Modulation of Bradykinin Receptor Ligand Binding Affinity and Its Coupled G-proteins by Nitric Oxide*

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To determine whether nitric oxide (NO) can modulate bradykinin (BK) signaling pathways, we treated endothelial cells with an NO donor, N-nitroso-L-glutathione (GSNO), to determine its effect(s) on G-proteins (G, and G) that are coupled to the type II kinin (BK) receptor. Radioligand binding assays and Western analyses showed that GSNO (10–500 μM, 0–72 h) did not alter the expression of BK receptor, G, or G. However, GSNO caused a 6-fold increase in basal cGMP production and decreased high affinity BK bindings sites and GTPase activity by 74 and 85%, respectively. The cGMP analogue, dibutyryl-cGMP, also inhibited BK-stimulated GTPase activity by 74% suggesting that some of the effects of NO may be mediated through activation of guanylyl cyclase. The NO synthase inhibitor, N-nitroso-L-arginine, inhibited endogenous NO synthase activity and cGMP production by 91 and 78%, respectively, but increased BK-stimulated GTPase activity by 61%. To determine which G-proteins are affected by NO, we performed GTP binding assays with [35S]GTP-S followed by immunoprecipitation with specific G-protein antisera. Both GSNO and dibutyryl-cGMP increased basal G-protein GTP binding activities by 18–26%. However, GSNO decreased BK-stimulated G, G, and G GTP binding activity by 93, 61, and 90%, respectively, whereas epinephrine-stimulated G GTP binding activity was unaffected. These results suggest that NO can modulate BK signaling pathways by selectively inhibiting G-proteins of the G and G family.

The vasoactive nonapeptide, bradykinin (BK), is released during immune hypersensitivity reactions and contributes to the inflammatory process by modulating endothelial cell permeability, vascular tone, and neutrophil chemotaxis (1, 2). The cellular effects of BK are mediated by seven transmembrane-spanning receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins) (3, 4). We have previously shown that bovine aortic endothelial cells contain predominately the type II kinin (BK) receptor that is coupled to G-proteins of the G and G family (5). Both G and G can activate phosphoinositide-specific phospholipase C, which mobilizes intracellular calcium via the hydrolysis of phosphatidylinositol 4,5-bisphosphate (5–7). This intracellular calcium signal is necessary for many of the vascular responses elicited by BK including the release of endothelial-derived nitric oxide (NO) (8).

The stimulation of Gi proteins and phospholipase A, by BK leads to the production of arachidonic acids and leukotrienes, which are important in mediating the inflammatory response (9). In addition, stimulation of Gi proteins by BK can potentially decrease cAMP production via inhibition of adenylyl cyclase activity (10). The cAMP-dependent pathway serves to counteract many of the clinical symptoms associated with immune hypersensitivity reactions (11). Indeed, β-adrenergic receptor agonists such as epinephrine, which activates the G-adenyl cyclase pathway, are often administered to alleviate anaphylactic reactions (12). Thus, factors that modulate BK receptor-coupled G-proteins may influence the course and outcome of BK-mediated inflammatory processes.

Sustained high levels of NO are produced during inflammatory conditions by cytokine-inducible type II NO synthase in resident and nonresident vascular cells (13–15). Although both BK and NO are released during immune hypersensitivity reactions, the effects of NO on BK-mediated responses are not known. Recent studies suggest that exogenous NO donors can activate mitogen-activated protein kinase pathways and stimulate p21 via S-nitrosylation of these signaling molecules (16, 17). A similar mechanism has been proposed for the activation of heterotrimeric G-proteins by NO in peripheral blood mononuclear cells (18). Although these studies demonstrated activation of basal heterotrimeric G-protein activity by NO, it is not known which G-proteins are affected and how NO affects agonist-stimulated G-protein activity.

Because BK and NO are important inflammatory mediators, the effects of NO on BK-mediated responses may have important clinical implications. The purpose of this study, therefore, is to determine whether NO can regulate BK signaling pathways via its effects on G-proteins that are coupled to the BK receptor.

