Pre-B Cell Colony-Enhancing Factor (PBEF/Nampt/Visfatin) Primes Neutrophils for Augmented Respiratory Burst Activity through Partial Assembly of the NADPH Oxidase

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Pre-B Cell Colony-Enhancing Factor (PBEF/Nampt/Visfatin) Primes Neutrophils for Augmented Respiratory Burst Activity through Partial Assembly of the NADPH Oxidase

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Pre-B cell colony-enhancing factor ([PBEF] also known as Nampt/visfatin) is a pleiotropic 52-kDa cytokine-like molecule whose activity has been implicated in multiple inflammatory disease states. PBEF promotes polymorphonuclear neutrophil (PMN) proinflammatory function by inhibiting constitutive PMN apoptosis. We investigated whether PBEF activates or primes for PMN respiratory burst. We found that although PBEF did not activate respiratory burst on its own, it primed for increased reactive oxygen species generation through the NADPH oxidase. PBEF promoted membrane translocation of cytosolic NADPH oxidase subunits p40 and p47, but not p67, induced p40 phosphorylation on Thr154, and activated the small GTPase Rac. Priming, translocation, and phosphorylation were dependent on activation of p38 and ERK MAPKs, but not of PI3K. Priming by PBEF occurred independent of its NAD-generating capacity because neither nicotinamide mononucleotide or NAD could recapitulate the effects, and a specific inhibitor of PBEF, APO-866, could not inhibit priming. Taken together, these results demonstrate that PBEF can prime for PMN respiratory burst activity by promoting p40 and p47 translocation to the membrane, and this occurs in a MAPK-dependent fashion. The Journal of Immunology, 2011, 186: 000–000.

As the primary early effectors of an innate immune response, polymorphonuclear neutrophils (PMN) are the first and most abundant cells to arrive at the site of infection or injury (1). Their potent antimicrobial activity is effected through proteolytic enzymes and the generation of reactive oxygen species (ROS) (2). The critical role of ROS in PMN antimicrobial defenses is revealed by patients with chronic granulomatous disease in whom defective ROS production results in greater susceptibility to recurrent infection (3). However, ROS released by PMN are also implicated in the bystander tissue injury that accompanies an inflammatory response. PMN-induced cell injury has been observed in a number of inflammatory diseases, including sepsis and acute respiratory distress syndrome (4), and a mouse model of liver injury demonstrated that PMN ROS activity has been observed in a number of inflammatory disease states, PBEF promotes polymorphonuclear neutrophil (PMN) proinflammatory function by inhibiting constitutive PMN apoptosis. We investigated whether PBEF activates or primes for PMN respiratory burst. We found that although PBEF did not activate respiratory burst on its own, it primed for increased reactive oxygen species generation through the NADPH oxidase. PBEF promoted membrane translocation of cytosolic NADPH oxidase subunits p40 and p47, but not p67, induced p40 phosphorylation on Thr154, and activated the small GTPase Rac. Priming, translocation, and phosphorylation were dependent on activation of p38 and ERK MAPKs, but not of PI3K. Priming by PBEF occurred independent of its NAD-generating capacity because neither nicotinamide mononucleotide or NAD could recapitulate the effects, and a specific inhibitor of PBEF, APO-866, could not inhibit priming. Taken together, these results demonstrate that PBEF can prime for PMN respiratory burst activity by promoting p40 and p47 translocation to the membrane, and this occurs in a MAPK-dependent fashion. The Journal of Immunology, 2011, 186: 000–000.

The surge in oxygen consumption leading to ROS production, termed the respiratory or oxidative burst, is a consequence of increased activity of the NADPH oxidase. The NADPH oxidase, first described in phagocytosing cells from guinea pigs (6), is a multi-protein complex consisting of three subunits found in the cytosol —p40, p47, and p67 (7–9)—that assemble with gp91 and p22 subunits in the cell membrane upon oxidation activation (10, 11). Once assembled, the enzyme couples electron transfer from cytoplasmic NADPH to oxygen, generating superoxide radical (O2−) and other species through the activity of specific downstream enzymes (12). Early studies of the NADPH oxidase demonstrated that chemotactic factors and other substances that lacked intrinsic capacity to activate the NADPH oxidase could amplify enzyme activity in response to activating stimuli such as formylated bacterial peptides (13); this process has been termed “priming”.

