

## A novel pathway for receptor-mediated post-translational activation of inducible nitric oxide synthase

Viktor Brovkovich<sup>a</sup>, Yongkang Zhang<sup>a</sup>, Svitlana Brovkovich<sup>a</sup>, Richard D. Minshall<sup>a, b</sup>, Randal A. Skidgel<sup>a, b, \*</sup>

<sup>a</sup> Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL, USA

<sup>b</sup> Center for Lung and Vascular Biology, University of Illinois College of Medicine, Chicago, IL, USA

Received: August 12, 2009; Accepted: November 17, 2009

### Abstract

Inducible nitric oxide synthase (iNOS) is a major source of nitric oxide during inflammation whose activity is thought to be controlled primarily at the expression level. The B1 kinin receptor (B1R) post-translationally activates iNOS beyond its basal activity *via* extracellular signal regulated kinase (ERK)-mediated phosphorylation of Ser<sup>745</sup>. Here we identified the signalling pathway causing iNOS activation in cytokine-treated endothelial cells or HEK293 cells transfected with iNOS and B1R. To allow kinetic measurements of nitric oxide release, we used a sensitive porphyrinic microsensor (response time = 10 msec.; 1 nM detection limit). B1Rs signalled through G $\alpha$ i coupling as ERK and iNOS activation were inhibited by pertussis toxin. Furthermore, transfection of constitutively active mutant G $\alpha$ i Q204L but not G $\alpha$ q Q209L resulted in high basal iNOS-derived nitric oxide. G- $\beta$ - $\gamma$  subunits were also necessary as transfection with the  $\beta$ -adrenergic receptor kinase C-terminus inhibited the response. B1R-dependent iNOS activation was also inhibited by Src family kinase inhibitor PP2 and transfection with dominant negative Src. Other ERK-MAP kinase members were involved as the response was inhibited by dominant negative H-Ras, Raf kinase inhibitor, ERK activation inhibitor and MEK inhibitor PD98059. In contrast, PI3 kinase inhibitor LY94002, calcium chelator 1,2-bis-(*o*-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), protein kinase C inhibitor calphostin C and protein kinase C activator PMA had no effect. Angiotensin converting enzyme inhibitor enalaprilat also directly activated B1Rs to generate high output nitric oxide *via* the same pathway. These studies reveal a new mechanism for generating receptor-regulated high output nitric oxide in inflamed endothelium that may play an important role in the development of vascular inflammation.

**Keywords:** nitric oxide • kinin B1 receptor • inducible nitric oxide synthase • endothelial cells • ACE inhibitors

### Introduction

Nitric oxide regulates diverse physiological and pathological responses in the cardiovascular system [1–3]. Three distinct isoforms of nitric oxide synthase (NOS) produce nitric oxide from the precursor amino acid L-Arg. The constitutively expressed endothelial and neuronal NOS (eNOS and nNOS) have low basal activity that is enhanced by receptor stimulation leading to increased intracellular calcium, phosphorylation at key residues and/or altered interactions with regulatory proteins [2, 4]. In contrast, inducible NOS (iNOS) is not constitutively expressed in most cells and the regulation of its activity is considered to be primarily

at the level of transcription and expression in response to cytokines and inflammatory mediators [5]. Furthermore, iNOS contains tightly bound Ca<sup>++</sup>/calmodulin that does not dissociate in resting cells, and it is constitutively active once synthesized [1, 6]. This has led to the prevailing view that iNOS generates unregulated high output nitric oxide (given sufficient substrate and cofactors) until the protein is degraded [1, 4–6]. Recent studies have suggested some mechanisms for post-translational regulation of iNOS that affect basal activity or protein expression. For example, iNOS dimerization and activity are inhibited by S-nitrosylation [7] and interaction with kalirin or NAP110 [4] but enhanced by interaction with hsp90 [8]. iNOS activity is also reduced by sequestration in aggresomes [9], and its degradation is enhanced by interaction with caveolin-1 [4]. The phosphorylation sites that are important in regulating eNOS activity [1, 2] are not conserved in iNOS and its regulation by phosphorylation has not been well studied. Recently it was reported that co-transfection of iNOS and activated Src results in increased phosphorylation of iNOS at Tyr<sup>151</sup>

\*Correspondence to: Randal A. SKIDGEL, Ph.D.,  
Department of Pharmacology, University of Illinois at Chicago College of  
Medicine, 835 South Wolcott, (M/C 868), Chicago, IL 60612, USA.  
Tel.: 312-996-9179  
Fax: 312-996-1225  
E-mail: rskidgel@uic.edu

and decreased activity [10] and phosphorylation of iNOS at  $\gamma^{1055}$  by Src results in increased iNOS half-life [11].