EXPERIMENTAL PROCEDURES

Materials—All standard culture reagents were obtained from JRH Bioscience. Bradykinin, HEPES, t-arginine, ascorbic acid, creatinine phosphate, phosphocreatine kinase, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, bacitracin, 1,10-phenanthroline, triethanolamine HCl, dithiothreitol, bovine serum albumin (BSA), ATP, GDP, GTP, sodium nitrite, and glutathione were purchased from Sigma. Captz peptides were obtained from E. R. Squibb & Sons, Inc. (Princeton, NJ). Hoe-140 (a BK receptor antagonist) was obtained from Hoechst Marion Roussel, Inc. (Cincinnati, OH). The NO synthase inhibitor, N′-monomethyl-L-arginine (LNMMA), was purchased from Calbiochem (San Diego, CA). N-Nitroso-L-glutathione (GSNO) was synthesized as described (19). The radioisotopes, [3H]arginine (40.5 Ci/mmol), [3H]BRK
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(121.6 Ci/mmol), [γ-32P]GTP (30 Ci/mmol), and [35S]GTP·S (1250 Ci/ mmol), and the polyclonal rabbit antisera to Goαi (EC/2), Goαq (QL), and Goαm (RM/1) were supplied by NEN Life Science Products. [35S]iGMP (>2500 Ci/µg) was obtained from Biotechnological Inc. (Stoughton, MA). The polyclonal rabbit antisera P4 was raised against a partially purified decapeptide corresponding to the COOH-terminal regions of Goαi (Research Genetics, Inc., Huntsville, AL), and its specificity has been previously verified (20). Protein molecular weight markers were purchased from Life Technologies, Inc. The chemiluminescence detection kit (ECL) was obtained from Amersham Corp. The polyvinylidene difluoride transfer membrane (pore size, 0.2 µm) was purchased from Bio-Rad.

Cell Culture—Bovine aortic endothelial cells were isolated and cultured in a growth medium containing Dulbecco’s modified Eagle’s medium, 5 mM l-glutamine (Life Technologies, Inc.), 10% fetal calf serum (Hyclone, Logan, UT), and antibiotic mixture of 100 units/ml penicillin/ 100 µg/ml streptomycin/250 ng/ml Fungizone as described previously (5). They were characterized by morphology using phase-contrast microscopy (Nikon, Optiphot 200) and by staining for Factor VIII-related antigens (21). All passages were performed with a disposable cell scraper (Costar Inc., Cambridge, MA), and only endothelial cells of less than 6 passages were used. Confluent endothelial cells (5×10^5) were treated with various concentrations of GSNO, dibutyryl-cGMP, and LNAME for the indicated time intervals. Treatment with LNAME was terminated by the addition of BS (10^6) for 20 min at 22 °C and terminated after 30 min with gentle shaking. All reaction tubes and filters were pretreated with 3H[HK] (1 pulse to 10 nM) in a buffer containing Tris-HCl (100 mM, pH 7.4), MgCl2 (5 mM), EDTA (0.6 mM), bacitracin (140 µg/ml), Captorplage (1 µM), 1 mM dithiothreitol, 1 mM 1,10-phenanthroline, and 0.1% BSA in a total volume of 0.1 ml. The assay mixture was incubated at 4 °C for 90 min with gentle shaking. All reaction tubes and filters were pretreated overnight with 0.1% BSA and 0.1% polyethyleneimine, respectively, to decrease nonspecific binding. The assays were terminated by vacuum filtration on Whatman GF/C filters. Each filter was counted for 2 min in a liquid scintillation counter (Beckman LS 1800). Bovine aortic endothelial cell membrane contain only one kinin receptor, the BK subtype (22). Nonspecific binding was determined in the presence of 10 µM of HOE-140 (IC50 of 0.1 µM) and accounted for approximately 8% of total binding. The BK2 receptor density (Bmax) and affinity (Kd) were determined by the Ligand Program of Munson and Rodbard (23). All assays were performed three times in duplicate.

