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NADPH oxidase activity is required for endothelial cell proliferation and migration

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Abstract NADPH oxidase has been shown to play an important role in cardiovascular biology. The goal of the present study was to determine whether NADPH oxidase activity is important for endothelial cell growth and migration. In proliferation assays, growth factor- or serum-induced DNA synthesis in three different types of human endothelial cells was abrogated by inhibitors of NADPH oxidase, but not by inhibitors of xanthine oxidase or nitric oxide synthase. Moreover, vascular endothelial growth factor-induced migration of human endothelial cells was suppressed in the presence of NADPH oxidase inhibitors. These results support a potential role for NADPH oxidase in mediating angiogenesis.

Key words: NADPH oxidase; Reactive oxygen species; Endothelial cell; Proliferation; Migration

1. Introduction

Superoxide and other reactive oxygen species (ROS) have been implicated in the initiation and progression of several vascopathic disease states. At the same time, ROS are now recognized to play an important role as signal transduction intermediates [1–4]. Several intracellular sources contribute to the production of ROS, including cyclooxygenases, cytochrome P450, endothelial nitric oxide synthase (NOS), lipoxygenases, mitochondrial respiration, NADPH oxidase, and xanthine oxidase.

Recent studies have underscored the importance of NADPH oxidase-derived ROS in vascular biology. Many components of the leukocyte NADPH oxidase complex, including p22phox, p47phox, p67phox and gp91phox (or a related homologue), have been identified in endothelial cells or vascular smooth muscle cells (VSMC) [5–9]. In VSMC, NADPH oxidase activity has been shown to increase in response to thrombin [8], angiotensin II [10,11], platelet-derived growth factor (PDGF) [12], and tumor necrosis factor-α [13]. In endothelial cells, activation of NADPH oxidase has been reported to occur in response to oscillatory and steady state shear stress [14], cyclical strain [15], ischemia [16,17], or high concentrations of K^+ [16].

In the present study, we show that endothelial cell proliferation and migration require NADPH oxidase activity. These results suggest that endothelial cell growth is tightly coupled to the redox state of the cell. Moreover, the findings imply that the inhibition of NADPH oxidase activity may provide a foundation for anti-angiogenic therapy.

2. Materials and methods

2.1. Cell proliferation assays

Human coronary endothelial cells (HCAEC), human dermal microvascular endothelial cells (HDMVEC) and human umbilical vein endothelial cells (HUVEC) (Clonetics) were grown to confluence in Endothelial Cell Growth Medium-2-MV BulletKit (Clonetics) and seeded in 24-well plates at a density of 4×10^4/well. The following day, the cells were serum-starved for 7 h in Endothelial Cell Basal Medium-2 (EBM-2) containing 0.5% fetal bovine serum (FBS) and then treated for 24 h with 0.5% FBS, 10 ng/ml vascular endothelial growth factor (VEGF) (Peprotech), 5 ng/ml bFGF (Peprotech) or 5% FBS in the presence or absence of the NADPH oxidase inhibitors, DPI (Biomol) [18], apocynin (Aldrich) [19], or 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Sigma) [20]; the xanthine oxidase inhibitor, allopurinol (Sigma); or the NOS inhibitor L-NAME (Sigma) at the doses indicated. [^{3}H]Thymidine (1 μCi/well, 20 Ci/mmol) was added during the last 4 h of incubation. Cells were rinsed with phosphate buffer solution, trypsinized and radioactivity was extracted with 0.2 N NaOH. [^{3}H]Thymidine incorporation was measured by counting an aliquot of cell extract in an automated liquid scintillation counter.