We found that activation of kinin B1 receptors (B1Rs) by peptide agonist or angiotensin converting enzyme (ACE) inhibitors led to generation of iNOS-derived high output nitric oxide in cytokine-pretreated human lung microvascular endothelial cells (HLMVEC) [12–14], suggesting a novel mode of acute post-translational regulation of iNOS that fundamentally differs from known mechanisms. Indeed, we discovered that B1R stimulation activated the extracellular signal-regulated kinase (ERK) which phosphorylated iNOS on Ser<sup>745</sup>, leading to a 2.5 to 5-fold further increase in nitric oxide production over basal activity [15]. More recently, platelets were found to constitutively express iNOS and platelet agonists at low doses (*e.g.* thrombin) acutely stimulated iNOS-mediated nitric oxide production to enhance platelet secretion and aggregation [16]. These findings indicate a novel mode of acute activation of iNOS *via* G protein coupled receptor (GPCR)-mediated signalling pathways that are still to be delineated.

We undertook the present study to investigate the upstream signal transduction pathway and mitogen activated protein kinase (MAPK) components activated by B1R stimulation that leads to post-translational activation of iNOS-dependent high-output nitric oxide. Here we show that B1R-mediated activation of iNOS requires  $G_{\alpha i}$  and  $\beta\gamma$ -dependent activation of Src, Raf, Ras, MAPK/ERK kinase (MEK) and ERK. This newly described signalling pathway for high output nitric oxide production may play an important role in the development of vascular inflammation.

## Materials and methods

### Materials

Reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Low-glucose Dulbecco's modified Eagle's medium (DMEM) without phenol red and custom-made L-Arg-free DMEM were from Invitrogen (Carlsbad, CA, USA). 5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone (Raf kinase inhibitor), Ste-MPKKPTPIQLNP-NH<sub>2</sub> (ERK activation inhibitor peptide 1), 2'-Amino-3'-methoxyflavone (PD 98059), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), SH-6, and myr-SIRKALNILGYPDYD-OH (m-SIRK) were obtained from Calbiochem (San Diego, CA, USA). Enalaprilat, the active form of the ACE inhibitor enalapril, was from Merck. Human iNOS cDNA in pcDNA3 was a gift from Dr. Timothy Billiar, Univ. of Pittsburgh. Human B1R cDNA was a gift from Dr. Fredrik Leeb-Lundberg, Univ. of Lund, Sweden. Adenoviral vector (type 5) encoding the C-terminus of  $\beta$ -adrenergic receptor kinase 1 (Adv-ct- $\beta$ ARK) or empty vector control (Adv-EV) were generous gifts from Dr. Walter J. Koch (Jefferson Medical College, Philadelphia, PA, USA). Dominant-negative (DN) Src (Y527F, K295M) in vector pSM was a gift from Dr. Silvio Gutkind (National Institute of Dental Research, National Institutes of Health, Bethesda, MD, USA),  $G_{\alpha i}$  Q205L,  $G_{\alpha q}$  Q209L and DN-H-Ras (S17N), were from Missouri University of Science and Technology cDNA Resource Center (Rolla, MO, USA). Antibodies to ERK and phospho-ERK were from New England Biolabs (Ipswich, MA, USA). Antibodies to human iNOS,  $G_{\alpha q}$  and eNOS were from BD Transduction Laboratories (San Jose,

CA, USA) and anti- $G_{\alpha i}$  antibody was from Calbiochem (San Diego, CA, USA). Antibodies for  $\beta$ -actin and GAPDH were from Ambion (Austin, TX, USA).