Western Blotting—Membrane proteins (25 µg) and molecular weight markers were separated by SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose (Nytran, Schleicher & Schuell) in a transfer buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% (v/v) methanol. The blotted membranes were incubated overnight at 4 °C with blocking solution (5% nonfat dry milk and 0.1% Tween 20 in PBS) prior to the addition of the following dilutions of specific rabbit polyclonal antisera: P4 (1:400), EC/2 (1:1000), QL (1:1000), and RM/1 (1:1000). The membranes were washed twice with PBS containing 0.1% Tween 20 and incubated for 30 min at 30 °C in a buffer containing [35S]GTP (20 nM), MgCl2 (5 mM), EDTA (0.1 mM), NaCl (50 mM), creatine phosphate (50 mM), creatine phosphate kinase (50 mM), dithiothreitol (1 mM), bacitracin (140 µg/ml), Captorplage (1 µM), leupeptin (10 µg/ml), aprotinin (50 µg/ml), 1,10-phenanthroline (1 mM), BSA (0.2%), and triethanolamine HCl (50 mM, pH 7.4) for 10 min. The samples were removed from the membranes and placed in a scintillation counter. The supernatant was removed, and the cells were washed twice with PBS followed by incubation at 37 °C for 30 min in a buffer containing indomethacin (10 µM), 3-isobutyl-1-methylxanthine (1 mM), Captorplage (10 µM), NaCl (154 mM), KCl (5.6 mM), CaCl2 (2.0 mM), MgCl2 (1.0 mM), NaHCO3 (3.6 mM), glucose (5.6 mM), and HEPES (10 mM, pH 7.4). The endothelial cells were then washed with BS (100 µg/ml) for 5 min, the medium was rapidly removed, and the reaction was terminated with 100 µl of trichloroacetic acid (10%). Cells were disrupted by a probe sonicator and centrifuged for 10 min at 3000 x g. The supernatant was extracted twice with three volumes of water-saturated ether prior to lyophilization and reconstitution in a sodium acetate buffer (50 mM, pH 6.2). The GMP production was determined by a radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA) using [35S]GTP·S and expressed as picomoles/106 cells. Each experiment was performed in triplicate with corresponding standard curve in acetate buffer.

Immunoprecipitation of [35S]GTP·S-labeled G-proteins—Membrane proteins (30 µg) from control and GSNO-treated endothelial cells were incubated for 30 min at 30 °C in a buffer containing [35S]GTP·S (20 nM), GTP (2 µM), MgCl2 (5 mM), EDTA (0.1 mM), NaCl (50 mM), creatine phosphate (50 mM), creatine phosphate kinase (50 mM), dithiothreitol (1 mM), bacitracin (140 µg/ml), Captorplage (1 µM), leupeptin (100 µg/ml), aprotinin (50 µg/ml), 1,10-phenanthroline (1 mM), BSA (0.2%), and triethanolamine HCl (50 mM, pH 7.4) for 10 min. The assay was initiated by the addition of BK (10 nM) and terminated after 30 min with unlabelled GTP·S (100 µM). Samples were then resuspended in 100 µl of immunoprecipitation buffer containing Triton X-100 (1%), SDS (0.1%), NaCl (150 mM), EDTA (5 mM), Tri-HCl (25 mM, pH 7.4), leupeptin (10 µg/ml), aprotinin (10 µg/ml), and phenylmethylsulfonyl fluoride (2 mM). The following G-protein antisera with their corresponding final dilutions were added to the mixture: α2 (P4, 1:20), αq (EC/2, 1:100), αq (QL, 1:100), and αm (RM/1, 1:100).