Pre-B cell colony-enhancing factor (PBEF), also known as visfatin and Nampt, is a highly conserved 52-kDa protein that exerts pleiotropic biologic activities (14). Orthologs of the PBEF gene are present in both prokaryotes and eukaryotes. In the former, PBEF functions as a Nampt catalyzing the generation of NAD (15). A similar activity has been identified in eukaryotic cells, including human cells, in which PBEF/Nampt is the ratelimiting step in a salvage pathway of NAD biosynthesis (16). However, PBEF also has well-characterized activity as an extracellular cytokine-like molecule (14), as well a more controversial activity as an alternate ligand for the insulin receptor, synthesized by visceral fat cells and so termed visfatin (17).

We have previously shown that PBEF promotes PMN inflammatory activity in response to a broad array of microbial and host-derived inflammatory mediators through the inhibition of PMN apoptosis (18); whether PBEF can modulate other inflammatory functions in PMN is unknown. We now report that PBEF can prime for PMN oxidative burst activity, and that this occurs through activation of Rac and incomplete assembly of the NADPH oxidase. We further demonstrate that PMN priming by
PBEB is mediated by MAPK activation and occurs independent of its enzymatic Nampt activity.

Materials and Methods

Reagents

Low-glucose DMEM, HBSS, penicillin/streptomycin solution, and FCS were purchased from Invitrogen (Burlington, ON, Canada). LPS from Escherichia coli 0111:B4, PMA, IML, diphenylhexatrienone (DPI), apocynin, GW5074, phosphoribosyl pyrophosphate (PPP), nicotinamide, nicotinamide mononucleotide (NAM), and NAD were purchased from Sigma-Aldrich. SB203580 and AP0-866 (also known as FK866) were purchased from Cayman Chemical (Ann Arbor, MI). PD98059, anti-p-ERK (serine 202/204, α, and phosphorylated in β 1:1 HSS), PMD-2/3 were then loaded with naling Technology (Boston, MA). Anti-p-47, -gp91, -Hsp70, -p-Akt (serine 473), -p-Akt (threonine 308), -Akt, and -GST were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p67 was from BD Biosciences (Mississauga, ON, Canada), and HRP-conjugated anti-rabbit and anti- mouse secondary Abs were from GE Healthcare (Mississauga, ON, Canada). Unless otherwise indicated, all other reagents were purchased from Sigma-Aldrich.

Neutrophil isolation

PMN were isolated as described previously with minor modifications (19). Briefly, fresh blood from consenting healthy human donors was collected into heparinized tubes and combined with 3% dextran to allow for RBC sedimentation. The lymphocyte-rich upper layer was layered onto Ficoll- Paque Plus (GE Healthcare) and centrifuged (400 × g, 30 min). Following erythrocyte lysis, PMN were suspended in FCS-supplemented DMEM (106 cells/ml). PMN purity was determined using flow cytometry size and granularity characteristics and was consistently >95%.

Generation of rPBEB

GST-tagged rPBEB was generated as described previously (18) with slight modifications. Briefly, full-length PBEB was cloned into the GST Gene Fusion System pGEX-4T-3 vector and transformed into DH5α-competent cells (Invitrogen). Successfully transformed bacteria were cultured in Luria-Bertani medium with 2% glucose and 100 μg/ml ampicillin at 37°C to an A600 of ~1.0. Protein generation was induced with 100 μM isopropyl-β-thiogalactopyranoside for 1 h at room temperature. Bacteria were pelleted, resuspended in cold STE buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 150 mM NaCl) with lysozyme (100 μg/ml), 5 mM DTT, and 2.5% sarkosyl and then sonicated on ice. Bacterial debris was pelleted and the supernatant incubated with glutathione-Sepharose 4B beads (GE Healthcare) and incubated for 1 h at room temperature. Bound protein was eluted using 20 mM glutathione in 50 mM Tris-HCl (pH 9.0) for 30 min and eluted protein was dialyzed for 24 h against PBS. Endotoxin was removed by phase separation with Triton X-114 and residual detergent cleared by centrifugation at 12,000 rpm for 1 min at 4°C. Protein identity was confirmed by SDS-PAGE probing with anti-GST and anti-PBEB Abs.