2.2. Cell migration assays

Migration assays were carried out in a modified Boyden chamber (Neuro Probe). HCAEC were serum-starved overnight in EBM-2 medium containing 0.5% FBS. The bottom 48-well plate was filled with EBM-2 medium containing 0.2% FBS with or without 50 ng/ml VEGF in the presence or absence of DPI, apocynin, AEBSF, allopurinol or L-NAME at the doses indicated. A polycarbonate filter pretreated with 13.5 μg/ml fibronectin (Sigma) was laid over the bottom plate and then covered with the top plate. HCAEC were pre-incubated in EBM-2 medium containing 0.2% FBS in the presence or absence of the same inhibitors for 10 min and then added to the top well at a density of 7500 cells/well. The chamber was incubated at 37°C for 4 h. The cells were fixed and stained with Diff-Quik (Dade Behring). The cells on the top of the membrane were removed and the cells that had migrated to the bottom side were counted under light microscopy. Counts are reported as an average of cell number per mm^2 ($n=6$–12 per condition).

2.3. ROS generation

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) to fluorescent dichlorofluorescein (DCF) in fluorescence-activated cell sorting (FACS) assays as previously described [21]. HCAEC were grown to 95% confluence, incubated with control media (C) or 50 ng/ml VEGF for 1 h in the absence or presence of 100 μM DPI, 500 μM L-NAME or 100 μM

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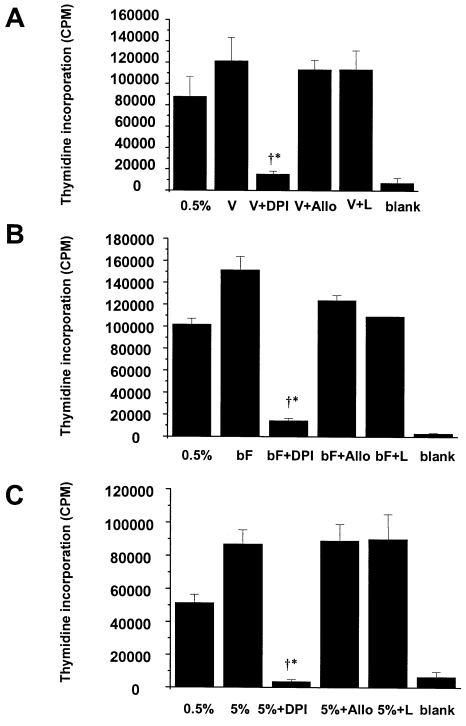

Fig. 1. Thymidine incorporation in HCAEC is decreased by NADPH oxidase inhibitor. [^{3}H]Thymidine incorporation was measured in untreated HCAEC (0.5%) or in HCAEC treated with (A) 10 ng/ml VEGF (V), (B) 5 ng/ml bFGF (bF) or (C) 5% FBS (5%) in the absence or presence of 10 μM DPI (+DPI), 100 μM allopurinol (+Allo) or 500 μM L-NAME (+L). Blank represents background counts from unseeded wells. [^{3}H]Thymidine incorporation is expressed as mean ± S.D. from four samples. $^*P < 0.05$ compared with control, untreated cells. $^\dagger P < 0.05$ compared with treated cells.

allopurinol. The cells were washed with HBSS and incubated with DCFH-DA at 37°C for 30 min. DCF fluorescence distribution of 20 000 cells was detected by FACS analysis at an excitation wavelength of 485 nm and at an emission wavelength of 530 nm. Propidium iodide-positive cells were excluded from the analysis.

3. Results

3.1. Endothelial cell proliferation is dependent on NADPH oxidase activity

The incubation of serum-starved HCAEC with 10 ng/ml VEGF resulted in a 1.4-fold induction of thymidine uptake (Fig. 1A). Pretreatment of HCAEC with 10 μM DPI (NADPH oxidase/NOS inhibitor) resulted in a dramatic reduction in VEGF-induced thymidine incorporation below basal levels, whereas pretreatment with 100 μM allopurinol (xanthine oxidase inhibitor) or 500 μM L-NAME (NOS inhibitor) had no significant effect (Fig. 1A). bFGF- or serum-mediated induction of cell proliferation (1.5-fold and 1.7-fold, respectively) was also abrogated by pre-incubation with 10 μM DPI, but not with allopurinol or L-NAME (Fig. 1B,C). DPI-mediated inhibition of DNA synthesis was dose-dependent (Fig. 2A). Moreover, proliferation of HCAEC was suppressed in a dose-dependent manner by two other inhibitors of NADPH oxidase, apocynin and AEBSF (Fig. 2B,C). In HDMVEC, VEGF-, bFGF- and serum-induced thymidine incorporation was significantly blunted by 10 μM DPI, but not by allopurinol or L-NAME (Fig. 3A–C). Similar results were obtained with HUVEC cells (Fig. 3D shows serum response). Taken together, these results suggest that both basal and stimulated endothelial cell proliferation are dependent upon NADPH oxidase activity.