The samples were allowed to incubate for 16 h at 4 °C with gentle mixing. The antibody-G-protein complexes were then incubated with 50 µl of protein A-Sepharose (1 mg/ml, Pharmacia Biotech Inc.) for 2 h at 4 °C, and the precipitate was collected by centrifugation at 12,000 x g for 10 min. Preliminary studies indicated that all α2, αm, and αq were completely precipitated by this procedure because Western blot analysis of the supernatant with the P4, EC/2, QL, and RM/1 antisera did not reveal the presence of 40–41 KD proteins. The pellet was washed three times in a buffer containing PBS and 50 mM HEPES (50 mM, pH 7.4), NaF (10 µM), sodium phosphate (50 mM), NaCl (100 mM), Triton X-100 (1%), and SDS (0.1%). The final pellet containing the immunoprecipitated [35S]GTP·S-labeled G-protein was counted in a liquid scintillation counter (LS 1800, Beckman Instruments, Inc., Fullerton, CA). Nonspecific activity was determined in the presence of unlabeled GTP·S (100 µM).
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RESULTS

Cell Culture—Relatively pure (>95%) bovine aortic endothelial cell cultures were confirmed by morphologic features and immunofluorescent staining with Factor VIII antibodies (results not shown). There were no observable adverse effects of GSNO, LNMA, or dibutyl-cGMP on cell number, morphology, or immunofluorescent staining.

Effect of NO on $BK_2$ Receptor Density—Untreated bovine aortic endothelial cell membranes contain 94 ± 8 fmol/mg of $BK_2$ receptor with a $K_d$ of 0.48 ± 0.07 nm. Treatment of endothelial cells with GSNO (10–500 μM) for 24 h did not affect total $BK_2$ receptor density ($B_{max}$ of 95 ± 7 fmol/mg) or overall $BK_2$ receptor affinity ($K_d = 0.52 ± 0.1$ nm) ($p > 0.05$ for both).

Effect of NO on G-protein Expression—In a concentration-dependent manner, treatment with GSNO did not significantly affect the amount of $G_{α_{i2}}, G_{α_{i3}},$ and $G_{α_{q/11}}$ after 24 h as determined by densitometric analysis of immunoblots. There were no observable adverse effects of GSNO, LNMA, or dibutyl-cGMP on cell number, morphology, or immunofluorescent staining.

Data Analysis—Band intensities were analyzed densitometrically with the NIH Image program (26). All values are expressed as means ± S.E. compared with controls and among separate experiments. EC$_{50}$ and IC$_{50}$ values were calculated by linear or logarithmic extrapolation.

Effect of NO on $BK_2$ Receptor Affinity—In a concentration-dependent manner, treatment with GSNO (10–500 μM) had no effect on $G_{α_{i2}}, G_{α_{i3}},$ and $G_{α_{q/11}}$ after 24 h (Fig. 1A). Similarly, in a time-dependent manner, GSNO (500 μM) had no effect on $G_{α_{i2}}, G_{α_{i3}},$ and $G_{α_{q/11}}$ protein levels for up to 72 h (Fig. 1B). The P4 ($α_{i2}$), EC2 ($α_{i3}$), and QL ($α_{q/11}$) antisera were quite specific because recognition of their respective α subunits could be blocked only in the presence of excess decapeptides from which they were derived (5, 21).

Treatment with the NO synthase inhibitor, LNMA (5 mM), also did not affect the amount of any G-protein α subunits. In addition, the amount of $α_i$ and common $β$ subunit as determined by the RM/1 and SW/1 antisera, respectively, was also unaffected by GSNO (500 μM) or LNMA (5 mM).

Effect of NO on $BK_2$ Receptor-G-protein Coupling—We have previously shown that the type II kinin ($BK_2$) receptor is the predominant $BK_2$ receptor subtype in bovine aortic endothelial cells (22). Radioligand binding studies showed that untreated endothelial cell membranes contain two $BK_2$ receptor binding sites (Fig. 2A). The high affinity agonist binding site that constitutes 32% of the total $BK_2$ receptor sites has a $K_d$ of 14 ± 3 pm and a $B_{max}$ of 27 ± 5 fmol/mg. The low affinity $BK_2$ binding site that constitutes 68% of the total $BK_2$ receptor sites has a $K_d$ of 480 ± 42 pm and a $B_{max}$ of 67 ± 6 fmol/mg.