Assessment of oxidative burst and priming

Intracellular ROS production was measured as described previously as the conversion of dihydroorudamine (DHR)123 to rhodamine in the presence of H2O2 (20). PMN were loaded with DHR123 (1 μM) (Molecular Probes/Invitrogen) for 5 min at 37°C and 5% CO2 and then stimulated under various conditions for appropriate times. For priming studies detecting intracellular ROS generation, PMN were pretreated with the priming stimulus for 1 h unless otherwise indicated and then loaded with 1 μM DHR123 for 5 min at 37°C in 5% CO2. IMLF (100 nM) or PMA (500 nM) was added for 5 min and then cells were placed on ice and intracellular PMN fluorescence was quantified on the FL3 channel of a FACScanto flow cytometer and analyzed using FACSDiva software (BD Biosciences, San Jose, CA). A minimum of 10,000 events were recorded and analyzed. Extracellular ROS was measured using enhanced isoluminol chemiluminescence as described by Lundqvist and Dahlgren (21). Briefly, 105 PMN were pretreated with PBEF for 1 h or left untreated, centrifuged for 5 min at 1,000 × g, and resuspended in 1 ml HSS. PMD-2/3 were then loaded with HRP (4 U/ml; Sigma-Aldrich), isoluminol (56 μM; TCI America, Portland, OR), and in some instances superoxide dismutase (50 U/ml; Sigma-Aldrich) to quench extracellular ROS. After allowing cells to equilibrate for 5 min at 37°C in 5% CO2 (IMLF (100 nM) was added to appropriate conditions to activate the cells. Light emission was recorded every 5 s in a Lumat LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN) using disposable 5-ml glass tubes.

Membrane extraction

Membrane proteins were isolated using the ProteoJET membrane protein extraction kit (Fermentas Life Sciences, Burlington, ON, Canada). Briefly, PMN were treated as indicated and incubated at 37°C and 5% CO2. For all membrane extraction studies, inhibitors were added to PMN cultures for 30 min and then cells were incubated with PBEF for a further 8 min, a time shown in preliminary work to result in optimal evidence of translocation. Ten minutes before the end of treatment, 100 nM serine/threonine phosphatase inhibitor calycin A (Cell Signaling Technology) was added to each tube. The tubes were placed on ice and PMN centrifuged for 5 min at 400 × g and washed twice with cell wash solution. Cells were permeabilized with a mild, nondenaturing detergent provided in the ProteoJET kit supplemented with 1 mM diisopropyl fluorophosphate (EMD Chemicals, Gibbstown, NJ), 1 mM PMSE, 1 mM Na2VO4, 1× Protease Inhibitor Cocktail (BD Biosciences), 1× PhosSTOP phosphatase inhibitor mixture (Roche, Laval, QC, Canada), and 100 mM calycin A.

GST-p21-binding domain bead preparation

Beads were prepared as described previously (22). Briefly, a pGEX-2T vector containing an insert corresponding to the p21-binding domain of p21-activated kinase (aa 67–150) fused to GST (provided by Dr. G. Bokoch, Scripps Institute, La Jolla, CA) was transformed into E. coli cells. Bacteria were grown to appropriate density and protein production induced using 100 μM isopropyl-β-thiogalactopyranoside. Pelleted bacteria were lysed in the presence of 1× Protease Inhibitor Cocktail, 1 mM DTT, and 1 mM PMSE and then sonicated. The clarified sonicate was incubated with glutathione-Sepharose 4B beads for 45 min, washed four times, and a 50% slurry was generated using Hanks’ buffered saline supplemented with 1× Protease Inhibitor Cocktail, 5 mM MgCl2, and 1 mM DTT. Prepared beads were analyzed by SDS-PAGE for concentration and purity.

