml aprotinin and 10 μg/ml leupeptin. Insoluble cell debris was removed by centrifugation and the supernatant mixed with GST-tagged CRIB domain of PAK (bound to glutathione-sepharose) at 4 °C for 1 h. Resin was washed four times in 50 mM Tris–HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM AEBSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Bound proteins were eluted in SDS sample buffer and analysed by immunoblotting for Rac_1.

Membrane and cytosolic localisation of Rac_1 and $p47^{phox}$ was quantified by cell fractionation. Briefly, cells were washed in PBS and lysed by sonication (5-7 Hz; 3 × 10 s bursts) in pre-chilled hypotonic lysis buffer (10 mM Tris–HCl (pH 7.4), 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 0.2 mM $NaVO_4$, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM AEBSF). Nuclei and intact cells were removed by centrifugation at 3000 rpm at 4 °C for 5 min. Supernatants were subjected to ultra-centrifugation at 100,000 × *g* at 4 °C for 45 min using Beckman Optima L-90K Ultracentrifuge (Beckman Instruments Inc., USA). The pellet was defined as the membrane fraction and the supernatant as cytosolic fraction. The membrane pellets were dissolved in 80 μl of SDS-lysis buffer and 200 μl of cytosolic fractions were also mixed with 50 μl of SDS-lysis buffer. Equal protein amounts of membrane and cytosolic fractions were subjected to western blot analysis for $p47^{phox}$ and Rac_1. Membranes were re-probed with anti-α/β tubulin antibody (Upstate, USA) to assess the efficiency of cell fractionation.

2.6. Data analysis

The data were tested for normality by inspecting histograms and by applying the Kolmogorov–Smirnov test (automatically applied by Sigma Stat™ as part of the procedure for producing ANOVA results). In all cases, the data did not deviate sufficiently from normality to warrant non-parametric statistics. All data represent the means of triplicate determinations; each experiment was repeated at least six times. Both one-way and two-way analysis of variance (ANOVA) was used to determine statistical significance. Two-way ANOVA tests were employed where two conditions existed and one-way ANOVA was used when comparing effects of drug treatments with untreated controls. Statistical significance was assumed at a value of $P<0.05$.

3. Results

Sixteen-hour incubation of hVSMCs with the thromboxane A_2 analogue, U46619 elicited an increase in $O_2^{\cdot-}$ formation (Fig. 1). Incubation with iloprost or NONOate for 1 h, following a 16 h incubation with U46619, completely blocked U46619-stimulated $O_2^{\cdot-}$ formation in concentration-dependent manners (Fig. 1). The inhibitory effect of iloprost on U46619-stimulated $O_2^{\cdot-}$ formation was blocked by PKA peptide inhibitor but not by PKG peptide inhibitor (Fig. 2A). By contrast, the inhibitory effect of NONOate was blocked by the PKG peptide inhibitor and not by PKA peptide inhibitor (Fig. 2B). Acutely, incubation with U46619 for 1 h augmented $O_2^{\cdot-}$ formation from hVSMCs, an effect blocked by iloprost, NONOate and apocynin (Fig. 3).

Acute incubation with *C. difficile* toxin B, a Rho-GTPase inhibitor, for 1 h completely inhibited the increase in $O_2^{\cdot-}$ release from hVSMCs following a 16-h incubation with U46619 (Fig. 4). Incubation with U46619 for 16 h also increased Rac_1 activity in hVSMCs (Fig. 5). Acute incubation with iloprost and NONOate for 1 h reversed increased Rac_1 activity to basal level (Fig. 5). These inhibitory effects of iloprost or NONOate on Rac_1 activity were blocked by PKA or PKG peptide inhibitor, respectively (Fig. 5). Acute incubation with iloprost and NONOate for 1 h also inhibited $p47^{phox}$ and Rac_1 translocation to the plasma membrane in hVSMCs when co-incubated with U46619 for 16 h (Fig. 6).