Treatments with increasing concentrations of GSNO (1, 10, 50, 100, 500, and 1000 μM) for 24 h progressively decreased the amount of $BK_2$ receptor high affinity binding site (IC$_{50}$ value of 54 ± 11 μM) (Fig. 2B). Maximal decrease in $BK_2$ receptor high affinity binding site occurred at a GSNO concentration of 500 μM, which converted 74% of $BK_2$ receptor high affinity agonist binding sites (20 fmol/mg) to low affinity binding sites ($K_d$ of 520 ± 40 pm, $B_{max}$ of 87 ± 5 fmol/mg) (Fig. 2A). Complete
conversion of BK<sub>2</sub> receptor high affinity agonist binding sites to low affinity sites (<i>K<sub>d</sub></i> of 520 ± 64 pM, <i>B<sub>max</sub></i> of 94 ± 5 fmol/mg) was observed in the presence of the nonhydrolyzable GTP analogue, GTP<sub>γS</sub> (10 μM).

Effect of NO on BK-stimulated GTPase Activity and cGMP Production—In a concentration-dependent manner, stimulation of endothelial cell membranes with of BK (0.1–100 nM) produced a progressive increase in GTPase activity with maximal activity (15.0 ± 2.0 pmol/min/mg) occurring at a BK concentration of 10 nM (Fig. 3A). The EC<sub>50</sub> value for BK-stimulated GTPase activity was 2.4 ± 0.4 nM. When membranes from endothelial cells were treated with increasing concentrations of GSNO (10–500 μM), there was a progressive decrease in BK-stimulated GTPase activity (Fig. 3B). The calculated IC<sub>50</sub> for GSNO by logarithmic extrapolation was 32 ± 6 μM. At a GSNO concentration of 500 μM, a maximal 85% reduction in BK-stimulated GTPase activity (2.3 ± 0.7 pmol/min/mg) was observed (p < 0.01).

Fig. 3. A, the concentration-dependent effects of BK (10 pM to 1 μM) on GTPase activity in endothelial cell membranes. B, the concentration-dependent effects of GSNO on BK-stimulated GTPase activity. Membranes (30 μg) from endothelial cells treated with the indicated concentrations of GSNO for 24 h were used in GTPase assay. The line drawn through the data points represents BK-stimulated GTPase activity as an inverse logarithmic function of GSNO concentration.

Untreated or control endothelial cells have a basal cGMP production of 0.24 ± 0.08 pmol/10<sup>6</sup> cells. Stimulation with increasing concentrations of BK (0.1–100 nM) produced a progressive increase in cGMP levels with an EC<sub>50</sub> of 1.9 ± 0.3 nM and a maximal 10.3-fold increase in cGMP production at a BK concentration of 10 nM (2.5 ± 0.3 pmol/10<sup>6</sup> cells, p < 0.001) (Fig. 4A). Inhibition of endothelial NO synthase by 5 mM of LNMA (IC<sub>50</sub> of 0.8 ± 0.1 mM) resulted in 63 and 92% reductions in the corresponding basal and BK-stimulated cGMP levels (0.09 ± 0.04 and 0.21 ± 0.07 pmol/10<sup>6</sup> cells, respectively) (p < 0.05 for both) (Fig. 4B). Endothelial cells treated with 500 μM of GSNO (EC<sub>50</sub> of 44 ± 6 μM) showed a maximal 6-fold increase in basal cGMP levels after 24 h (0.24 ± 0.08 pmol/10<sup>6</sup> cells to 1.5 ± 0.2 pmol/10<sup>6</sup> cells, p < 0.01). However, stimulation with BK (10 nM) did not result in any further increase in cGMP production from basal levels in GSNO-treated cells (1.8 ± 0.2 pmol/10<sup>6</sup> cells, p >