Rac activation assay

The Rac activation assay was conducted as reported previously (22) with minor modifications. Briefly, 105 PMN were treated with PBEF (100 ng/ml) or left untreated for 1 h at 37°C and then stimulated with IMLF (100 nM) for 5 min. Cells were pelleted at 4°C and lysed on ice with lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 100 μM glyceraldehyde-3-phosphate dehydrogenase, native 4B beads (GE Healthcare) and incubated for 1 h at room temperature. Bound protein was eluted using 20 mM glutathione in 50 mM Tris-HCl (pH 9.0) for 30 min and eluted protein was dialyzed for 24 h against PBS. Endotoxin was removed by phase separation with Triton X-114 and residual detergent removed with S/D solvent detergent removal resin (Sigma-Aldrich). Endotoxin levels were tested using the chromogenic Limulus amebocyte lysate endpoint assay (Lonza, Basel, Switzerland) and preparations showing <1.0 endotoxin unit/μg protein were used. Protein identity was confirmed by SDS-PAGE probing with anti-GST and anti-PBEB Abs.

Densitometry and normalization

Densitometry analyses were performed using a Bio-Rad GS800 densitometer and Quantity One software. For differential extraction studies assessing membrane association of subunits, whole cell lysate and cytosolic protein extracts had protein normalized to Hsp70 while membrane protein extracts were normalized to gp91. Adjacent blots showing membrane and cytosolic protein extracts were loaded in 1:1 proportion with a change in color between bands to require different exposures to optimally visualize bands. For membrane extraction studies assessing p40 phosphorylation on Thr54, phospho-p40 was normalized to p40, and this was then normalized to the appropriate
loading control indicated above depending on which protein extract was being analyzed. Fold increase reflects changes when compared with control set as 1.

**Immunoprecipitation**

PMN (10⁷) were treated as indicated and 100 nM calyculin A was added to cells 10 min prior to the end of treatment. Cells were washed with ice-cold PBS and lysed with lysis buffer on ice. Lysates were spun at 12,000 rpm for 10 min to remove insoluble debris with a small portion of each lysate boiled in Laemmli buffer to confirm equal protein content. Lysates were precleared using protein G-Sepharose beads (GE Healthcare) for 30 min and then incubated with appropriate primary Ab overnight at 4°C. Washed protein G beads were added to the lysates for 1 h and then washed three times and boiled in Laemmli buffer. Samples were analyzed using SDS-PAGE with appropriate Abs.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.0 software and significance determined using a Student paired t test or one-way ANOVA with a Tukey post hoc test as required. Significance is noted as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

**PBEF primes for ROS production in PMN via NADPH oxidase**

Increasing amounts of rPBEF alone had no effect on intracellular ROS generation as assessed by DHR123 uptake but induced dose-dependent priming of respiratory burst in response to the for- mylated bacterial peptide fMLF, similar to LPS (100 ng/ml) (Fig. 1A). Similar to intracellular priming, PBEF enhanced extracellular ROS generation ∼3-fold as assessed by isoluminol chemilumi-nescence (Fig. 1B). PMN treated for similar durations and doses with GST tag alone showed no evidence of priming (data not shown). Priming of oxidative burst activity peaked at 1 h and, similar to LPS-induced priming, the influence of PBEF was a transient phenomenon and was substantially reduced following 4 h PBEF exposure (data not shown). To exclude the possibility that PBEF directly alters interactions between fMLF and its receptor, we examined receptor-independent PBEF priming in response to PMA. PBEF also primed the PMA-induced oxidative burst (Fig. 1C). Pretreatment with the nonspecific inhibitor of NADPH oxidase, DPI, or the highly specific inhibitor, apocynin, abolished the fMLF-induced and PBEF-enhanced ROS generation in PMN. When cells were pretreated with the highest concentrations of either inhibitor, no significant difference could be observed between ROS generation in response to fMLF alone and ROS generation in PBEF-primed cells (Fig. 1D, 1E).

