Endothelial Nitric-oxide Synthase Activation Generates an Inducible Nitric-oxide Synthase-like Output of Nitric Oxide in Inflamed Endothelium*

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High levels of NO generated in the vasculature under inflammatory conditions are usually attributed to inducible nitric-oxide synthase (iNOS), but the role of the constitutively expressed endothelial NOS (eNOS) is unclear. In normal human lung microvascular endothelial cells (HLMVEC), bradykinin (BK) activates kinin B2 receptor (B2R) signaling that results in Ca2+-dependent activation of eNOS and transient NO. In inflamed HLMVEC (pretreated with interleukin-1 β and interferon-γ), we found enhanced binding of eNOS to calcium-calmodulin at basal Ca2+ levels, thereby increasing its basal activity that was dependent on extracellular L-Arg. Furthermore, B2R stimulation generated prolonged high output eNOS-derived NO that is independent of increased intracellular Ca2+ and is mediated by a novel Goiγ-i, MEK1/2-, and JNK1/2-dependent pathway. This high output NO stimulated with BK was blocked with a B2R antagonist, eNOS siRNA, or eNOS inhibitor but not iNOS inhibitor. Moreover, B2R-mediated NO production and JNK phosphorylation were inhibited with MEK1/2 and JNK inhibitors or MEK1/2 and JNK1/2 siRNA but not with ERK1/2 inhibitor. BK induced Ca2+-dependent eNOS phosphorylation at Ser1177, Thr495, and Ser114 in cytokine-treated HLMVEC, but these modifications were not dependent on JNK1/2 activation and were not responsible for prolonged NO output. Cytokine treatment did not alter the expression of B2R, Goi112, Goi122, JNK, or eNOS. B2R activation in control endothelial cells enhanced migration, but in cytokine-treated HLMVEC it reduced migration. Both responses were NO-dependent. Understanding how JNK regulates prolonged eNOS-derived NO may provide new therapeutic targets for the treatment of disorders involving vascular inflammation.

Nitric oxide (NO) is synthesized by three isoforms of nitric-oxide synthase (NOS) as follows: neuronal NOS (nNOS,3 NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). nNOS and eNOS are constitutively expressed and require increased intracellular Ca2+ ([Ca2+]i) for activation, whereas iNOS expression is induced in response to inflammatory stimuli and, once synthesized, is tightly bound to Ca2+-calmodulin and is thereby constitutively active (1–3). The primary source of NO in the vasculature is eNOS under normal conditions, whereas iNOS becomes prominent under inflammatory conditions.

Nitric oxide can exert either beneficial or pathological effects on the cardiovascular system, dictated by the amount of NO produced, subcellular localization, and protein interactions of NOS, in addition to the redox state of the cellular environment (4, 5). Under physiological conditions, eNOS generates low levels of NO for maintenance of vascular homeostasis (6), and alterations in NO production contribute to the pathogenesis of vascular disorders such as hypertension, diabetes, heart failure, and atherosclerosis (7).

Many vascular diseases are accompanied by inflammation, resulting in a general increase in NO production which, under conditions of oxidative stress, can lead to generation of peroxynitrite and protein nitration, DNA damage, and apoptosis (8, 9). Moreover, it is generally held that vascular inflammation leads to reduced bioavailability of eNOS-derived NO and eNOS “uncoupling,” accompanied by induction of endothelial iNOS.

3 The abbreviations used are: nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; B2R, bradykinin B2 receptor; B1R, bradykinin B1 receptor; BK, bradykinin; BK-analog, [Phe8-ψ(CH-NH)-Arg9]bradykinin; DAKD, des-Arg10-kallidin; DALKD, des-Arg10-Leu9-kallidin; MGTA, DL-2-mercaptopimethyl-3-quandialin-3-oxethyliopropionic acid; L-NNA, L-Nω-nitro-l-arginine; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N′,N′,N′-tetraacetic acid tetra(acetoxy-methyl) ester; PTX, pertussis toxin; [Ca2+]i, intracellular calcium concentration; HLMVEC, human lung microvascular endothelial cells; Ca2+-CaM, calcium-calmodulin; PEG-SOD, polyethylene glycol-superoxide dismutase; JBD, JNK binding domain; DAN, 2,3-diaminophosphalene; HBSS, Hanks’ balanced salt solution.

Background: In healthy endothelium, agonist-induced eNOS activation results in transient, calcium-dependent NO production.

Results: In inflamed endothelium, bradykinin stimulates prolonged eNOS-derived NO that depends on Goiγ, MEK1/2, and JNK, resulting in reduced migration.

Conclusion: eNOS activation is mediated differently in normal and inflamed endothelium, resulting in divergent NO production and effects.

Significance: High eNOS-derived NO may impair angiogenesis and wound healing in inflammation.
expression, which is the source of high, uncontrolled NO that further exacerbates the disease process (10). However, we have found that iNOS activity in inflamed human endothelium can be increased 3–5-fold beyond its basal level by G protein-coupled receptor signaling (11–13), and accumulating evidence indicates that eNOS could also play an “iNOS-type” role in inflammation, but the mechanisms have not been defined (14).

The kallikrein-kinin system plays a prominent role in vascular inflammation, and its biological effects are mediated by release of kinin peptides that activate two different G protein-coupled receptors named B1 (B1R), whose expression is induced by inflammatory mediators, and B2 (B2R), which is constitutively expressed (15, 16). The B2R is selectively activated by bradykinin (BK) or kallidin (KD), which can be further processed by plasma carboxypeptidase N or membrane carboxypeptidase M to generate des-Arg9-BK or des-Arg10-KD, which no longer activate the B2R, but instead are specific B1R agonists (16–18). Stimulation of the B2R or B1R differentially activates eNOS or iNOS, respectively (19). In healthy endothelium, the kinin B2R activates eNOS resulting in a short burst of Ca2+-dependent NO production (20, 21). Under inflammatory conditions, endothelial B1R stimulation leads to acute activation of iNOS via ERK1/2-dependent phosphorylation at Ser745 and prolonged and high output NO production (11–13). However, signaling pathways for endothelial B2R-dependent NO production under inflammatory conditions have not been investigated.

Increased Ca2+-calmodulin binding is a major mechanism for eNOS activation, but several other post-translational modifications govern its activity, including lipid attachment, phosphorylation, and S-nitrosylation (22, 23). Phosphorylation modulates eNOS activity by altering enzyme localization, facilitating protein-protein interactions, or inducing conformational changes that abet or impede efficient flow of electrons between the reductase and oxygenase domains (23, 24). The best characterized phosphorylation sites on eNOS modified in response to BK (and many other agonists) stimulation in control endothelial cells include dephosphorylation of the inhibitory Thr(P)495 by calcineurin and phosphorylation at the stimulatory Ser1177 by Akt (primarily) and also protein kinase A (PKA) and calcium-calmodulin-dependent kinase II (25, 26). Additionally, B2R activation alters phosphorylation at Tyr81, Ser615, and Ser633 but not Ser114 (27–29).

In this study, we discovered that B2R stimulation in cytokine-treated human lung microvascular endothelial cells (HLMVEC) induced prolonged high output NO by activating eNOS. Similar to iNOS, we found that eNOS binds calcium-calmodulin at basal Ca2+ levels in cytokine-treated endothelial cells, enhancing its basal activity and priming it for activation independent of increased [Ca2+]. Although BK-induced Ca2+-mobilization is critical for eNOS activation in healthy endothelium, we found that BK stimulates a novel Gαi/o-mediated and MEK1/2-→JNK1/2-dependent activation of eNOS in inflamed endothelium. Moreover, we established that prolonged B2R-mediated NO production reduced endothelial cell migration, a response opposite that generated by B2R activation and NO production in control endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bradykinin (BK; RPPGFSPFR), bradykinin pseudopeptide analog (Phe9-ψ(CH-NH)-Arg3)BK, 2-aminoethyldiphenyl borane, tempol, MEK1/2 inhibitor U0126, and its corresponding negative control U0124 were from Tocris Bioscience/R&D Systems, Inc. (Minneapolis, MN). Des-Arg10-kallidin (DAKD), calcium ionophore (A23187), icatibant acetate (HOE-140), des-Arg10-Leu6-kallidin (DALKD), and polyethylene glycol-superoxide dismutase (PEG-SOD) were from Sigma. NOS inhibitors (l-N6-nitro-l-arginine (l-NNA), 1400W, N6-propyl-l-arginine, and the nitrate/nitrite fluorometric assay kit (780051) were from Cayman Chemicals (Ann Arbor, MI). dl-2-Mercaptomethyl-3-guanidinoethyliopanoic acid (MGTA), BAPTA-AM, Akt inhibitor III SH-6, EGF receptor tyrosine kinase inhibitor PD153035, JNK inhibitor II, JNK inhibitor VIII, ERK inhibitor II (FR180204), and ERK inhibitor II negative control (FR180289) were from Calbiochem/EMD Millipore (Billerica, MA). MEK1/2 inhibitors CI-1040 (PD184352) and PD98059 were from Selleck Chemical (Houston, TX) and Cell Signaling Technology (Danvers, MA), respectively. Pertussis toxin (PTx) was from List Biological Laboratories (Campbell, CA). Antibodies to human eNOS, eNOS (Thr(P)1495), eNOS(Ser(P)634), and nNOS were from BD Transduction Laboratories (San Jose, CA); rabbit anti-iNOS (H-174) was from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies to human eNOS(Ser(P)1177), total-SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), total MEK1/2, and phospho-MEK1/2 (Ser217/Ser221) were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies to Gαi1,2 and anti-Gαq/11 were from Sigma, and anti-β-actin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Ambion/Invitrogen. Mouse monoclonal anti-calmodulin antibody (05-173) was purchased from Millipore (Temecula, CA).