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4. Discussion and conclusions

The present study demonstrates firstly that U46619 (a stable analogue of thromboxane A_2) augments $O_2^{\cdot-}$ formation in human VSMCs following a 16-h incubation. This effect of U46619 was blocked by the NOX inhibitor, apocynin, indicating that U46619 up-regulates NOX, which is the source of $O_2^{\cdot-}$ under these conditions. This is consistent with previous studies in porcine and rabbit vascular cells, in which U46619 was shown to upregulate the expression of NOX subunits, including gp91^{phox} and p47^{phox} [7-9,11]. In subsequent experiments, cells were primed by incubating with U46619 and acute effects of NONOate and iloprost were then studied. This model is of relevance as acute cardiovascular events are associated with an a priori upregulation of NOX by vasculopathic factors [1-4].

U46619 also acutely promoted the formation of $O_2^{\cdot-}$, an effect again blocked by apocynin indicating that TXA_2 acutely activates NOX in human VSMCs. This too is consistent with a previous study in isolated rabbit cavernosal smooth muscle cells in which it was demonstrated that U46619 increases $O_2^{\cdot-}$ formation through activation of NOX [11]. In this latter study, however, the acute effects of iloprost, NO donor, the role of PKA or PKG, Rac_1 or the translocation of p47^{phox} was not studied.

In the present study, both NONOate and iloprost inhibited the acute formation of $O_2^{\cdot-}$ following priming and upregulation of NOX activity with U46619 as well as acutely (over 1 h without priming). It should be stressed that direct quenching could not account for these effects as NONOate and iloprost were washed clear of the system before measuring $O_2^{\cdot-}$. Furthermore, the effect of iloprost was blocked by PKA inhibitors but not by inhibitors of PKG and the effect of NONOate was blocked by inhibition of PKG but not by inhibition of PKA. This demonstrates that the inhibition of $O_2^{\cdot-}$ formation is mediated by classic pathways: adenylyl cyclase–cAMP–PKA for iloprost and guanylyl cyclase–cGMP–PKG pathway for NONOate. These novel effects adds to the repertoire of vasculoprotective attributes of NO and PGI_2.

The acute activation of NOX involves a complex sequence of events that is controlled by activation and translocation of both Rac_1 and p47^{phox} to the plasma membrane [13]. In the present study, pharmacological inhibition of Rac_1 with toxin B completely inhibited $O_2^{\cdot-}$ formation, consolidating that Rac_1 plays an obligatory role in mediating acute NOX activity in hVSMCs. Using adenoviruses, we have also previously demonstrated that over-expression of Rac_1 in hVSMCs promotes NOX activity but infection with a dominant negative adenovirus blocks NOX activity [20]. In the present study, iloprost and NONOate also blocked the translocation of both Rac_1 and p47^{phox} to the plasma membrane, effects that were again blocked by PKA inhibition (for iloprost) and PKG inhibition (for NONOate) as for $O_2^{\cdot-}$ formation above (Fig. 7; [42-44]).

These data are consistent with another study in which it was demonstrated that cAMP inhibits Rac_1 activity, which in turn inhibits the replication and migration of VSMCs [18,19]. Other functions associated with vasculopathy that are elicited by Rac_1 activation but inhibited by both NO and PGI_2 are adhesion molecule expression [21-23], calcium mobilisation [24-26], metalloproteinase expression [27-29] and VSMC migration [30-32]. In turn, $O_2^{\cdot-}$ derived from NOX also augments these diverse vascular events, suggesting that a common denominator for NOX–NO and PGI_2 interactions is Rac_1.

The mechanisms underlying the regulation of Rac_1 activity by PKA and or PKG are unknown but may involve phosphorylation of one or more of the large family of guanine nucleotide exchange factors (GEFs) and/or GTPase activating proteins (GAPs) that govern functional status of all GTPases in the cell [33]. The GTPases are activated by GEFs, which

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catalyse loading with GTP, and inactivated by GAPs, which accelerate hydrolysis of the bound nucleotide to form GDP [34]. Exploring this area is beyond the scope of the present study but warrants further investigation.