**PBEF priming involves p40 and p47 cytosolic subunit translocation to the membrane**

Activation of the NADPH oxidase requires that the cytosolic subunits p40, p47, and p67 translocate to the plasma or vacuolar membrane where they complex with the membrane-bound components gp91 and p22 (23). Priming stimuli can induce phosphorylation of the oxidase subunits and/or their translocation from cytosol to membranes. LPS, for example, promotes p47 phosphorylation and translocation but does not affect p67 or p40 (24). Alternatively, platelet-activating factor induces p40 and p67 phosphorylation (25) and further promotes p67 translocation (26). We first investigated whether p40, p47, or p67 translocated to the membrane upon PBEF stimulation (Fig. 2A), and whether translocation in response to fMLF was augmented by PBEF pretreatment. PBEF induced membrane translocation of the p40 (Fig. 2B) and p47 (Fig. 2C) subunits, but not of p67 (Fig. 2D). Additionally, PBEF increased the fMLF-induced translocation of p40 and p47, whereas fMLF-induced membrane translocation of p67 was not enhanced by PBEF pretreatment (Fig. 2B–D).

The small GTPase, Rac2, in its GTP-bound and activated form is necessary for NADPH oxidase activity (27–29). PBEF induced an increase in GTP-bound Rac and further augmented Rac activation in response to fMLF (Fig. 2E).

Taken together, these studies show that PBEF induces changes in the cellular localization of key components of the NADPH oxidase. We next sought to determine whether it also modified their activational state.

**PBEF induces threonine phosphorylation of p40**

p40 is phosphorylated in resting cells, but the initiation and extent of further phosphorylation has been found to correlate with ROS generation (30). Phosphorylation of Thr154 of p40 has been shown during NADPH oxidase activation (31), and thus we sought to determine whether PBEF treatment induced phosphorylation of p40 Thr154 in PMN. PBEF alone induced threonine phosphorylation of p40 and it further augmented fMLF-induced p40 phosphorylation in PMN whole cell lysates. Phosphorylated p40 was found in both membrane and cytosolic PMN protein extracts, suggesting that p40 phosphorylation alone may not be sufficient to induce membrane translocation (Fig. 3A).

p40 plays a nonessential role in extracellular ROS generation in response to fMLF and PMA; however, intracellular ROS generation induced by phagocytosis is defective in individuals with p40 deficiency (32). Furthermore, the presence of p40 increases p47 interactions with membrane-bound oxidase subunits and enhances oxidase activity (33). Phosphorylated p40 in resting endothelial cells can compensate for p47 deficiency in maintaining basal ROS production, and p40 dephosphorylation is a prerequisite for PMA-induced p47 phosphorylation and ROS generation (34). Thus, the role of p40 in NADPH oxidase activation is complex and the consequences of its interactions with p47 remain incompletely understood. We found that p47 coimmunoprecipitated with phosphorylated p40 following PBEF treatment. Additionally, PBEF further enhanced the fMLF-induced association of the two subunits (Fig. 3B). Due to technical issues with the Ab against p40, total p40 coimmunoprecipitated with p47 could not be detected.