Fig. 4. A, the concentration-dependent effects of BK (0.1–100 nM) on intracellular cGMP production in endothelial cell. B, basal (no stimulation) and BK (10 nM)-stimulated intracellular cGMP levels in untreated endothelial cells (Control) or endothelial cells pretreated with LNMA (5 mM) or GSNO (500 μM) for 24 h. *, represents a significant difference compared with unstimulated (Basal) untreated (Control) cells (p < 0.05). **, represents a significant difference between BK stimulation and basal (no stimulation) for each treatment condition (p < 0.05).
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GTPase and NO synthase activity were observed when endo-BK-stimulated GTPase activity (24 ± 80 pmol/min/mg, p < 0.05), suggesting that endogenous endothelial NO production (EC<sub>50</sub> of 45 ± 6 μM) was decreased by 42 ± 3, 52 ± 6, and 66 ± 6% in the presence of P4 (α<sub>i2</sub>), EC/2 (α<sub>i3</sub>), and QL (α<sub>i11</sub>) respectively (p < 0.05) for all (Fig. 6B). The combination of P4, EC/2, and QL reduced BK-stimulated GTPase activity by 95 ± 3% (p < 0.01). The RM/1 antibody (α<sub>i</sub>) had no effect on BK-stimulated GTPase activity but did decrease isoproterenol (10 μM)-stimulated GTPase activity by 79 ± 4% in control cells (p < 0.01). In cells treated with GSNO (500 μM, 24 h), BK-stimulated GTPase activity was reduced by 78 ± 3% (p < 0.01), whereas isoproterenol-stimulated GTPase activity was completely unaffected (p > 0.05). The addition of P4 or QL antibody to membranes from GSNO-treated cells did not cause any further decrease in BK-stimulated GTPase activity (79 ± 2 and 81 ± 3% decreases, respectively, p < 0.05), whereas the addition of EC/2 or the combination of all three antibodies did produce a further decrease in BK-stimulated GTPase activity (84 ± 2 and 96 ± 2%, respectively, p < 0.05). These findings indicate that treatment with GSNO produced similar inhibitory effects on BK-stimulated GTPase activity as the combination of P4, EC/2, and QL antibodies. The addition of RM/1 antisera inhibited isoproterenol-stimulated GTPase activity in both control and GSNO-treated cells (79 ± 4 and 80 ± 3% decreases, respectively, p < 0.05).

Effects of NO on Agonist-stimulated GTP Binding Activity—Immunoprecipitation of [35S]GTP-γ-S-labeled G-proteins with antisera directed against specific subunits demonstrated that treatment with 500 μM of GSNO (EC<sub>50</sub> of 42 ± 6 μM) alone for 24 h produced a maximal increase in basal α<sub>i2</sub>, α<sub>i3</sub>, and α<sub>i11</sub> GTP binding activity (18 ± 2, 22 ± 3, 24 ± 2, and 26 ± 3%, respectively). These findings indicate that both NO and cGMP stimulate the basal activities of all G-proteins but paradoxically inhibit only BK-stimulated G<sub>i</sub> and G<sub>q</sub> without affecting epinephrine-stimulated G<sub>i</sub>.

In untreated endothelial cell membranes, α<sub>i2</sub>, α<sub>i3</sub>, and α<sub>i11</sub> accounted for 27% (2.8 ± 0.1 fmol/min/mg or 3880 ± 125 cpm), 29% (3.0 ± 0.5 fmol/min/mg or 4160 ± 693 cpm), and 44% (4.5 ± 0.6 fmol/min/mg or 6240 ± 832 cpm) of BK-stimulated α<sub>i2</sub> subunit GTP binding activities, respectively (Fig. 7A). In membranes pretreated with GSNO (500 μM) for 24 h, BK-stimulated α<sub>i2</sub> GTP binding activity was reduced by 93% (0.2 ± 0.1 fmol/min/mg or 280 ± 130 cpm, p < 0.01). Similarly, treatment with GSNO (0.5 mM) for 24 h resulted in 61 and 90% decreases in BK-stimulated α<sub>i2</sub> (1.2 ± 0.2 fmol/min/mg or 1660 ± 277 cpm, p < 0.05) and α<sub>i11</sub> (0.4 ± 0.1 fmol/min/mg or 5540 ± 139 cpm, p < 0.01) GTP binding activity, respectively.