From a clinical perspective, the acute activation of intravascular NOX and the resultant burst in endogenous $O_2^{\cdot -}$ formation promotes events that include thrombosis, vasoconstriction, adhesion molecule expression and ischaemia reperfusion injury [1-4]. These events are central to the aetiology of diverse disorders that include angina pectoris, myocardial infarction and sepsis. In turn, both PGI_2 and NO donors have been shown to prevent these events in pre-clinical models and in man [35,36]. It is not unreasonable to suggest, therefore, that the acute therapeutic action of NO and iloprost are mediated, at least in part, through inhibition of acute NOX activation. The corollary to this is that over-expression of NOX and excess $O_2^{\cdot -}$ formation leads to a negation of the endogenous vasculoprotective actions of NO and PGI_2 which in turn leads to a greater expression of NOX [4-7,37,38]. Therapeutically, PGI_2 mimetics and NO donors may act by breaking this cycle. Furthermore, drugs that augment the biological actions of NO and PGI_2, namely phosphodiesterase inhibitors, have been shown to block the upregulation of NOX in vascular cells through protection of PKA and PKG activation driven by NO and PGI_2 [11,39-41]. Thus, it is reasonable to suggest that co administration of PDE inhibitors may augment the therapeutic potential of NO donors and PGI_2 mimetics through enhanced suppression of intravascular oxidative stress.

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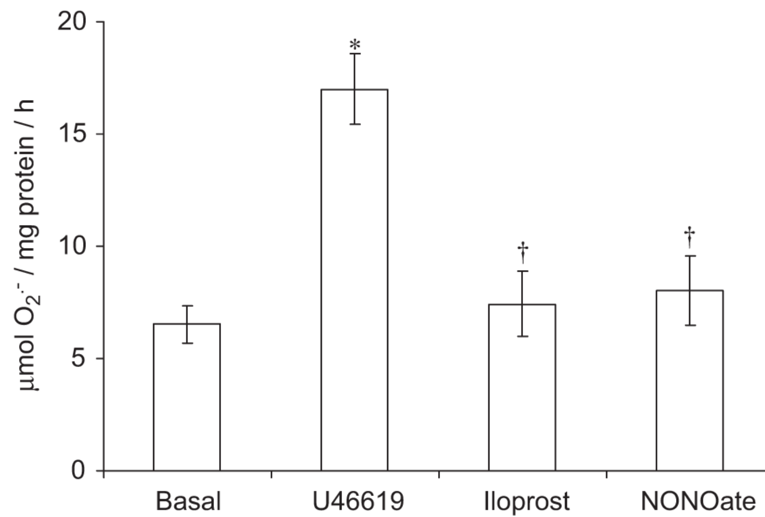

Fig. 1.

Acute effect (1 h) of iloprost (100 ng/ml) and NONOate (10 µM) following a 16-h incubation with U46619 (100 nM) on superoxide (O_2^-) formation by human isolated vascular smooth muscle cells (hVSMCs). Each point represents the mean (±S.E.M.), $n = 6$. *P<0.05, significantly greater than basal. †P<0.01, significantly reduced compared to U46619.