**PBEF priming of oxidative burst is ERK- and p38-dependent, but not PI3K-dependent**

Priming stimuli including TNF-α and GM-CSF require activation of the MAPKs ERK and p38 to promote NADPH oxidase activity (35). Additionally, it has been reported that priming by GM-CSF (36) and oxidative burst activity in response to fMLF (37) require the generation of PtdIns(3,4,5)P3 through the activity of PI3K. PBEF induced phosphorylation of both ERK and p38 in PMN (Fig. 4). Priming stimuli including TNF-α and GM-CSF require activation of the MAPKs ERK and p38 to promote NADPH oxidase activity (35). Additionally, it has been reported that priming by GM-CSF (36) and oxidative burst activity in response to fMLF (37) require the generation of PtdIns(3,4,5)P3 through the activity of PI3K. PBEF induced phosphorylation of both ERK and p38 in PMN (Fig. 4A). Inhibition of either the upstream activator of ERK, MEK1, with PD98059 (10 μM) or inhibition of the MEK activator c-Raf1 with GW5074 (10 μM) blocked PBEF priming without inhibiting fMLF-induced activation of oxidative burst activity. A selective inhibitor of p38 activation, SB203580 (10 μM), similarly prevented PBEF priming, but not fMLF-induced activation (Fig. 4B, 4C).

**ERK and p38 activation are necessary for PBEF-induced p40 and p47 membrane translocation**

We next evaluated the role of p38 and ERK in PBEF-induced NADPH oxidase subunit translocation. ERK inhibition with PD98059 completely abolished p40 and p47 translocation and attenuated p40 Thr154 phosphorylation (Fig. 5). Similarly, p38 inhibition with SB203580 prevented both p40 and p47 translocation and p40 Thr154 phosphorylation (Fig. 6). An inhibitor of
PI3K, LY29004, however, had no effect on p40 or p47 translocation and on p40 phosphorylation (data not shown).

Both ERK and p38 MAPKs were activated by PBEF, and inhibition of either prevented translocation of p40 and p47 and phosphorylation of p40. When we inhibited ERK using PD98059, we found significantly decreased PBEF-induced p38 activation (Fig. 7A). On the other hand, when we inhibited p38 with SB203580, we saw no decrease in ERK activation but rather an increase (Fig. 7B), suggesting that ERK activation precedes p38 activation. When we treated PMN with PBEF and immunoprecipitated p38, we found that Thr154-phosphorylated p40 interacted physically with activated p38, and a lack of phosphorylated p40 in control cells was evident when none was coimmunoprecipitated with unactivated p38 from control cells. When ERK was immunoprecipitated, however, no phosphorylated p40 was found in association with activated ERK (Fig. 7C). Taken together, these data suggest that ERK activation precedes p38 activation following PBEF exposure, and that interactions between activated p38 and p40 may be a proximate step in p40 recruitment to the nascent NADPH oxidase.

Priming of oxidative burst does not require PBEF enzymatic activity

To determine whether the priming effect of PBEF was dependent on its Nampt activity, we first assessed the effects of the addition of the two Nampt substrates, PPP and nicotinamide, during PBEF treatment and found that increasing amounts of substrate did not increase priming by PBEF (Fig. 8A). Importantly, nicotinamide was present in the cell media prior to exogenous addition but was at a concentration much lower (32.8 μM) than the $K_d$ of PBEF.
Neither the immediate product of the Nampt reaction, NAM, nor its secondary product, NAD, primed for oxidative burst (Fig. 8B, C), but rather nicotinamide, NAM, and NAD partially abrogated PBEF priming, whereas NAM and NAD even inhibited fMLF activation of oxidative burst (Fig. 8A–C).

Pretreatment of PMN with inhibitory concentrations of APO-866, a small molecule inhibitor of Nampt activity (38), did not attenuate PBEF priming of oxidative burst (Fig. 8D). Similarly, incubation of PBEF with the inhibitor prior to treatment of PMN was without effect (Fig. 8E). Consistent with this, pretreatment of PMN with APO-866 did not affect p40 or p47 membrane translocation in response to PBEF (data not shown).

To determine whether PBEF Nampt activity was responsible for PBEF-induced MAPK activation, PMN were treated with PBEF.
in conjunction with PPP and nicotinamide or with NAM or NAD alone. Addition of exogenous substrates did not enhance PBEF-induced ERK or p38 phosphorylation, and activation of MAPKs could not be recapitulated with NAM or NAD alone. Whether APO-866 could block MAPK activation was also assessed by pretreatment of PMN or PBEF with the inhibitor, and it showed no attenuation of PBEF-induced ERK or p38 activation (Fig. 8F).