3.2. Endothelial cell migration is dependent on NADPH oxidase activity

HCAEC migration was induced 1.5–3-fold in the presence of 50 ng ml VEGF (Fig. 4A–C). This response was abrogated

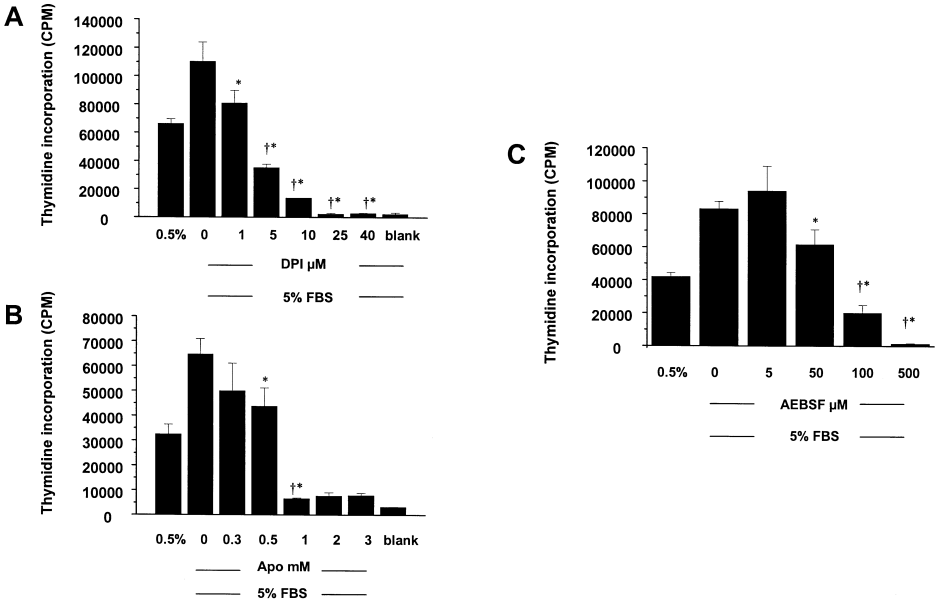

Fig. 2. Decrease in thymidine incorporation by NADPH oxidase inhibitors is dose-dependent. [^{3}H]Thymidine incorporation was measured in untreated HCAEC (0.5%) or in HCAEC treated with 5% FBS in the presence or absence of increasing doses of (A) DPI, (B) apocynin or (C) AEBSF.