**Cell Culture and Transfection**—HLMVEC from Clonetics were used from passage 4–6 and were cultured in dishes precoated with 0.1% (v/v) gelatin using endothelial cell basal medium-2 supplemented with growth factors from the EGM-2 MV bullet kit (Lonza; Walkersville, MD), 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin solution (Cellgro; Manassas, VA). Human embryonic kidney (HEK293) cells from ATCC (Manassas, VA) were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. HEK293 cells were transiently transfected using Effectene (Qiagen; Valencia, CA) reagent according to the manufacturer’s instructions. Unless otherwise indicated, all experiments using HLMVEC or HEK293 cells were performed in phenol red-free DMEM Nutrient Mix F-12 (Ham) from Invitrogen, supplemented with 0.5% (v/v) FBS.

**Cytokine Treatment**—HLMVEC or HEK293 cells were treated with 5 ng/ml interleukin-1β (IL-1β) (Calbiochem/EMD Millipore; Billerica, MA) and 100 units/ml interferon-γ (IFN-γ) (Invitrogen) for 16–24 h to stimulate inflammation. Because cytokine treatment induces expression of kinin B1 receptors...
(B1R) and carboxypeptidase M, which can convert BK to the B1R agonist des-Arg9-BK, cells were routinely pretreated with B1R antagonist DALKD (1 μM) and carboxypeptidase M inhibitor MGTA (20 μM) for 30 min to block the B1R pathway, unless otherwise indicated.

RNA Interference—Small interfering RNA (siRNA) from Sigma was synthesized using previously published and validated sequences for eNOS (30), MEK1 (31), MEK2 (31), and JNK1/2 (32). Sequences (sense) are as follows: eNOS, 5'-GCGCGAACUCAAAGAC(dT)(dT)-3'; MEK1, 5'-AAGUCGUGAAAGGUGCAG(dT)(dT)-3'; MEK2, 5'-AAGUGGCGCCGAAUCAAGAC(dT)(dT)-3'; and JNK1/2, 5'-TGAAGAAGTTGCTCTACCTT(dT)(dT)-3'. Scrambled RNA duplex (sc-37007) was from Santa Cruz Biotechnology and used as a negative control. HLMVEC were electroporated with 100 nM siRNA using the Amaxa Biosystems Nucleofector system (Lonza; Walkersville, MD) based on the manufacturer’s kit and protocol (S005) optimized for HLMVEC. Cells were treated with cytokines 48–72 h later. Approximately 72–96 h post-transfection, total cell lysates were collected, and protein knockdown efficiency was assessed by Western blot analysis.

Western Blotting—HLMVEC were washed once with ice-cold phosphate-buffered saline (PBS) containing 1 mM Na2VO₃. Cells were then lysed in ice-cold 2X Laemmli buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.004% (w/v) bromophenol blue) supplemented with fresh 3% (v/v) β-mercaptoethanol, 1 mM Na2VO₃, 20 mM β-glycerophosphate, 1 mM phenylmethlysulfonyl fluoride (PMSF), and 1% (v/v) protease inhibitor solution (Sigma). HEK293 cells were washed once with ice-cold PBS containing 1 mM Na2VO₃ and lysed with ice-cold buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, and 0.5% (w/v) sodium deoxycholate supplemented with fresh 3% (v/v) β-mercaptoethanol and inhibitors as stated above. Cells were lysed on ice for 30 min, collected, and centrifuged at 14,000 × g for 15 min at 4 °C. Cell lysates were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to Immobilon PVDF membranes (EMD Millipore; Billerica, MA). Blots were blocked with 5% (w/v) evaporated nonfat milk in PBS containing 0.1% (v/v) Tween 20 and then incubated with the appropriate primary antibody, and the bands were visualized by enhanced chemiluminescence (Pierce/Thermo Scientific).

Co-immunoprecipitation Assay—Control and cytokine-treated HLMVEC (~2.5 × 10⁶ cells per condition; not stimulated with BK) were washed once with ice-cold PBS containing 1 mM Na2VO₃ and lysed with ice-cold buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, and 0.5% (w/v) sodium deoxycholate supplemented with fresh 3% (v/v) β-mercaptoethanol and inhibitors as stated above. Cells were lysed on ice for 30 min, collected, and centrifuged at 14,000 × g for 15 min at 4 °C. Cell lysates were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to Immobilon PVDF membranes (EMD Millipore; Billerica, MA). Blots were blocked with 5% (w/v) evaporated nonfat milk in PBS containing 0.1% (v/v) Tween 20 and then incubated with the appropriate primary antibodies followed by horseradish peroxidase-labeled secondary antibody, and the bands were visualized by enhanced chemiluminescence (Pierce/Thermo Scientific).

Nitrate/Nitrite Fluorometric Assay—Control and cytokine-treated HEK-B2R/eNOS cells (~2.5 × 10⁶ cells/ml) were washed twice with pre-warmed HBSS (InVitrogen 14025) and then incubated with HBSS containing 1 mM l-Arg, 4 μM 1400W, 1 μM DALKD, and 20 μM MGTA. Cells were allowed to equilibrate for 10 min at 37 °C prior to collecting the first aliquot (time, 0 min) of HBSS. The removed HBSS was replenished, and cells were then stimulated with vehicle (HBSS) or 1 μM BK. Post-BK stimulation (60 min), HBSS was collected and centrifuged at 250 × g for 10 min to remove potential cell debris. NO2⁻/NO3⁻ accumulation present in 20 μl of HBSS was detected by following the manufacturer’s instructions (Cayman Chemicals; Ann Arbor, MI). In brief, 2,3-diaminonaphthalene (DAN) reacts with nitrate (NO3⁻) yielding the fluorescent product, 1-(H)-naphthotriazole, which is detected with a plate reader capable of measuring fluorescence using an excitation wavelength of 365 nm and an emission wavelength of 430 nm. Because the DAN reagent only reacts with NO3⁻, nitrate (NO3⁻) was converted to NO2⁻ by incubating samples with nitrate reductase for 1 h at room temperature. To determine BK-induced NO2⁻/NO3⁻ accumulation, basal NO2⁻/NO3⁻ accumulation was subtracted from values obtained with BK stimulation conditions.

Ca²⁺ Measurements—Increased [Ca²⁺]i was measured with the Ca²⁺-sensitive fluorescent probe fura-2/AM as described (18) with minor modifications. Control and cytokine-treated HLMVEC were grown to confluence on glass coverslips and were incubated with 1 μM l-Arg, 4 μM 1400W, 1 μM DALKD, and 20 μM MGTA for 30 min prior to addition of 100 nM BK or 100 nM BK-analog. Basal NOS activation was assessed by incubating cells in l-Arg-free DMEM/F-12 medium (without FBS) for 2 h prior to stimulation with 1 mM l-Arg. To directly measure NO production in real time, a highly sensitive porphyrinic microsensor was positioned with a micromanipulator close to the cell culture surface (20 ± 1 μM) and used as described previously (21, 33). A computer-based Gamry VP600 potentiostat was used to measure the current generated, which was proportional to the NO released. Each electrode was calibrated with NO standard. The concentration of NO achieved at maximum (control cells) or 20 min (cytokine-treated cells) after addition of agonist was used to quantitate the results.

Quantitative Real Time-PCR—Total RNA was extracted from 2.5 × 10⁶ cells from control and cytokine-treated HLMVEC using the TRIzol® reagent (Invitrogen) according to the
eNOS Masquerades as iNOS in Inflamed Endothelium

Kinin B2 Receptor (B2R) Activation Generates Prolonged NO Production in Inflamed Endothelial Cells—B2R-mediated eNOS activation in bovine aortic endothelial cells and human umbilical vein endothelial cells has been well characterized (20, 26). However, B2R-stimulated NO production has not been studied in endothelial cells under inflammatory conditions that are classically associated with activation of the kallikrein-kinin system and generation of B2R agonist kinins (15, 16). To investigate this, HLMVEC or human embryonic kidney cells transiently expressing B2R and eNOS (HEK-B2R/eNOS) were pretreated with 5 ng/ml interleukin-1β (IL-1β) and 100 units/ml interferon-γ (IFN-γ) for 16–24 h (“cytokine-treated”). Unless otherwise indicated, cells were then preincubated for 30 min with B1R antagonist DALKD (1 μM) and carboxypeptidase inhibitor MGTA (20 μM). This pretreatment blocks B1R signaling by antagonizing the receptor and inhibiting the carboxypeptidase-mediated conversion of bradykinin (BK) to B1R agonist kinins. After preincubation, the cells were exposed to BK (100 nM) in the presence or absence of B1R antagonist DALKD (1 μM) and carboxypeptidase inhibitor MGTA (20 μM) for 5 min and then returned to control conditions. Maximal and basal NO production was determined by measuring nitrite production in the medium using the Griess reagent at various time points. Maximal NO production was defined as the concentration at 5 min, and basal NO production was assessed at 5 min after BK stimulation. The data are presented as the percentage of maximal NO production (%M) at 5 min, with the control set at 100%.