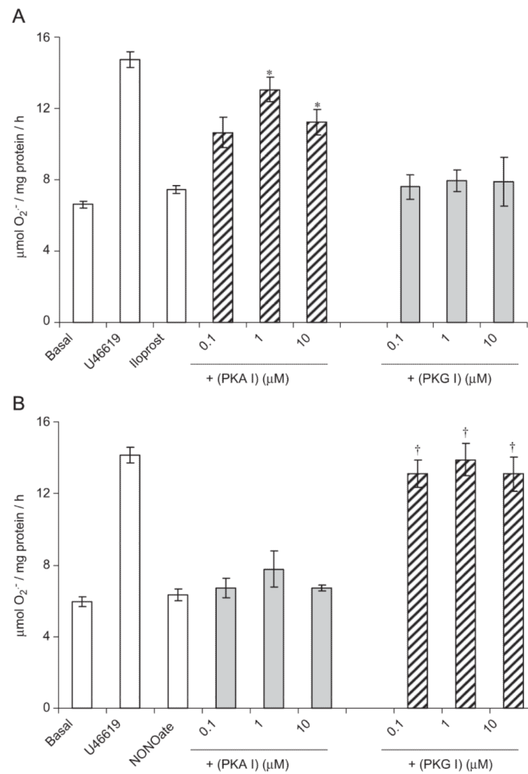

Fig. 2.

Effect of PKA inhibitor (PKA I; 14-22 amide peptide sequence) or PKG inhibitor (PKG I; DT-3 peptide) on the acute inhibition of O_2^{-} formation by hVSMCs pre-incubated for 16 h with 100 nM U46619 (100 nM) by (A) iloprost (100 ng/ml) and (B) NONOate (10 µM). Each point = mean±S.E.M., $n = 6$. *P<0.01, significantly increased compared to iloprost alone. †P<0.01, significantly increased compared to NONOate alone.

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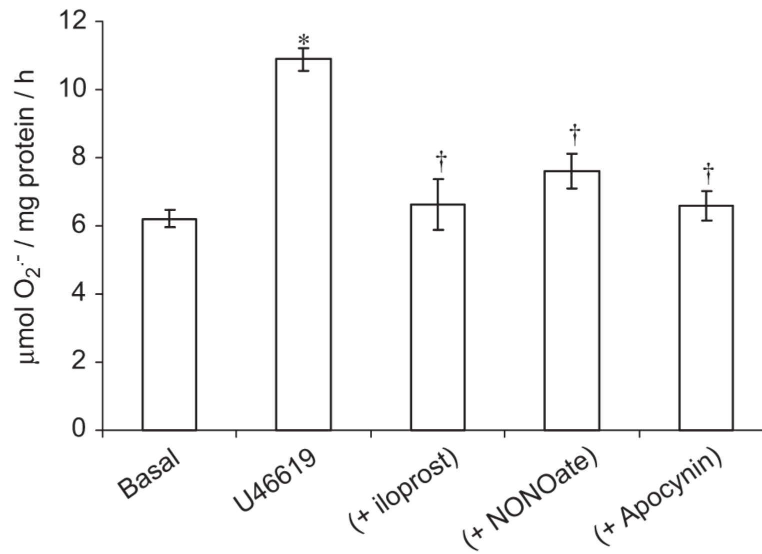

Fig. 3.
Acute (1-h incubation) effect of U46619 (100 nM) [±iloprost (100 ng/ml), NONOate (10 μM) or apocynin (10 μM)] on superoxide ($O_2^{\cdot-}$) formation by human isolated vascular smooth muscle cells (hVSMCs). Each point represents the mean (±S.E.M.), $n = 6$. *$P<0.01$, significantly increased compared to basal. †$P<0.01$, significantly reduced compared to U46619 alone.