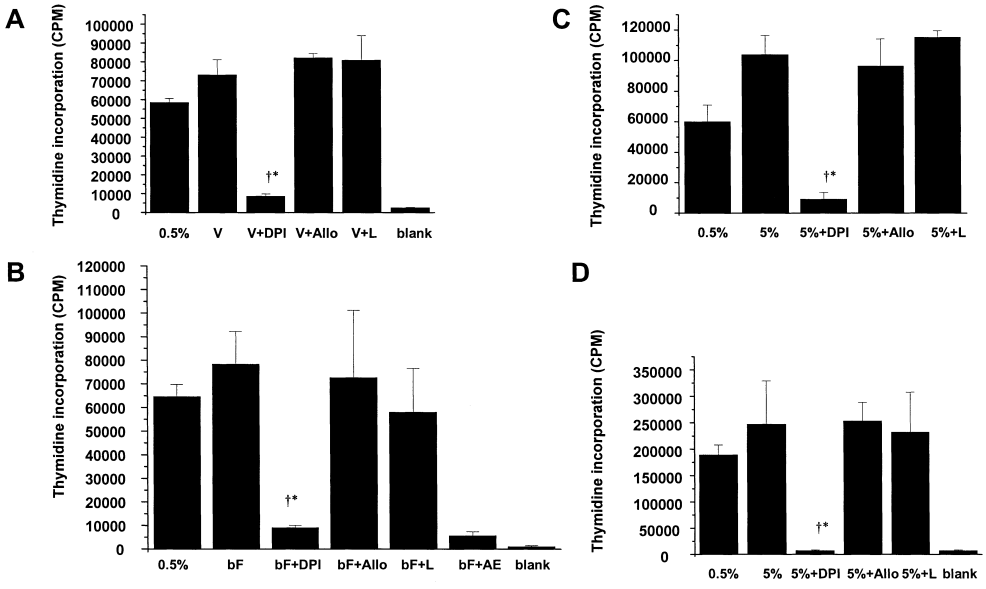

Fig. 3. Thymidine incorporation in HDMVEC and HUVEC is decreased by NADPH oxidase inhibitor. [^{3}H]Thymidine incorporation was measured in untreated HDMVEC (0.5%) treated with (A) 10 ng/ml VEGF (V), (B) 5 ng/ml bFGF (bF) or (C) 5% FBS (5%) in the absence or presence of 10 μM DPI (+DPI), 100 μM allopurinol (+Allo) or 500 μM L-NAME (+L). (D) [^{3}H]Thymidine incorporation was measured in untreated HUVEC (0.5%) or in HUVEC treated with 5% FBS in the absence or presence of the same dose of inhibitors.

by the addition of 10 μM DPI or 300 μM apocynin (Fig. 4A,B). Moreover, both basal and VEGF-induced migration were inhibited by 250 μM AEBSF (Fig. 4C). HCAEC migration was inhibited by DPI (Fig. 5A) and AEBSF (Fig. 5B) in a dose-dependent manner. In contrast, the addition of L-NAME or allopurinol had no effect on chemotaxis (Fig. 4B,C).

3.3. *ROS generation in VEGF-treated HCAEC is inhibited specifically by NADPH oxidase inhibitors*

We have previously shown that VEGF does not induce ROS generation in endothelial cells (Abid et al., unpublished observations). To determine whether the results of the proliferative assays correlated with changes in intracellular ROS generation, we measured DCF fluorescence in VEGF-treated HCAEC in the presence or absence of ROS antagonists. The arithmetic means (control cells arbitrarily set at 100) ± S.D. of DCF fluorescence from three independent experiments were as follows: no treatment, 100 ± 3.5; VEGF, 83 ± 6; VEGF+DPI, 23.5 ± 2; VEGF+L-NAME, 93.6 ± 2.8; VEGF+allopurinol, 78.2 ± 6. Representative histograms are shown in Fig. 6.

4. Discussion

Previous studies have established an important role for ROS in cell proliferation. For example, in VSMC, ROS have been implicated in the mitogenic response to PDGF