RESULTS

Wound Healing Assay—Wound healing was assessed using the in vitro scratch assay technique as described previously (35). Briefly, a cell-free zone, within a confluent monolayer of control cells, was created using a pipette tip. Cells were then washed twice with serum-free media and sustained in growth factor-free EGM-2 medium supplemented with 10% (v/v) FBS, 1 μM DALKD, 20 μM MGTA, without or with 10 μM HOE-140, 4 μM l-NNA, or 10 μM tempol. Using phase contrast microscopy, 5–10 distinct images of unstimulated cells along the cell-free zone were taken, and the coordinates were documented. Thereafter, cells were stimulated with 1 μM BK or 15 μM of vehicle (basal DMEM/F-12 medium), and images were collected 12 h later at the original coordinates. Wound closure was quantified by measuring the area of the cell-free zone before and 12 h after BK stimulation. Using ImageJ software, % wound closure was calculated as \( \Delta \text{Area of wound} = (T_{\text{Initial}} - T_{\text{Final}}) / \text{area of wound} \times 100 \).

ApoTome Assay—ApoTome was evaluated using the in situ cell death detection kit (Roche Applied Science) according to the manufacturer’s instructions. Cytokine-treated HLMVEC were grown to confluence on glass coverslips. Samples were incubated for 20 min in DMEM/F-12 medium supplemented with 10% (v/v) FBS, 20 μM MGTA, and 10 μM DALKD, followed by a 12-h incubation with or without 1 μM BK. Medium was then carefully removed, and cells were air-dried. Coverslips were not rinsed with PBS to prevent inadvertent removal of apoptotic cells. Next, samples were fixed with 4% (v/v) paraformaldehyde in PBS, pH 7.4, permeabilized with 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate solution, and then treated with TUNEL reaction mixture consisting of enzyme solution and label solution provided by the manufacturer. The positive control sample was treated with 50 units/ml of recombinant DNase I prior to labeling, whereas the negative control consisted of cells treated in the absence of terminal transferase (enzyme solution). Coverslips were then rinsed with H2O2, air-dried, and prepared with ProLong Gold antifade mounting reagent containing DAPI (Invitrogen). Finally, samples were analyzed with a Zeiss ApoTome microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm.

Modified Boyden Chamber Assay—Endothelial transmigration was performed using 24-well Millicell hanging cell culture inserts containing PET membranes with 8.0-μm pores (Millipore; Billerica, MA). Briefly, membranes were precoated overnight with 0.1% (v/v) gelatin and then air-dried. The bottom wells were filled with EGM-2 medium supplemented with growth factors from EGM-2 MV Bullet kit and 10% (v/v) FBS, and the Millicell cell culture insert was inserted into the top. A cell suspension (~1.0 × 10^6 control or cytokine-treated HLMVEC in 400 μl of EGM-2 medium supplemented with 10% (v/v) FBS), without or mixed with agonist and/or inhibitors, was added to the top chamber. Transmigration through the membrane was allowed to proceed for 8 h at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The bottom surface of the membrane was then washed once with PBS and fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature. Cells on the upper surface of the membrane were carefully removed with a cotton tip. The membranes were then excised from the insert, washed twice with H2O2, air-dried, and mounted with ProLong Gold antifade reagent with DAPI. Cells present on the lower surface of the membrane were counted under a Zeiss ApoTome microscope.
and more prolonged NO, reaching a maximum in ~40 min and not returning to base line until ~80 min.

To confirm that the prolonged NO response was mediated via B2R (and not B1R) activation, we used a pseudopeptide BK analog, [Phe⁸-Arg⁹(CH₂-NH)Arg⁹]bradykinin, that has a nonpeptide C-terminal bond (36). This BK analog cannot be cleaved by carboxypeptidases, thus preventing its conversion into a des-Arg⁹ B1R agonist, allowing experiments to be done in the absence of DALKD and MGTA. As shown in Fig. 1C (top panel), the BK analog also stimulated prolonged NO production in cytokine-treated HLMVEC. The responses to BK (Fig. 1A, bottom panel) and BK analog (Fig. 1C, bottom panel) were both blocked with B2R antagonist HOE-140 (1 μM). Thus, prolonged NO stimulated by BK in cytokine-treated HLMVEC depends on activation of the B2R and not the B1R.

Cellular NO consumption is an important factor regulating intracellular NO signaling (37, 38). Because electrode-based measurements of NO detect changes from base line, detection of increased NO could reflect either enhanced NO output by a NOS or a decrease in consumption of basally generated NO. As one way to address this, we used DAN to measure total nitrite (NO₂⁻) accumulation after conversion of nitrate (NO₃⁻) to NO₂⁻ with nitrate reductase. Cells were preincubated with iNOS inhibitor 1400W to block possible NO₂⁻/NO₃⁻ generation by iNOS, whose expression is induced by cytokine treatment. We detected a significant increase in BK-stimulated NO₂⁻/NO₃⁻ accumulation (after 60 min) in medium from cytokine-treated HEK-B2R/eNOS cells (64 ± 15 nM, n = 3), whereas there was no detectable increase in NO₂⁻/NO₃⁻ in control HEK-B2R/eNOS cells stimulated with BK, suggesting an increase in eNOS-derived NO under cytokine-treated conditions. For unknown reasons, the same assay did not give reproducible results in HLMVEC, so in these cells we used a previously published approach (37) using the long half-life (20 h) NO donor DETA-NONOate to test whether BK stimulation results in decreased NO consumption. DETA-NONOate provides a constant source of NO that, after a few minutes of incubation with cells, rises to a plateau (as measured with a microelectrode) whose amplitude is inversely correlated with the rate of NO consumption and varies, depending on cell type (37). Cytokine-treated HLMVEC were preincubated with 4 μM l-NNa and 4 μM 1400W to block eNOS and iNOS, and then DETA-NONO-

FIGURE 1. BK and BK-analog stimulate prolonged B2R-mediated, eNOS-derived NO production in cytokine-treated HLMVEC and HEK-B2R/eNOS cells. A, top panel, comparison of tracings of real time measurement of BK-stimulated NO production in untreated (control) HLMVEC versus HLMVEC pre-treated with 5 ng/ml IL-1β and 100 units/ml IFN-γ (cytokine-treated) for 16–24 h. Bottom panel, B2R-mediated NO production in control and cytokine-treated HLMVEC pretreated in the absence or presence of 200 mM HOE-140, eNOS selective inhibitor (4 μM l-NNa), or iNOS-selective inhibitor (4 μM 1400W) 30 min prior to stimulation with 100 nM BK. B, top panel, comparison of tracings of real time measurement of BK-stimulated NO production in control and cytokine-treated HEK cells transiently expressing the B2R and eNOS (HEK-B2R/eNOS). Bottom panel, B2R-mediated NO production in control and cytokine-treated HEK-B2R/eNOS pretreated in the absence or presence of 4 μM l-NNa or 4 μM 1400W 30 min prior to stimulation with 100 nM BK. C, top panel, comparison of tracings of real time measurement of BK-analog-stimulated NO production in control and cytokine-treated HLMVEC. Bottom panel, B2R-mediated NO production in control and cytokine-treated HLMVEC pretreated in the absence or presence of 1 μM HOE-140, 4 μM l-NNa, or 4 μM 1400W 30 min prior to stimulation with 100 nM BK analog. Data are expressed as mean NO concentration (% of vehicle) ± S.E. achieved at the peak maximum in control cells and at 20 min in cytokine-treated cells. (n = 3; ***, p < 0.001; *, p < 0.05 compared with vehicle-treated cells.)
ate (5 μM) was added to the cells. NO, measured continuously with the microsensor, achieved a steady-state concentration of ~260 nM after ~8 min. Addition of 100 nM BK at 10 min did not change the steady-state concentration of NO that, when measured at 20 min, was 250 ± 25 nM with NO donor alone versus 275 ± 20 nM with NO donor + BK (n = 3), indicating that BK stimulation does not inhibit NO consumption.

One other potential mechanism by which BK might increase NO is by enhancing nitrite reductase activity to convert NO$_2^-$ to NO, which has also been reported to be catalyzed by eNOS, although only under hypoxic and/or acidic conditions (39). To test this, cytokine-treated HLMVEC were preincubated in L-Arg-free medium (as 300 μM L-Arg can inhibit eNOS nitrite reductase activity (39)) with 4 μM 1400W to block iNOS. Addition of 10 μM NO$_2^-$ alone or 10 μM NO$_2^-$ + 100 nM BK did not generate significant NO after 20 min as measured with the porphyrinic microsensor (10 ± 10 nm NO or 15 ± 15 nm NO, respectively; n = 3). Thus, the BK-stimulated NO response is not due to activation of nitrite reduction. It is of interest that the lack of a response in the absence of extracellular L-Arg is usually considered to be a property of iNOS regulation but not eNOS (40). The above data support the conclusion that the prolonged NO output stimulated by BK-mediated B2R activation in cytokine-treated cells is due to increased NO activity.