Treatment with GSNO (500 μM) for 24 h, however, did not significantly affect epinephrine-stimulated α<sub>i</sub> GTP binding activity (Fig. 7B). In untreated endothelial cell membranes, epinephrine (0.1 mM) produced a 3-fold increase in α<sub>i</sub> GTP binding.
activity from $2.3 \pm 0.3$ fmol/mg/min (3120 ± 360 cpm) to $6.9 \pm 0.5$ fmol/mg/min (9490 ± 624 cpm) ($p < 0.005$). Treatment with GSNO (0.5 mM) alone for 24 h caused a 30% increase in basal $\alpha_s$ GTP binding activity (3.0 ± 0.4 fmol/mg/min or 4090 ± 513 cpm, $p$, 0.05). In membranes treated with GSNO, stimulation with epinephrine did not produce a significant change in $\alpha_s$ GTP binding activity compared with that of untreated membranes (6.8 ± 0.8 fmol/min/mg or 9480 ± 1070 cpm, $p > 0.05$).

**DISCUSSION**

The findings in this study indicate that a brief 24-h exposure of endothelial cells to exogenous NO attenuates BK-stimulated $G_i$ and $G_q$ protein activity. The BK$_2$ receptor-G-protein coupling was inhibited by GSNO treatment as demonstrated by reductions in BK-stimulated high affinity binding sites and GTPase activity. This inhibitory effect of NO was relatively specific because epinephrine-stimulated $\alpha_s$ GTP binding activity was relatively unaffected. There were no observable changes in the density of BK$_2$ receptor or the amounts of $G_i$, $G_q$, or $G_s$, suggesting that NO inhibited G-protein function rather than expression. These findings, therefore, suggest that NO can preferentially inhibit the function of G-proteins that are coupled to the BK$_2$ receptor in endothelial cells.

In this study, GSNO was selected as the NO donor because of its relatively long half-life compared with other shorter acting NO donors such as sodium nitroprusside and 3-morpholinosydnonimine (27). In addition, sodium nitroprusside and 3-morpholinosydnonimine can also release cyanide and superoxide anion in addition to NO and therefore are relatively more toxic than GSNO at comparable concentrations (28). Furthermore, the precursors of GSNO, sodium nitrite and glutathione, have no effect on BK-stimulated G-protein function at GSNO concentrations comparable with those used in this study. Inhibition of endogenous endothelial NO synthase activity with LNMA resulted in an increase in BK-stimulated GTPase activity, suggesting that constitutive endothelial NO produc-

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2. A. Miyamoto and J. K. Liao, unpublished observation.
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stimulated G-protein activity, we found that they also nonspecifically increased basal G-protein activity by approximately 20%. The direct activation of heterotrimeric G-proteins by NO donors, however, may not occur exclusively via the stimulation of cGMP production because treatment with dibutyryl-cGMP produced a lower level of G-protein activation compared with that of GSNO. Indeed, previous studies in peripheral blood mononuclear cells showed that NO donors can directly activate heterotrimeric G-proteins and p21\textsuperscript{ras}, via S-nitrosylation of these signaling molecules (17, 18). Furthermore, S-nitrosylation of terminal cysteine residues of the neuronal heterotrimeric G-protein, G\textsubscript{q}, renders G\textsubscript{q} less susceptible to ADP-ribosylation by pertussis toxin (30). It remains to be determined, however, whether S-nitrosylation of G-proteins has any affect on agonist-stimulated G-protein activity.