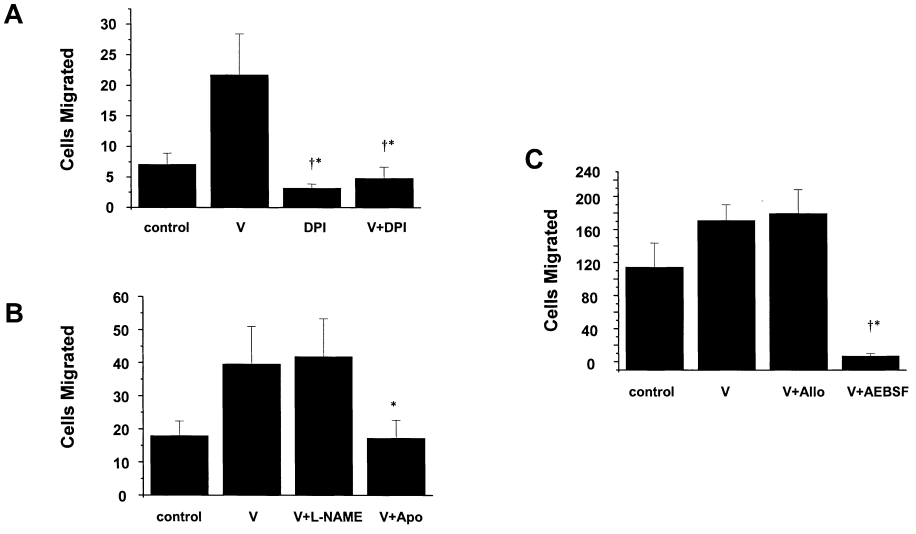

Fig. 4. VEGF-mediated chemotaxis of HCAEC is abrogated by NADPH oxidase inhibitors. A modified Boyden chamber was used to assess migratory activity in control HCAEC or in HCAEC exposed to 50 ng/ml VEGF (V) in the absence or presence of (A) 10 μM DPI (V+DPI), (B) 500 μM L-NAME (V+L-NAME) or 300 μM apocynin (V+Apo), (C) 100 μM allopurinol (V+Allo) or 250 μM AEBSF (V+AEBSF).

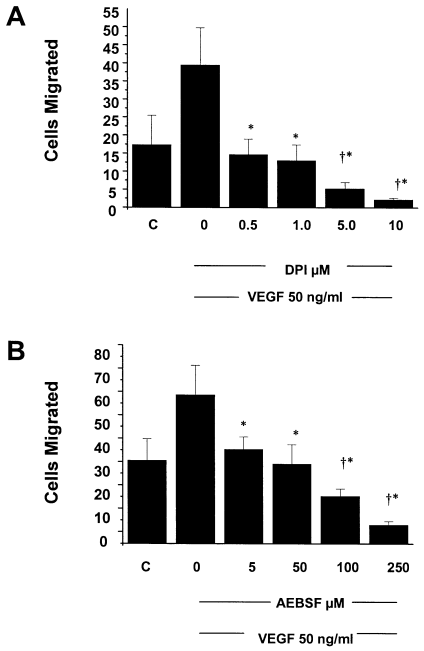

Fig. 5. Decrease in VEGF-mediated chemotaxis by NADPH oxidase inhibitors is dose-dependent. Migration in control HCAEC (C) or in HCAEC exposed to 50 ng/ml VEGF in the absence or presence of (A) increasing doses of DPI or (B) increasing doses of AEBSF.

[22], phenylephrine [23], and thrombin [8], as well as the hypertrophic effect of angiotensin II [10]. Moreover, the addition of exogenous ROS to VSMC was reported to induce cell proliferation [24], while suppression of ROS resulted in increased apoptosis [25,26]. In contrast to these findings in VSMC, little is known about the role of ROS in endothelial cell growth and/or survival.

In the present study, we have demonstrated that the mitogenic and chemotactic effects of VEGF were abrogated by three structurally unrelated inhibitors of NADPH oxidase, but not by inhibitors of xanthine oxidase or NOS. We have previously shown that VEGF does not induce ROS generation in HCAEC (Abid et al., unpublished observations). We have demonstrated here that ROS generation in VEGF-treated HCAEC is inhibited by DPI, but not by allopurinol or L-NAME. Taken together, these findings suggest that VEGF-mediated proliferation and migration of endothelial cells are dependent upon ambient, rather than incremental, levels of NADPH oxidase-derived ROS.

As an extension of the above findings, we have shown that NADPH oxidase activity is also a critical determinant of bFGF- and serum-induced endothelial cell proliferation. Indeed, at higher concentrations of NADPH oxidase inhibitors, thymidine uptake decreased well below control values, suggesting that NADPH oxidase-derived ROS are required for endothelial cell proliferation under both basal and stimulated states. Finally, we have demonstrated that NADPH oxidase inhibitors suppress the proliferation of multiple types of endothelial cells. These results are the first to show that NADPH oxidase activity is a universal requirement for endothelial cell proliferation.