**Prolonged B2R-mediated NO Production Is Mediated by eNOS Activation**—We previously reported that in cytokine-treated HLMVEC, B1R activation results in post-translational activation of iNOS leading to very high and prolonged NO production (11, 12). Interestingly, the time course of B2R-mediated NO production in cytokine-treated HLMVEC (Fig. 1A, top) is strikingly similar to that generated by B1R-mediated activation of iNOS (11, 12). However, the total NO released by B2R activation (based on area under the curve for the total NO response) was ~53% of the B1R-stimulated iNOS response (11) in cytokine-treated HLMVEC (300 ± 25 area units for B1R/iNOS versus 160 ± 20 area units for B2R-stimulated NO; n = 3).

To determine which NOS isoform was being activated, we used L-NNA to inhibit eNOS and 1400W to inhibit iNOS. (Based on $K_i$ values, L-NNA is ~100-fold more selective for eNOS/nNOS than iNOS, whereas 1400W is ~7000-fold more selective for iNOS than eNOS). Using the porphyrinic microsensor, B2R-mediated NO production was inhibited 60–80% by 4 μM L-NNA, whereas 4 μM 1400W had only a minor effect in HLMVEC (Fig. 1A, bottom panel) and HEK-B2R/eNOS (Fig. 1B, bottom panel).

Similar results were obtained with the BK-analog [Phe$^8$-ψ(CH$_2$-NH)$^3$bradykinin in the absence of DALKD and MGTA (Fig. 1C, bottom panel). NOS inhibitor selectivity was verified by stimulating receptor-independent NOS activity in human embryonic kidney (HEK) cells overexpressing either iNOS or eNOS alone. We confirmed that 4 μM L-NNA inhibited the Ca$^{2+}$-ionophore (10 μM A23187)-stimulated eNOS activity but not basal L-Arg-stimulated iNOS activity, whereas 4 μM 1400W inhibited L-Arg-stimulated iNOS activity but not A23187-stimulated eNOS activation (data not shown). When BK was used to stimulate HEK cells transfected with only the B2R (in the absence of eNOS) in both control and cytokine-treated cells, there was no NO response as detected with the porphyrinic microsensor nor NO$_3^-$/NO$_2^-$ accumulation measured with the DAN reagent (data not shown).

Because L-NNA can also inhibit nNOS, we explored whether B2R stimulation might activate nNOS in cytokine-treated HLMVEC. Although nNOS is typically localized in neuronal tissue, its expression was also reported in endothelial cells (41, 42). We analyzed eNOS, iNOS, and nNOS protein expression in total cell lysates collected from control and cytokine-treated HLMVEC. As shown in Fig. 2A, cytokine treatment increased expression of iNOS but did not alter eNOS or nNOS protein expression. We also measured BK-stimulated NO production from cytokine-treated HLMVEC pretreated with NOS inhibitor $N^o$-propyl-L-arginine (0.01–10 μM) that has ~8-fold selectivity for nNOS ($K_i$ = 5 nM) over eNOS ($K_i$ = 39 nM). As shown in Fig. 2B, substantial inhibition of B2R-mediated NO production was only seen with high concentrations of $N^o$-propyl-L-arginine (1–10 μM), consistent with suppression of eNOS activity.

To confirm that B2R-mediated NO production was derived from eNOS, we transfected HLMVEC with siRNA targeting human eNOS (30) followed by cytokine treatment. As shown in Fig. 2C, we achieved ~85% knockdown of eNOS expression, and B2R-mediated NO production was reduced by 61% in cytokine-treated HLMVEC transfected with eNOS siRNA compared with cells transfected with nonspecific control siRNA. The residual NO production was due to the remaining eNOS, as it was blocked by the eNOS inhibitor (L-NNA) but not with iNOS inhibitor 1400W (Fig. 2C). Similar inhibition of NO production was obtained when we knocked down eNOS expression in control HLMVEC and used A23187 to stimulate receptor-independent eNOS activity (Fig. 2D). Overall, our data show that cytokine treatment induces B2R-mediated and eNOS-dependent prolonged NO production.

**Cytokine Treatment Induces a Time-dependent Change in Kinetics of B2R-mediated NO Production**—Cytokines can alter cell functions by promoting changes in gene expression and protein synthesis over a time course of several hours (43). We previously showed that IL-1β and IFN-γ increased expression of carboxypeptidase M, B1R, and iNOS in HLMVEC (21, 44). To determine whether prolonged B2R-mediated NO production was acutely affected by cytokine treatment or required a prolonged incubation, we measured the kinetics of NO production in HLMVEC treated with IL-1β and IFN-γ for 2, 6, 10, or 16 h prior to stimulation with 100 nM BK. As shown in Fig. 3, the kinetic profile of B2R-mediated NO production did not differ from untreated cells at 2 h but was noticeably changed 6 h post-cytokine treatment, resulting in two phases of NO production. The first peak is similar to that observed in untreated cells, reaching maximal NO levels in 5 min, but instead of returning to base line, the initial rapid peak is superimposed on a slower developing second phase of NO production. Pretreatment with cycloheximide for 10 or 16 h prior to BK stimulation almost eliminated the initial transient peak while leaving the prolonged phase intact.

**Divergent G Protein-coupled Signaling Pathways Mediate B2R-dependent eNOS Activation in Control and Cytokine-treated HLMVEC**—The kinin B2R is a prototypical $G_{αq/11}$-coupled receptor whose activation leads to stimulation of phos-
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FIGURE 2. B2R-mediated NO production is more sensitive to inhibition by eNOS-selective inhibitor (L-NNA) than nNOS-selective inhibitor (N\(^{\text{N}}\)-propyl-L-arginine) and is attenuated in cells electroporated with siRNA targeting eNOS. A, Western analysis was used to detect eNOS, nNOS, iNOS, and GAPDH (loading control) protein expression. B, B2R-mediated NO production measured in cytokine-treated HLMVEC pretreated with increasing doses of L-NNA and N\(^{\text{N}}\)-propyl-L-arginine in the presence of 1 µM DALKD and 20 µM MGTA prior to stimulation with BK. Data are expressed as mean NO production (% of vehicle) ± S.E. achieved at 20 min. (n = 3; \(*, p < 0.05; **, p < 0.01; ***, p < 0.001\) compared with vehicle-treated cells.) C, HLMVEC were transfected by electroporation with vehicle (mock electroporation), 100 nM eNOS siRNA, or 100 nM negative control (NC) siRNA. Post-transfection (48–72 h), cells were cytokine-treated, and B2R-mediated NO production was measured in the absence or presence of 4 µM L-NNA or 4 µM 1400W. D, HLMVEC were electroporated as described above. Receptor-independent NO production was measured in control HLMVEC in the absence or presence of 4 µM L-NNA or 4 µM 1400W prior to stimulation with 10 µM A23187. Data are expressed as mean [NO], nM.

pholipase C (PLC) and increased [Ca\(^{2+}\)], but it can also couple through Go\(_{q/11}\) (16, 45). To determine whether Go\(_{q/11}\)-mediated Ca\(^{2+}\) release was required for B2R-mediated eNOS activation, we measured NO production from control and cytokine-treated HLMVEC (Fig. 4A) and HEK-B2R/eNOS (Fig. 4B) pretreated with the [Ca\(^{2+}\)]\(_{\text{i}}\) chelator BAPTA-AM (25 µM) for 30 min. In control cells, B2R-mediated NO production was reduced by BAPTA-AM, consistent with a Go\(_{q/11}\)-coupled response. In contrast, prolonged B2R-mediated NO production in cytokine-treated cells was not inhibited by 25 µM BAPTA-AM using either BK (Fig. 4A) or [Phe\(^{\text{4}}\)-ψ(CH\(_2\)-NH)]bradykinin (Fig. 4C) as agonist. However, the remaining small initial transient peak was blocked (Fig. 5A), indicating it is a remnant of the B2R response found in control HLMVEC as indicated in Fig. 3. A higher concentration of BAPTA-AM (37.5 µM) was also ineffective in inhibiting prolonged B2R-mediated NO production in cytokine-treated HLMVEC (data not shown), and the response was unaffected by pretreatment with thapsigargin (2 µM) to deplete [Ca\(^{2+}\)], stores or a combination of BAPTA-AM and thapsigargin (Fig. 5B). Furthermore, the prolonged B2R-mediated response in cytokine-treated HLMVEC was still present in cells that were preincubated in Ca\(^{2+}\)-free media in the absence or presence of 2-aminoethoxydiphenyl borate (2-ABP; 50 µM) to block the inositol 1,4,5-trisphosphate receptor (Fig. 5C). However, 2-aminoethoxydiphenyl borane did inhibit the initial small transient NO peak as seen with BAPTA-AM.