The ability of NO to modulate BK receptor ligand binding affinity via effects on specific G-protein activities could have important biochemical and physiological consequences. Because both NO and bradykinin are released under certain inflammatory conditions, NO may function as an important autocrine and paracrine inhibitor of BK-mediated processes including the release of BK-stimulated NO from vascular endothelial cells. The conversion of high to low affinity BK receptor sites by NO is similar to the effects of nonhydrolysable GTP analogues such as GTP\textsubscript{S}, which uncouples the BK receptor from its G-proteins (5, 20–22). Thus, it is conceivable that NO may modify critical cysteine residues on \( \alpha \) and \( \gamma \) but not \( \beta \), which are important in regulating GTP binding and hydrolysis. Alternatively, we cannot exclude the possibility that NO affects \( \beta\gamma \) subunits whose association with the \( \alpha \) subunit is required to generate the formation of high affinity BK ligand binding sites (31). However, the role of \( \beta\gamma \) subunit in mediating the inhibitory effects of NO is less likely given that specific antibodies to the carboxyl terminus of \( \alpha \) subunits produce similar inhibitory effects as NO. Finally, it is possible that NO may directly modify the BK receptor but not the \( \beta_2\)-adrenergic receptor, particularly in the region of the third cytoplasmic loops and carboxyl terminus, which are known to interact with G-proteins (32). It remains to be determined, however, whether such modifications, if any, could alter BK receptor-G-protein coupling.

Many BK-mediated inflammatory processes such as mucous hypersecretion and smooth muscle contraction occur via phosphatidylinositol 4,5-bisphosphate hydrolysis and elevation of intracellular calcium (33, 34). We have previously shown that the G-proteins of the G\textsubscript{i} and G\textsubscript{q} family couple the BK\textsubscript{2} receptor to the stimulation of phospholipase C and generation of inositol 1,4,5-trisphosphate in endothelial cells (5). Thus, the findings of this study suggest that NO may counteract many of the inflammatory responses elicited by BK through inhibition of BK\textsubscript{2} receptor-coupled G-proteins. Interestingly, a recent study indicates that NO can also inhibit growth factor-mediated phospholipase C activation via a cGMP-dependent protein kinase I pathway (35). Thus, NO-induced increases in intracellular cGMP levels may not only modulate BK signaling pathways at the level of heterotrimeric G-proteins but also may affect downstream effectors such as the \( \beta \) and \( \gamma \) isoforms of phospholipase C.

Clinically, NO may have a bronchoprotective role in allergy-induced asthma, in part, by alleviating BK-mediated bronchoconstriction (36, 37). NO causes bronchial smooth muscle relaxation through direct stimulation of soluble guanylyl cyclase (38). Furthermore, NO may block BK-mediated inflammatory responses and bronchial smooth muscle contraction by inhibiting BK\textsubscript{2} receptor-coupled G-proteins. Because NO inhibits G\textsubscript{i}, but not G\textsubscript{q}, it could also facilitate bronchial smooth muscle

![Graph](image-url)

**Fig. 7.** Specific G-protein activity as determined by immunoprecipitation of BK-stimulated [\(^{35}\)S]GTP\textsubscript{S} labeling of G\textsubscript{a12}, G\textsubscript{a13}, and G\textsubscript{a1/11} (A) and basal and epinephrine-stimulated [\(^{35}\)S]GTP\textsubscript{S}-labeling of G\textsubscript{a} from untreated (Control) or GSNO (500 \( \mu \text{M}, 24 \text{ h} \))-treated endothelial cells (B).
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relaxation through its permissive action of the G_s-adenylyl cyclase pathway. Indeed, a recent randomized double-blind placebo-controlled trial showed that bronchoconstriction after BK inhalation is attenuated by endogenous NO production in the bronchial airways (39).

In summary, we have identified a potentially important effect of NO on BK signaling pathways. Our findings indicate that NO can attenuate BK receptor ligand binding affinity and its coupled G-proteins via cGMP-dependent pathway(s). It remains to be determined how NO actually inhibits Gi and Gq but not G_s and whether these effects are mediated through cGMP-dependent or redox-sensitive pathways.

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