The mechanisms by which ROS mediate cell growth are presently unknown. ROS have been shown to activate multiple signaling pathways such as ERK1/2 [22,27] and Akt kinase [28]. Activation of ERK1/2 and Akt has been implicated in VEGF-induced endothelial cell growth and/or survival [29,30]. In addition, ROS have been shown to activate the transcription factor, NFκB [31,32]. Increased activation of NFκB, in turn, has been reported to increase endothelial cell survival [33]. Whether one or more of these signaling pathways are involved in mediating the effect of NADPH oxidase-derived ROS on endothelial cell proliferation and/or migration remains to be determined.

The requirement of NADPH oxidase activity for endothelial cell proliferation and migration may have important clin-

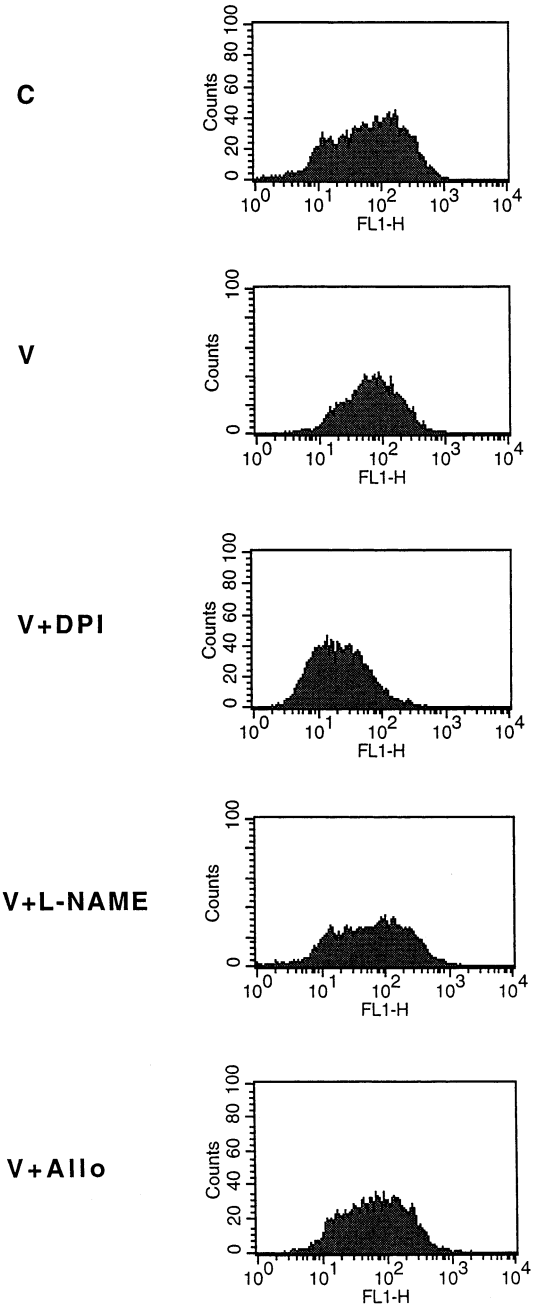

Fig. 6. ROS generation in VEGF-treated cells is inhibited by NADPH oxidase inhibitor. Representative DCF fluorescence distribution of untreated HCAEC (C) or VEGF-treated HCAEC in the absence (V) or presence of 100 μM DPI (V+DPI), 500 μM L-NAME (V+L-NAME) or 100 μM allopurinol (V+Allo).

ical implications. The growth of tumors is dependent on new blood vessel formation. In recent years, there has been an effort to develop novel anti-angiogenesis strategies that inhibit tumor growth. For example, specific inhibitors of matrix metalloproteinases, vasostatin [34], endostatin [35], angiostatin [36,37], and anti-thrombin [38] have been shown to inhibit angiogenesis. In addition, neutralizing antibodies that inhibit the VEGF–VEGF receptor axis [39,40], and antisense targeting of bFGF and FGF receptor 1 [41] have been shown to suppress tumor growth. The results of the present study suggest that the NADPH oxidase complex may serve as a novel target for anti-angiogenic therapy.

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