These results show that in cytokine-treated HLMVEC, prolonged B2R-mediated eNOS activation is independent of increases in [Ca\(^{2+}\)], induced by either release from intracellular Ca\(^{2+}\) stores or extracellular Ca\(^{2+}\) influx.

To determine whether the lack of inhibition of the NO response in cytokine-treated HLMVEC by BAPTA-AM was due to the inability of B2R activation to increase [Ca\(^{2+}\)], we measured the increase in [Ca\(^{2+}\)] in response to BK. As shown in Fig. 5D, not only did BK increase [Ca\(^{2+}\)] in cytokine-treated HLMVEC, but the peak Ca\(^{2+}\) response was ~4.5-fold greater than in control HLMVEC. The increase in peak [Ca\(^{2+}\)] was not mediated by Go\(_{q/11}\) signaling as PTx did not inhibit the Ca\(^{2+}\) response in cytokine-treated HLMVEC (Fig. 5E). Thus, the lack of Ca\(^{2+}\) dependence of the eNOS response to BK in cytokine-treated HLMVEC is not due to the inability of BK to stimulate increased [Ca\(^{2+}\)].

To determine whether the prolonged B2R-mediated NO response in cytokine-treated cells was dependent on coupling
through \( \text{G}_{\alpha/\iota} \), we measured NO production from control and cytokine-treated HLMVEC pretreated with 1.5 g/ml PTx for 3 h prior to stimulation with BK. As shown in Fig. 4, A and B, PTx had no effect on B2R-mediated NO production in control HLMVEC or HEK-B2R/eNOS, but it substantially inhibited the response in cytokine-treated cells, indicating that it is mediated by B2R coupling through \( \text{G}_{\alpha/\iota} \). PTx also inhibited the prolonged B2R-dependent NO stimulated by BK-analog [Phe-NH(CH\(_{2}\)-NH)Arg]\( ^{\mu}\)bradykinin (Fig. 4C).

Because PI3K-mediated Akt activation is involved in the regulation of eNOS by many agonists, we measured the effect of Akt inhibitor SH-6 on B2R-mediated NO production from control and cytokine-treated HLMVEC (Fig. 4A) or HEK-B2R/eNOS (Fig. 4B). SH-6 (15 \( \mu \)M) significantly reduced B2R-mediated NO generation in control cells, but it had no effect on cytokine-treated HEK-B2R/eNOS cells and inhibited only

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**FIGURE 3.** Time course of cytokine-induced change in the pattern of B2R-mediated NO production in HLMVEC pretreated without (0 h) or with 5 ng/ml IL-1\( \beta \) and 100 units/ml IFN-\( \gamma \) for 2, 6, 10, and 16 h prior to stimulation with 100 nM BK.

**FIGURE 4.** B2R-dependent eNOS activation is mediated by divergent signaling pathways in control and cytokine-treated cells. B2R-mediated NO production was measured from control and cytokine-treated cells pretreated with vehicle, calcium chelator (BAPTA-AM, 25 \( \mu \)M), \( \text{G}_{\alpha/\iota} \) inhibitor (PTx, 1.5 \( \mu \)g/ml, 3 h), Akt inhibitor (SH-6, 15 \( \mu \)M, or MK2206, 5 \( \mu \)M), and MEK1/2 inhibitors (PD98059, 50 \( \mu \)M, or U0126, 10 \( \mu \)M), for 30 min at 37 °C. Cell type and agonist conditions are as follows: A, control and cytokine-treated HLMVEC stimulated with 100 nM BK in the presence of 1 \( \mu \)M DALKD and 20 \( \mu \)M MGTA; B, control and cytokine-treated HEK-B2R/eNOS stimulated with 100 nM BK in the presence of 1 \( \mu \)M DALKD and 20 \( \mu \)M MGTA; C, control and cytokine-treated HLMVEC stimulated with 100 nM BK-analog. Data are expressed as mean NO concentration (% of vehicle) ± S.E. achieved at the peak maximum in control cells and at 20 min in cytokine-treated cells. (\( n = 3 \); ***, \( p < 0.001 \); **, \( p < 0.01 \); *, \( p < 0.05 \) compared with vehicle-treated cells.)
20% of the response in cytokine-treated HLMVEC. To further study the possible role of Akt in HLMVEC, we treated cells with a different highly specific and potent allosteric Akt inhibitor, MK2206 (5 μM) and found, as with SH-6, that it inhibited B2R-mediated NO generation in control cells, but this inhibitor had no effect in cytokine-treated HLMVEC (Fig. 4A), even at higher concentrations up to 50 μM (not shown). Thus, the B2R-mediated NO response in cytokine-treated HLMVEC is independent of Akt activation.

**Effect of Cytokine Treatment on Ca^{2+}-CaM Binding and Basal eNOS Activity**—Both eNOS and nNOS typically require an increase in intracellular Ca^{2+} for activation, whereas iNOS binds Ca^{2+}-CaM tightly at low basal Ca^{2+} levels and is therefore constitutively active (1–3). To determine whether cytokine treatment enhances basal eNOS affinity for Ca^{2+}-CaM, we immunoprecipitated total eNOS from control and cytokine-treated HLMVEC and immunoblotted for co-immunoprecipitated CaM. Interestingly, there was an ∼4-fold increase in Ca^{2+}-CaM association with eNOS in cytokine-treated cells compared with control cells (Fig. 6A). Total cell lysates collected from control and cytokine-treated HLMVEC indicated no difference in either eNOS or CaM protein expression. Enhanced affinity for Ca^{2+}-CaM binding is responsible for constitutive iNOS activity, which depends on the presence of extracellular L-Arg (46). Because we found that BK did not stimulate increased NO in cytokine-treated HLMVEC in the absence of extracellular L-Arg either in the presence (see above) or absence (not shown) of extracellular NO_{2}, we wondered whether increased Ca^{2+}-CaM binding would result in enhanced basal L-Arg-dependent eNOS-derived NO, similar to that found with iNOS. To assess this, we depleted control and cytokine-treated HLMVEC of L-Arg and then added back 1 mM L-Arg to stimulate basal NOS activity (Fig. 6B). As shown previously (11), L-Arg stimulated basal iNOS-derived NO produc-
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Complete inhibition of the further addition of eNOS inhibitor L-NNA (Fig. 6B). Conversely, in control HLMVEC (which express low levels of iNOS; Fig. 2A) basal eNOS activity was not detectable as L-Arg stimulated a smaller NO response that was inhibited by iNOS inhibitor 1400W, but not by eNOS inhibitor L-NNA (Fig. 6B). Thus, eNOS-derived NO can be generated by the addition of extracellular L-Arg and is consistent with our finding of enhanced eNOS binding to Ca2+-CaM at basal Ca2+ levels in cytokine-treated endothelial cells.

Effect of Cytokine Treatment on B2R and G Protein Expression—Typically, B2Rs are constitutively expressed, whereas B1R expression is induced by inflammatory cytokines (16). However, IL-1β was reported to induce up-regulation of both the B1R and B2R in murine airways (47). To investigate whether cytokine treatment with IL-1β and IFN-γ increased B2R expression, we measured B1R and B2R mRNA levels in control and cytokine-treated HLMVEC using quantitative real time RT-PCR. Consistent with our previous studies (21, 44), cytokine treatment increased B1R mRNA expression (control cells B1R/GAPDH = 0.72 ± 0.088; cytokine-treated cells B1R/GAPDH = 1.17 ± 0.023; n = 3). However, B2R mRNA did not change after cytokine treatment (control cells B2R/GAPDH = 1.18 ± 0.008; cytokine-treated cells B2R/GAPDH = 1.12 ± 0.005; n = 3). In addition, cytokine treatment did not alter protein expression of Gαq/11 or Gα1,2 (data not shown).

B2R-mediated NO Production Is Regulated by a MEK1/2- and JNK1/2-dependent Pathway in Cytokine-treated Endothelial Cells—We previously showed that B1R-dependent activation of prolonged NO output via iNOS in cytokine-treated HLMVEC is dependent on activation of the MEK1/2-ERK1/2 MAPK pathway (11–13). Because B2R stimulation has been reported to activate ERK (16, 48), we evaluated the role of this pathway in B2R-mediated NO production in control and cytokine-treated HLMVEC using three structurally distinct MEK1/2 inhibitors as follows: PD98059 (25 μM), U0126 (10 μM), and CI-1040 (2 μM). As shown in Fig. 7A, all three inhibitors significantly reduced B2R-mediated NO production from cytokine-treated HLMVEC but had little effect in control endothelial cells. The negative control compound, U0124 (10 μM), a structurally similar but inactive analog of U0126, did not alter B2R-mediated NO production in control or cytokine-treated HLMVEC. MEK1/2 inhibitors also significantly blocked the prolonged NO production stimulated by BK in cytokine-treated HEK-B2R/eNOS (Fig. 4B) or the B2R agonist analog [Phe8,ψ(CH2-NH)Arg9]bradykinin in cytokine-treated HLMVEC (Fig. 4C).