**Discussion**

The unique capacity of PMN to respond rapidly to microbial invasion or tissue injury is of fundamental importance to vertebrate survival. PMN are rapidly recruited from the circulation to the site of injury or microbial invasion; once there, they engulf and digest pathogens, killing them through the activation of both oxidant-dependent and oxygen-independent mechanisms (39). However, this potent but nonspecific response results in significant bystander injury, and so contributes to the pathogenesis of a group of inflammatory disorders, including arthritis, inflammatory bowel disease, acute lung injury, and, in the event of disseminated PMN activation, sepsis. The effectiveness of the initial response and the extent of the inadvertent tissue injury reflect the capacity of PMN to activate antimicrobial defenses and to prolong functional survival by inhibiting the expression of a constitutive apoptotic program. PMN harvested from septic patients demonstrate prolonged survival in conjunction with enhanced respiratory burst capacity (40). We have previously shown PBEF plays a pivotal role in inhibiting PMN apoptosis (18). We now show that PBEF can also prime PMN for augmented oxidative burst activity in response to activating stimuli such as bacterial formyl peptides and PMA.
Priming of the PMN oxidative burst is a complex process that involves both phosphorylation of subunits of the NADPH oxidase and their incomplete membrane translocation. TNF-α, for example, stimulates phosphorylation of p47 and p67 in a p38-dependent fashion, but it does not promote membrane localization of either subunit (25, 35, 41). Alternatively, platelet activating factor primes oxidative burst capacity rapidly and reversibly by inducing phosphorylation of p67 but not p47 (25, 42). Phosphorylation of p67 is p38-dependent (25) and precedes membrane translocation (26). We found that priming of fMLF-induced respiratory burst activity by PBEF was associated with translocation of cytosolic NADPH oxidase components p40 and p47 to the membrane, while translocation of the third cytosolic constituent, p67, was unaffected. Priming by rPBEF was also associated with phosphorylation of p40 on Thr154. Previous studies have shown that p40 can be phosphorylated in a protein kinase C-dependent fashion by activating agents such as PMA (31, 43), but rPBEF is the first priming agent that has been shown to induce p40 threonine phosphorylation. The recent emergence of a novel form of chronic granulomatous disease involving defective p40 demonstrated the importance of p40 for intracellular ROS generation in response to phagocytosis but not extracellular generation induced by fMLF or PMA (32). Thus, although we show that PBEF can induce posttranslational modifications in p40, the biologic consequence of these specific changes remains uncertain. Additionally, although we show translocation of oxidase subcomponents to cell membranes, the technique of differential extraction we used to obtain these fractions for analysis does not enable us to determine whether translocation is to the plasma membrane or to a granule membrane.

PBEF/Nampt/visfatin is a highly conserved protein whose pleiotropic biologic activities are incompletely understood. Orthologs
of PBEF are expressed both in prokaryotes (15) and eukaryotes as primitive as sponges and mollusks (14, 44). PBEF has been reported to exert at least three distinct biologic activities. In both bacteria and mammalian cells, PBEF functions as an intracellular Nampt, catalyzing the conversion of nicotinamide to NAD. In mammals, PBEF is the rate-limiting step in a salvage pathway of NAD biosynthesis, and in this capacity it plays an important role in cellular energetics. In controversies studies that were retracted because of inconsistencies in replicating the data, PBEF was reported to be an adipokine produced by visceral fat cells that could bind insulin receptor and exert both agonistic and antagonistic activity (17). This activity earned the gene the alternate designation visfatin, and although the index publication was withdrawn, the interaction of PBEF with the insulin receptor has been reported by others (45), and altered PBEF levels have been described in diabetes or in diabetogenic states such as obesity and the metabolic syndrome (14). Finally, a number of authors have documented an extracellular cytokine-like role for PBEF. The initial identification of PBEF described its cytokine-like role as a differentiation factor for early stage B cells (46). Plasma PBEF levels are elevated in patients with inflammatory bowel disease (47) and chronic obstructive pulmonary disease (48), while amniotic fluid PBEF levels are elevated in patients with chorio-amnionitis (49). Importantly, the PBEF concentration used in most of our studies was physiologically relevant and coincided with plasma levels from patients with rheumatoid arthritis (50). Recombinant PBEF induces expression of proinflammatory mediators IL-6 and MCP-1 in HUVECs (51).