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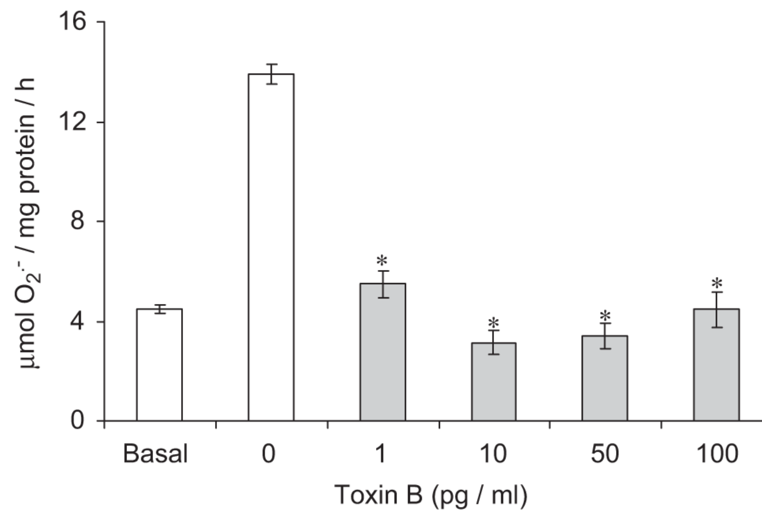

Fig. 4.

Acute effect (1-h incubation) of a Rho–GTPase inhibitor, *Clostridium difficile* toxin B, on U46619 (100 nM)-induced superoxide ($O_2^{\cdot-}$) formation (16 h) in human isolated vascular smooth muscle cells (hVSMCs). Each point represents the mean (±S.E.M.), $n = 6$. *$P<0.01$, significantly reduced compared to U46619.

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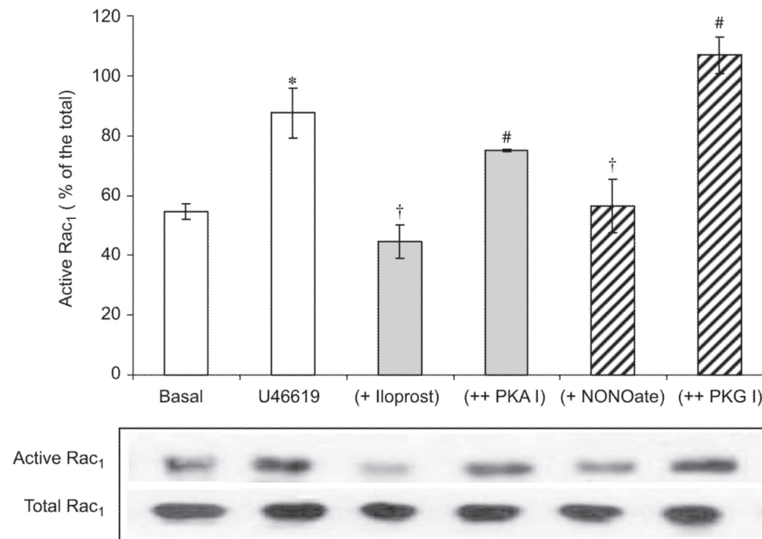

Fig. 5.

Acute effect (1-h incubation) of iloprost (100 ng/ml) or Deta-NONOate (10 μM)±PKA or PKG inhibitors (100 nmol) on Rac_1 activity following a 16-h incubation of human VSMC with U46619 (100 nmol). Rac_1 activity was quantified by Rac_1 pull down assays. Blots are representative of six independent experiments. *$P<0.05$, significantly increased compares to basal value. $^{\#}P<0.05$, significantly inhibited compared to U46619-treated value. $^{\dagger}P<0.01$, significantly increased compared to iloprost or Deta-NONOate only values.

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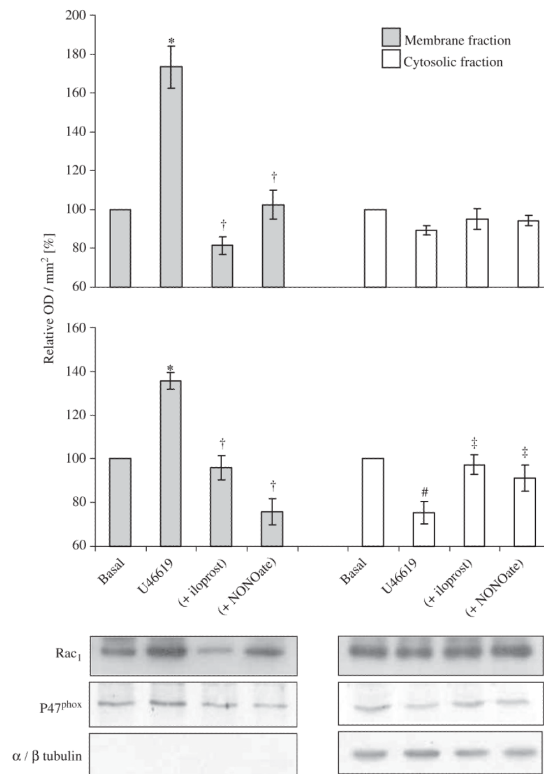