MEK1/2 is the dual specificity kinase directly upstream of ERK1/2 activation, and blockade of a signal by MEK1/2 inhibition is usually taken as prima facie evidence that the response requires activation of ERK1/2. To determine whether ERK1/2 was involved in the regulation of eNOS, we measured B2R-stimulated NO production in control and cytokine-treated HLMVEC preincubated in the absence or presence of a direct ERK1/2 inhibitor (FR180204; 50 μM) or a structurally similar inactive analog (FR180289; 50 μM). Surprisingly, FR180204 did not significantly inhibit B2R-mediated NO production in either control or cytokine-treated HLMVEC compared with cells pretreated with the negative control FR180289 (Fig. 7B). As a pos-

FIGURE 6. Cytokine treatment increases basal eNOS affinity for Ca2+-CaM and enhances L-Arg-stimulated eNOS activation. A, top panel, representative immunoblot (IB) showing the association between CaM and total eNOS immunoprecipitated (IP) from unstimulated (without BK) control and cytokine-treated HLMVEC. Total eNOS and CaM protein expression was analyzed in total cell lysates, and nonspecific binding was evaluated using the proper IgG and bead controls. Bottom panel, densitometry data expressed as mean fold increase relative to basal ± S.E. (n = 4; *p < 0.05 compared with basal). B, top panel, real-time NO measurements of substrate-depleted control (left panel) and cytokine-treated (right panel) HLMVEC stimulated with 1 mM L-Arg in the absence (trace 1) or presence of 4 μM 1400W without (trace 2) or with 4 μM L-NNA (trace 3). Bottom panel, data are expressed as mean NO concentration (% of vehicle) ± S.E. achieved at 20 min. (n = 3; ***, p < 0.001; *, p < 0.05 compared with vehicle-treated cells, and †, p < 0.05 compared with cells pretreated with 1400W.)
itive control to demonstrate the effectiveness of the ERK1/2 inhibitor in these cells, we measured NO production in cytokine-treated HLMVEC. We previously showed that B1R stimulation of high output NO results from ERK1/2-dependent phosphorylation of iNOS at Ser745 leading to its acute activation (11–13). As shown in Fig. 7C, FR180204 effectively attenuated B1R-mediated NO production in cytokine-treated HLMVEC. These data support the conclusion that B2R stimulation activates eNOS via a MEK1/2-dependent but ERK1/2-independent pathway.

ERK1/2 is a relatively specific substrate of MEK1/2, but it has been reported that MEK1/2 can also phosphorylate c-Jun

FIGURE 7. B2R-mediated NO production is dependent on MEK1/2 and subsequent JNK1/2 but not ERK1/2 activation in cytokine-treated HLMVEC. B2R-mediated NO production was measured in control and cytokine-treated HLMVEC with the following treatments. A, control or cytokine-treated HLMVEC were pretreated with vehicle or the following MEK1/2 inhibitors: 10 μM U0126, 25 μM PD98059, or 2 μM CI-1040 (30 min at 37 °C). Samples were pretreated with 10 μM U0124 as a negative control for MEK1/2 inhibitor U0126. Data are expressed as mean [NO] ± S.E. achieved at maximum in control cells and at 20 min in cytokine-treated cells. (n = 3; ***, p < 0.001 compared with vehicle-treated cells.) B, cells were pretreated with vehicle, ERK1/2 inhibitor (50 μM FR180204), or ERK1/2 inhibitor negative control (50 μM FR180289). (n = 3; n.s. = not significant, compared with cells treated with negative control.) C, as an ERK1/2 inhibitor-positive control, cytokine-treated HLMVEC were pretreated with vehicle or 50 μM FR180204 and then stimulated with 100 nM kinin B1 receptor (B1R) agonist DAKD (top panel), and the effect on NO production was compared with cells stimulated with 100 nM BK (bottom panel). D, cells were pretreated with vehicle, 20 μM JNK inhibitor II (SP600125), or 10 μM JNK inhibitor VIII. (n = 3; **, p < 0.01, compared with vehicle-treated cells.) Cytokine-treated (E) or control (F) HLMVEC were electroporated (EP) with 100 nM JNK1/2 siRNA or 100 nM negative control (NC) siRNA. (n = 3; ***, p < 0.001, n.s., not significant compared with negative control siRNA.) Total JNK1/2 (T-JNK1/2) and total eNOS protein expression were measured by Western analysis from control and cytokine-treated HLMVEC total cell lysates. IB, immunoblot.
N-terminal kinase (JNK) (49). To determine whether JNK1/2 was involved in the regulation of eNOS, we utilized two specific JNK1/2 inhibitors, JNK inhibitor II (SP600125; 20 μM) and JNK inhibitor VIII (10 μM). As shown in Fig. 7D, B2R-mediated NO production was significantly reduced in cytokine-treated HLMVEC pretreated with vehicle or 1.5 μg/ml PTx (3 h at 37 °C). Western analysis of BK-stimulated JNK1/2 and ERK1/2 phosphorylation in the absence or presence of 10 μM U0126 (15 min at 37 °C), D, HLMVECs were electrophoresed with 100 nm MEK1/2 siRNA or 100 nm negative control (NC) siRNA. Between 48 and 72 h, HLMVEC were treated with cytokines (16–24 h) and then stimulated with 100 nM BK for various times. Total MEK1/2 (T-MEK1/2) and JNK1/2 phosphorylation (P-JNK1/2) were assessed by Western blot analysis. Densitometry data for A–D are expressed as mean fold change relative to basal ± S.E. (n = 3; **, p < 0.01; *, p < 0.05 compared with basal). IB, immunoblot.

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To determine the effect of cytokine treatment on B2R-mediated JNK1/2 activation in HLMVEC, we measured the time course of JNK1/2 phosphorylation in response to BK. We found that JNK1/2 phosphorylation increased at 1 min, reaching a maximum 3.3-fold increase at 5 min and slowly decreasing thereafter, but it still remained elevated over basal at 30 and 60 min (Fig. 8A). In contrast, there was minimal change in JNK1/2 phosphorylation in response to BK in control HLMVEC (Fig. 8A). The activation of JNK1/2 in cytokine-treated HLMVEC required coupling through Goi/o as it was inhibited in cells preincubated with PTx (1.5 μg/ml; 3 h) (Fig. 8B). Furthermore, as was found with the NO response, B2R-mediated JNK1/2 activation in cytokine-treated HLMVEC was blocked by the MEK1/2 inhibitor U0126 (Fig. 8C) and was also reduced in cells in which MEK1/2 was knocked down with siRNA (Fig. 8D).

Because the B2R can transactivate the epidermal growth factor receptor (48, 50), we measured NO production from cytokine-treated HLMVEC pretreated in the absence or presence of the EGF receptor tyrosine kinase inhibitor PD153035 (200 nM; 30 min) prior to stimulation with BK. B2R-mediated NO production was not altered by the EGF receptor tyrosine kinase inhibitor (data not shown). The concentration of PD153035
used was effective, as it inhibited EGF-mediated eNOS-dependent NO production and JNK phosphorylation in cytokine-treated HLMVEC (data not shown). Overall, these data show that under inflammatory conditions, B2R stimulation induces eNOS activation and prolonged NO output by a Goi/o-mediated, MEK1/2, and JNK1/2-dependent pathway in HLMVEC.

B2R-mediated eNOS Phosphorylation at Ser1177, Thr495, Ser114, Ser615, Ser633, or Ser633. Does Not Play a Role in Prolonged eNOS-Derived NO in Cytokine-Treated HLMVEC—eNOS activity is tightly regulated by a variety of mechanisms, most notably by dephosphorylation at the inhibitory site Thr495 and PI3K/Akt-dependent phosphorylation at the stimulatory site Ser1177 (23–26). Additionally, eNOS activity is regulated by phosphorylation at Ser615, Ser633, and Ser114, depending on the cell type and stimulus utilized (26, 28, 29, 51, 52). We investigated B2R-mediated phosphorylation of eNOS at Ser1177, Thr495, Ser615, Ser633, and Ser114 in cytokine-treated HLMVEC. As shown in Fig. 9, A and B, maximum phosphorylation was observed 5 min post-BK stimulation at Ser1177, Thr495, and Ser114, but no significant phosphorylation was detected at Ser615 or Ser633. Phosphorylation at Ser1177 or Thr495 returned to baseline within 15 or 30 min of receptor activation, whereas phosphorylation at Ser114 declined but remained significantly elevated above baseline 30 min post-BK stimulation. To determine whether JNK has a role in B2R-mediated eNOS phosphorylation at the aforementioned sites, we preincubated cytokine-treated HLMVEC with JNK inhibitor II (SP600125; 20 μM) and analyzed BK-dependent phosphorylation. As shown in Fig. 9, C and D, B2R-mediated phosphorylation at Ser1177 and Ser114 was not significantly inhibited by JNK inhibitor II, at a concentration that inhibited NO production (Fig. 7D). Conversely, phosphorylation of these residues in response to BK was essentially abolished in the presence of BAPTA-AM (Fig. 9, C and D), which had no effect on prolonged NO production (Fig. 5A).