In support of a link between PBEF and glucose homeostasis, Revollo et al. (52) showed that haplodefiency and chemical inhibition of PBEF NAD-generating ability caused defects in NAD biosynthesis and glucose-stimulated insulin secretion both in vivo and in vitro. These defects, however, could be corrected by addition of the product of the PBEF enzymatic reaction, NAM (52), suggesting that the effects of PBEF may in fact be occurring through its enzymatic activity. We found no evidence to suggest that the priming effect of PBEF required its Nampt activity. Priming could not be induced by addition of nicotinamide or of the downstream products NAM or NAD. Furthermore, the specific antagonist of Nampt activity, APO-866, a small molecule inhibitor that competes with nicotinamide for binding to a pocket in PBEF dimers, failed to block the priming activity in a dose that inhibits NAD generation in PMN.

PBEF has been shown to have both intracellular and extracellular roles, although PBEF lacks a signal peptide and, to date, no specific cell surface receptor has been identified. A number of proteins that are normally present within the cell can function as damage-associated molecular patterns in the extracellular environment, activating proinflammatory gene expression through interactions with a pattern recognition receptor. The prototypical example of such a protein is the nuclear protein, HMGB1, that is released by injured cells and evokes a vigorous proinflammatory response through interactions with TLR-4 and RAGE (53), but similar activities have been described for proteins such as S100B and heat shock protein 70.

In summary, we have shown that in addition to its anti-apoptotic activity, PBEF also promotes the inflammatory function of PMN by priming for enhanced respiratory burst activity. This activity is independent of either the Nampt enzymatic activity of PBEF, and it is effected through the MAPks ERK and p38, which, in turn, promote the phosphorylation and membrane translocation of key cytosolic constituents of the NADPH oxidase. Dysregulated inflammatory responses are characteristic of a spectrum of disorders in which PBEF expression is increased, including rheumatoid arthritis, atherosclerosis, and sepsis, suggesting that increased extracellular PBEF at sites of inflammation may enhance the activation of recruited PMN. As a consequence, PBEF emerges as a promising therapeutic target to attenuate PMN-mediated tissue damage in inflammatory disease states.

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FIGURE 8. PBEF priming occurs independent of PBEF Nampt activity. PBEF priming of oxidative burst is not enhanced by addition of exogenous substrates of PBEF Nampt activity, nicotinamide and PPP (A), or product of Nampt activity including NAM (B) and NAD (C). PMN were treated with substrates/NAM/NAD alone or in conjunction with PBEF for 1 h, and priming of oxidative burst was measured by flow cytometry. Data shown are means of $n \geq 4$ experiments ± SE. Pretreatment of PMN (D) or PBEF (E) with the specific inhibitor of Nampt activity, APO-866, does not inhibit PBEF-induced priming of oxidative burst. APO-866 was incubated with PMN prior to PBEF treatment or incubated with PBEF prior to its addition to PMN, and priming was measured by flow cytometry. Data shown are means of $n \geq 4$ experiments ± SE. F, PBEF-induced MAPK activation is not dependent on PBEF Nampt activity. PMN were treated for 10–60 min with PBEF, PBEF in conjunction with substrates (nicotinamide and PPP), NAM, NAD, pretreated with APO-866 (APO) prior to PBEF treatment, or PBEF pretreated with APO-866 prior to addition to cells. Western blots of whole cell lysates shown are representative of $n = 3$ experiments.
Disclosures

The authors have no financial conflicts of interest.

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