Fig. 6.
Acute effect (1-h incubation) of iloprost (100 ng/ml) or Deta-NONOate (10 μM) on Rac_1 and $p47^{phox}$ membrane localisation following a 16-h incubation of human VSMC with 100 nmol U46619. Total cell lysates were fractionated and the membrane and cytosolic levels of (A) Rac_1 and (B) $p47^{phox}$ quantified by Western blotting. Blots are representative of at least six independent experiments. *P<0.05, significantly increased compares to basal value. $^\dagger P$<0.05, significantly inhibited compared to U46619-treated membrane fractions. $^\# P$<0.01, significantly reduced compares to basal value. $^\ddagger P$<0.01, significantly increased compared to U46619-treated cytosolic fractions.

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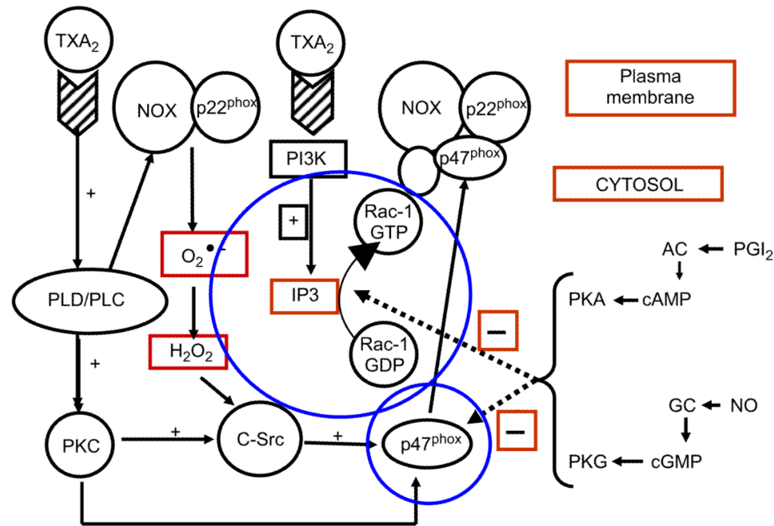

Fig. 7.

Working model for the acute activation thromboxane A_2 (TXA_2) of NADPH oxidase (NOX) activity and the inhibitory action of prostacyclin (PGI_2) and nitric oxide (NO) in human vascular smooth muscle cells. TXA_2 activates both phospholipase C (PLC) and D (PLD) and PI3 kinase (PI3 K) activity [42-44]. This has two consequences: (i) activation of protein kinase C (PKC) and c-Src which in turn triggers the translocation of 47^{phox}, a crucial catalytic subunit of the NOX complex to the plasma membrane and (ii) generation of inositol trisphosphate (IP3) which activates Rac_1 which then associates with the NOX complex, including NOX1 and 22^{phox} at the plasma membrane. These key events trigger the activation of NOX which then releases superoxide ($O_2^{\cdot-}$) which further augments NOX activation. By contrast, PGI_2 and NO, through activation of the cyclic GMP–PKG and the cyclic AMP–PKA systems, respectively, inhibit both the translocation of $p47^{phox}$ and the activation and translocation of Rac_1, thereby acutely blocking the activity of NOX and $O_2^{\cdot-}$ release.