Bradykinin Has Divergent Effects on Endothelial Wound Healing in Control and Cytokine-Treated Endothelial Cells—Endothelial NO production is known to regulate wound healing and angiogenesis (53, 54). To determine whether B2R-mediated NO production affects endothelial cell migration, we utilized the in vitro scratch assay (Fig. 10) and a modified Boyden Chamber assay (Fig. 11). Stimulation of control HLMVEC with BK significantly improved wound healing and increased transmigration (Figs. 10 and 11). However, BK stimulation of cytokine-treated HLMVEC were delayed wound healing and reduced transmigration of cells (Figs. 10 and 11). Furthermore, the changes in wound healing and transmigration in response to BK were mediated by B2R activation and were eNOS-dependent as both the stimulatory response in control cells and the inhibitory response in cytokine-treated cells were blocked by B2R antagonist HOE-140 or eNOS inhibitor 1-NNa. As a reduction in wound healing can also be caused by increased apoptosis, we utilized fluorescein-dUTP to label DNA at strand breaks as a marker of apoptosis. We found that B2R-mediated eNOS activation in cytokine-treated HLMVEC did not induce endothelial cell apoptosis (Fig. 10C).

NO itself enhances wound healing and angiogenesis (55), but enhanced levels of oxidative and nitrooxidative stress can alter the progression of wound healing (54). To determine whether

**FIGURE 9.** B2R-mediated eNOS phosphorylation at Ser1177, Thr495, and Ser114 is independent of JNK1/2 activation and requires increased Ca2+. A, cytokine-treated HLMVEC were preincubated with 1 μM DALKD and 20 μM MGTA and then stimulated with 100 nm BK for various times. Total cell lysates were prepared and immunoblotted with total eNOS and phospho-specific eNOS antibodies directed against Ser(P)1177, Thr(P)495, and Ser(P)114. Representative immunoblots are shown. B, densitometry of blots from three independent sets of lysates. Data are expressed as mean fold change relative to basal ± S.E. (n = 3; *** p < 0.001; ** p < 0.01; *, p < 0.05 compared with basal). C, cytokine-treated HLMVEC were preincubated in the absence or presence of 25 μM BAPTA-AM, 20 μM SP600125 (JNK inhibitor II), or vehicle for 30 min at 37 °C followed by stimulation with 100 nm BK in the presence of 1 μM DALKD and 20 μM MGTA. Total cell lysates were immunoblotted with total eNOS and phospho-specific eNOS antibodies directed against Ser(P)1177 and Ser(P)114. Representative immunoblots are shown. D, densitometry of blots from three independent sets of lysates. Data are expressed as mean fold change relative to basal ± S.E. (n = 3; *** p < 0.001; ** p < 0.01; *, p < 0.05 compared with basal). IB, immunoblot.
the presence of reactive oxygen species (ROS) reduces B2R-mediated NO bioavailability, we preincubated control and cytokine-treated HLMVEC without or with the superoxide scavengers tempol (10 μM) or polyethylene glycol-superoxide dismutase (PEG-SOD; 100 units/ml) 30 min prior to stimulation with 1 μM BK. We found that B2R-mediated NO production was increased by ~30% in the presence of tempol (Fig. 12A, middle panel) or PEG-SOD (Fig. 12B) in cytokine-treated HLMVEC but had no effect in control cells (Fig. 12A, left panel). Moreover, the reduction in wound healing mediated by B2R stimulation in cytokine-treated HLMVEC was inhibited in the presence of tempol (Fig. 12C and D). Overall, these data suggest that the presence of superoxide in inflamed endothelium reduces B2R-mediated NO bioavailability and explains part of the reduction in endothelial cell migration.

These data indicate that superoxide is generated in cytokine-treated cells and can reduce the NO detected by the microsensor. Although these results would argue against the likelihood that BK stimulation reduces superoxide-mediated consumption of NO, we assessed the maximal increase in basal NO that could be achieved by scavenging superoxide. In cytokine-treated HLMVEC (pretreated with 1400W to block iNOS activity), addition of 100 μM tempol generated only a small increase in detectable basal NO that returned to base line before 20 min (Fig. 12A, right panel). These results are consistent with our finding that eNOS is basally active in cytokine-treated endothelial cells (Fig. 6B), some of which is consumed by superoxide, and that the prolonged increase in NO after B2R stimulation does not arise from blocking the consumption of basally generated NO, but rather an increase in eNOS-derived NO production.

**DISCUSSION**

\( \text{iNOS is considered to be the primary source of "high output" NO in inflammation (3, 19, 56). This view arose from work done in rodent cells, where iNOS expression can be induced to high levels and from the fact that iNOS has basal activity that is not dependent on increased } \text{[Ca}^{2+}] \text{, (3, 19, 56). Our study challenges this notion, as we find that eNOS can generate high output NO in human endothelium under inflammatory conditions. We found a similar response in cytokine-treated HEK cells co-transfected with eNOS and B2R. Furthermore, this response is "iNOS-like" in that eNOS can generate high output in human endothelium using inflammatory conditions. We found a higher constitutive activity and lack of a requirement for an increase in } \text{[Ca}^{2+}] \text{, for agonist-stimulated activity (Fig. 13A). Instead, B2R-dependent eNOS activation is mediated by a novel G} \text{o}_{i/o-} \text{and MEK1/2 } \text{to JNK1/2-dependent pathway in cytokine-treated HLMVEC (Fig. 13B). The ability to generate high output eNOS-derived NO is likely more relevant in humans than in rodents because iNOS expression is highly induced in rodent cells in response to LPS or cytokines, but in human cells, iNOS expression is much lower and in many cases difficult to detect (3, 57) due to epigenetic silencing (58). As a result, basal iNOS-derived NO is much lower in human than in rodent cells, and high output eNOS-derived NO has the potential to play a more important role in human tissues.} \)
Electrode-based measurements of NO have the advantage of detecting real-time output of authentic NO with high sensitivity. However, as this method measures changes in NO concentration, it would not distinguish between a B2R and eNOS-dependent reduction in consumption of basally generated NO and increased NO production by eNOS. Cellular NO has a rel-

**FIGURE 11.** B2R-mediated NO production enhances transmigration of control HLMVEC but slows transmigration of cytokine-treated HLMVEC. The effect of BK on the migration of cytokine-treated (A) or control (B) HLMVEC was tested in a modified Boyden chamber assay in the absence or presence of 10 \( \mu M \) HOE or 4 \( \mu M \) L-NNA. All cells were pretreated with 1 \( \mu M \) DALKD and 20 \( \mu M \) MGTA prior to agonist stimulation. Results are expressed as mean fold change in migration of HLMVEC from the top to bottom side of the membrane relative to vehicle \( \pm \) S.E. (\( n = 3; ***, p < 0.001; **, p < 0.01 \)).

**FIGURE 12.** Scavenging ROS increases B2R-mediated NO bioavailability and impedes the BK-induced delay in wound healing. A, B2R-mediated NO production measured in control (left panel) and cytokine-treated (middle panel) HLMVEC pretreated without or with 10 \( \mu M \) tempol (30 min at 37 °C) prior to stimulation with 100 nM BK in the presence of 1 \( \mu M \) DALKD and 20 \( \mu M \) MGTA. Right panel, real-time NO measurements from cytokine-treated HLMVEC (preincubated with 4 \( \mu M \) 1400W) after addition of 100 \( \mu M \) tempol. B, B2R-mediated NO production measured in control and cytokine-treated HLMVEC pretreated without or with 100 units/ml PEG-SOD (30 min at 37 °C) prior to stimulation with 100 nM BK in the presence of 1 \( \mu M \) DALKD and 20 \( \mu M \) MGTA. Data are expressed as mean NO concentration \( \pm \) S.E. achieved at 20 min. (\( n = 3; *, p < 0.05 \) compared with vehicle-treated cells; n.s., not significant.) C, using the in vitro scratch assay technique, wound closure was monitored in cytokine-treated HLMVEC pretreated without or with 10 \( \mu M \) tempol prior to stimulation with 1 \( \mu M \) BK. D, data are expressed as percentage of wound closure compared with that of the control (vehicle) and shown as mean \( \pm \) S.E. (\( n = 3; *, p < 0.05; **, p < 0.01 \)).
eNOS Masquerades as iNOS in Inflamed Endothelium

In healthy (control) endothelial cells, eNOS is inactive and does not interact with calmodulin (CaM) at resting Ca^{2+} levels. Conversely, eNOS associates with CaM at resting Ca^{2+} levels in HLMVEC pretreated with IL-1β and IFN-γ (cytokine-treated) for 16–24 h. Consequently, basal L-Arg-dependent eNOS activity is enhanced in cytokine-treated but not control cells. eNOS in cytokine-treated HLMVEC, prolonged B2R-dependent NO production is mediated by PTx-sensitive 

CaM binding region. Three

phosphorylation at Ser1177, Ser633, and Ser615) or...

Angiogenesis

Wound healing

EC Migration

CaM

L-Arg

NO

O_2^-•

ONOO^-•

FIGURE 13. Proposed signaling pathway for basal and BK-mediated eNOS activation in inflamed endothelium. A, in healthy (control) endothelial cells, eNOS is inactive and does not interact with calmodulin (CaM) at resting Ca^{2+} levels. Conversely, eNOS associates with CaM at resting Ca^{2+} levels in HLMVEC pretreated with IL-1β and IFN-γ (cytokine-treated) for 16–24 h. Consequently, basal L-Arg-dependent eNOS activity is enhanced in cytokine-treated but not control cells. B, in cytokine-treated HLMVEC, prolonged B2R-dependent NO production is mediated by PTx-sensitive CaM at resting Ca^{2+} levels as follows: 1) an auto-inhibitory loop (amino acids 595–639) located within the FMN binding domain (63, 64); 2) a highly conserved loop (amino acids 1165–1178) present in the catalytic domain (65); and 3) a highly conserved loop (amino acids 1165–1178) present in the catalytic domain (65). Phosphorylation at several sites on eNOS can increase its sensitivity to ...
eNOS Masquerades as iNOS in Inflamed Endothelium

differences in its phosphorylation at relevant subcellular sites. For example, the phosphorylation state of eNOS was shown to differ depending on its subcellular localization, with eNOS phosphorylated at Thr^495 being restricted to caveolar fractions (67). Thus, the fraction of eNOS tightly associated with Ca^{2+}-CaM may be localized in noncaveolar fractions where Thr^495 is not phosphorylated, consistent with the reciprocal relationship between caveolin and Ca^{2+}-CaM binding to eNOS (22).

JNK is classically activated by upstream kinases MKK4 and MKK7, which is typically induced by cellular stress and/or activation of Gq/11-coupled receptors (68, 69). The B2R is classified as a prototypical Gq/11-coupled receptor but can also couple to Go/i/o (16). We found that B2R-mediated JNK1/2 phosphorylation and NO production were PTx-sensitive, showing that JNK1/2 is activated downstream of B2Rs coupled to Go/i/o, consistent with previous reports that Go/i/o, and associated Gβγ subunits can activate JNK (69–71). Surprisingly, we found Go/i/o-dependent JNK phosphorylation to be downstream of MEK1/2, although Go/i/o-dependent JNK activation has been reported to be independent of MKK4/7 (70), and one report showed MEK1/2 to phosphorylate JNK in a human astrocyte (U-251) cell line (49).

JNKs are proline-directed Ser/Thr kinases that phosphorylate many nuclear and cytosolic substrates (72). Human eNOS contains 15 Ser/Thr residues that are potential JNK phosphorylation sites as determined using ExPaSy ScanProsite. However, JNK often utilizes a docking domain ([R/K]2–3(X1–6)[L/I]) on the substrate or on a scaffold protein that facilitates JNK interaction with substrates lacking a JNK binding domain (JBD) (72, 73), which confers enhanced specificity of phosphorylation. Although human eNOS does not contain a typical JBD, JNK might interact with a novel JBD in eNOS or with a scaffold protein containing a JBD to facilitate eNOS phosphorylation. JNK has not been linked to eNOS activation but was recently reported to basally phosphorylate eNOS at Ser^116 (bovine numbering) to reduce eNOS-derived NO in bovine aortic endothelial cells (52). In contrast, we found that B2R-mediated JNK activation was required for stimulation of prolonged eNOS activity, which was independent of phosphorylation at the well known regulatory sites. These data suggest that JNK controls eNOS activation by a different mechanism, possibly via phosphorylation at a novel regulatory site. Alternatively, JNK may activate another kinase that phosphorylates eNOS (Fig. 13B) or the activation process may be independent of eNOS phosphorylation, for example by altering protein interactions with eNOS or eNOS localization (23, 74).

Enhanced B2R-mediated signaling and eNOS-dependent NO output after cytokine treatment could be facilitated by mechanisms in addition to increased Ca^{2+}-CaM binding. Cytokines alter cell functions by promoting significant changes in gene expression and protein synthesis (43). Although cytokine treatment did not alter B2R mRNA levels or the protein expression of Go/i/o, Gαq/11, eNOS, or JNK, B2R-mediated JNK activation was more robust in cytokine-treated HLMVEC. However, cytokine treatment might alter the membrane microdomain localization of signaling components without altering their overall expression. For example, Gαq/11 binds caveolin to concentrate in caveolae, whereas Gαi/o targets lipid rafts by default (75). B2Rs and eNOS localize to caveolae in healthy endothelial cells (76), but inflammation can induce changes in membrane phospholipid composition that affects cholesterol-rich microdomains to alter signal transduction (77). Thus, it is possible that cytokine treatment promotes segregation of B2Rs into lipid raft microdomains, enhancing its Go/i/o-dependent signaling and eNOS activation. Cytokine treatment could also alter post-translational modifications of the B2R that might facilitate Go/i/o coupling. Alternatively, it may not be B2R-Go/i/o coupling that is enhanced by cytokines but downstream components. For example, MAPKs rely on scaffolding proteins to amplify and modulate the specificity of their responses (72, 73). Thus, cytokine treatment might induce the expression of a scaffold protein or facilitate its binding to MEK1/2, JNK1/2, and eNOS to allow efficient activation to occur. Interestingly, although cytokine treatment enhanced signaling via Go/i/o and JNK, Ca^{2+} signaling through Go/i/o was also increased, indicating a major switch of B2R coupling from Go/i/o to Go/i/o is likely not the primary mechanism.

Endothelially derived NO and its derivatives play critical roles in regulating normal vascular functions but can also be involved in exacerbating or ameliorating cardiovascular diseases (8, 10, 56, 60). For example, NO is a major regulator of angiogenesis and plays important roles in wound healing (53). We found that B2R-mediated eNOS-derived NO has opposing effects on endothelial cell migration in control and cytokine-treated HLMVEC, promoting migration in control cells but inhibiting migration in cytokine-treated endothelial cells. Thus, prolonged B2R-mediated NO production might reduce angiogenesis as it was recently shown that caveolin1-deficient mice have impaired angiogenesis that is partially mediated by increased eNOS activation (78). The mechanism by which NO regulates endothelial cell migration is not understood, but VEGF-induced angiogenesis is dependent on NO production and subsequent increased cGMP andPKG activation, resulting in phosphorylation and activation of p38 and ERK1/2 (79). Although NO clearly plays a pro-migratory role, the balance between the concentration of NO and level of oxidative and nitrosative stress can differentially regulate its effect on the endothelium (54). For example, although NO itself is pro-migratory, under inflammatory conditions, combined with superoxide to generate ONOO^−, it could inhibit migration (54). This is consistent with our finding that scavenging of superoxide with tempol or PEG-SOD enhanced the NO detected in response to BK in cytokine-treated HLMVEC and reduced the inhibition of cell migration. However, it is interesting that in the presence of tempol, BK did not stimulate cell migration as it did in control HLMVEC. This could be due to the higher and more prolonged output of NO generated under inflammatory conditions.

HLMVEC in the lung circulation form the essential semi-permeable barrier that dynamically regulates the passage of fluid and protein (80). Sepsis is a leading cause of acute lung injury characterized by inflammation and increased lung microvascular endothelial permeability. The precise role of NO in sepsis and lung inflammation is still controversial. Studies have shown that NO production may have both salutary and harmful effects. A general theme is that low output NO pro-
motes barrier function, whereas high output NO, especially when combined with oxidants, is damaging and increases permeability (9, 14, 81, 82). For example, caveolin-1 depletion results in microvascular hyperpermeability, which may be due to increased eNOS-derived NO (82, 83), whereas decreased NO production caused by eNOS gene deletion or NOS inhibition also increased vascular permeability (84). Thus, a proper range of NO concentration is crucial in maintaining endothelial barrier function. NO regulates vascular permeability via activation of guanylate cyclase (85) or nitrosylation of cysteine residues on critical signaling molecules. For example, VEGF-mediated eNOS activation induces nitrosylation of β-catenin, thereby disrupting its association with VE-cadherin and increasing endothelial permeability through the paracellular pathway (86). VEGF also enhances endothelial permeability by stimulating β-catenin endocytosis that is dependent on β-arrestin 2 (87), and S-nitrosylation of β-arrestin 2 promotes its binding to clathrin heavy chain/β-adaptin, thereby inducing endocytosis (88). Under inflammatory conditions, ONOO− generation becomes an important mediator of enhanced endothelial permeability. ONOO−, via Tyr nitration, activates protein phosphatase 2A (89) or inactivates phosphatases such as PTP1B (60), all of which can disrupt the endothelial barrier.

Overall, the findings of this study blur the traditional distinctions between the functions of eNOS and iNOS in generating NO in inflammation. Furthermore, our previous studies have challenged the view that iNOS is only regulated at the level of expression or substrate supply, functioning as a constitutively active, uncontrolled generator of NO (1, 3, 56), i.e. the control of iNOS activity is more complex and "eNOS-like" than previously recognized. We found that stimulation of the kinin B1R in cytokine-treated HLMVEC or transfected HEK cells results in signaling through a Gαq/11, βγ, and β-arrestin 2-dependent pathway activating Src → Ras → Raf → MEK1/2 → ERK1/2 (11–13). ERK1/2 in turn phosphorylates iNOS at Ser745, leading to a 3–5-fold increase in iNOS activity over basal (11). The presence of both eNOS and iNOS-dependent high output NO pathways in inflamed endothelium could have important consequences in regulating endothelial function, and a better understanding of their roles could lead to new therapeutic approaches to ameliorate endothelial dysfunction caused by overproduction of NO in inflammatory vascular or lung disease.

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