### Cell culture and transfections

HLMVEC (Cambrex, East Rutherford, NJ, USA) were cultured in EBM-2 medium with growth factors from EGM-2 Bullet kits (CC-3162, Lonza, Rockland, ME, USA) and 10% fetal bovine serum (FBS). HLMVEC were used from passages 5 to 8 (at 85–100% confluence), and were pre-treated with 5 ng/ml interleukin-1 $\beta$  (IL-1 $\beta$ ) and 100 U/ml interferon- $\gamma$  (IFN- $\gamma$ ) for 16 to 18 hrs to induce expression of B1Rs and iNOS. HEK293 cells were maintained in DMEM containing 10% FBS, 100  $\mu$ g/ml streptomycin and 100 U/ml penicillin. Transfections of HLMVEC with B1R, iNOS,  $G_{\alpha i}$  Q204L,  $G_{\alpha q}$  Q209L, DN-H-RAS, or DN-Src were done using an Amaxa Nucleofector (Amaxa, Walkersville, MD, USA) with the manufacturer's optimized buffer kit and protocol (S005) for HLMVEC. HEK293 cells were transfected with iNOS, B1R,  $G_{\alpha i}$  Q204L mutant,  $G_{\alpha q}$  Q209L mutant, DN-H-Ras or empty plasmids with Effectene transfection reagent per manufacturer's instructions. After 24 hrs, cells were washed and maintained in DMEM/F-12 with 0.5% FBS overnight and then changed to the medium indicated for experiments. HLMVEC or HEK cells were infected with the adenovirus empty vector control (Adv-EV) or Adv-ct- $\beta$ ARK for 36 hrs in medium containing 10% FBS and then serum-deprived for 12 hrs before experiments [3, 17].

### Measurement of nitric oxide production

For direct measurement of nitric oxide generation in real time, a porphyrinic microsensor was used as described [12, 18]. The microsensor is highly sensitive for nitric oxide, and its response time is rapid, providing the ability to make kinetic measurements of nitric oxide generation. The porphyrinic sensor was positioned with a micromanipulator close to the cell culture surface ( $20 \pm 1 \mu$ m). To measure L-Arg-dependent basal iNOS-mediated nitric oxide generation, cells were incubated for 2 hrs in L-Arg-free media and then 1 mM L-Arg was added and the response (current *versus* time) recorded continuously. To initiate B1R-dependent iNOS activation and nitric oxide production, cells were incubated in L-Arg-containing media and then 100 nM des-Arg<sup>10</sup>-kallidin (DAKD) or 100 nM ACE inhibitor enalaprilat were added to initiate the response. Current generated was proportional to the nitric oxide released, and a computer-based Gamry VP600 potentiostat was used to monitor nitric oxide concentration over time. Each electrode was calibrated with a nitric oxide standard. The concentration of nitric oxide achieved 20 min. after addition of agonist or substrate was used to quantitate the results.

### Immunoblotting

Cells were washed with ice-cold PBS, collected and lysed for 30 min. on ice in lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10% glycerol, 1% NP-40, 1% [v/v] protease inhibitor cocktail [Sigma] and 1mM phenylmethylsulfonyl fluoride (PMSF)) and then centrifuged at  $16,000 \times g$  for 15 min. at 4°C. The resulting supernatant was resolved by SDS-PAGE on 8–16% gradient gels (ISC BioExpress) transferred to polyvinylidene difluoride (PVDF) membranes and detected with appropriate primary antibodies followed by horseradish peroxidase-labelled second antibody (Pierce, Rockford, IL, USA) and enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, Waltham, MA, USA).

## Detection of ERK activation

HLMVEC or HEK293 cells were serum-starved in DMEM/F12 for 3 hrs to decrease basal ERK phosphorylation and allow more sensitive detection of B1R-dependent activation. Cells were then treated with 100 nM DAKD as indicated, washed twice with ice-cold PBS containing 100  $\mu$ M sodium orthovanadate, collected by centrifugation and then lysed as above with lysis buffer also containing 20 mM p-nitrophenylphosphate, 25 mM NaF, 20 mM  $\beta$ -glycerolphosphate and 1 mM sodium orthovanadate to inhibit phosphatases. Samples were centrifuged (16,000  $\times$  g, 4°C, 15 min.) and the supernatants used. For detection of ERK, aliquots were added to an equal volume of 2 $\times$  SDS-PAGE sample buffer, boiled for 5 min. and subjected to Western blotting with antibody to total or phospho-ERK (New England Biolabs).

## Statistical analysis

Data are expressed as the mean  $\pm$  S.E. Statistical analysis was performed with the two-way ANOVA and Newman-Keuls test for multiple comparisons. The value of  $P < 0.05$  was considered significant.

## Results

### B1R agonist induces iNOS-dependent high output nitric oxide in endothelial and transfected HEK293 cells

In control HLMVEC, the addition of 1 mM L-Arg after 2 hrs incubation in L-Arg-free media resulted in a low basal output of nitric oxide (maximum concentration reached =  $90 \pm 5$  nM;  $n = 10$ ) (Fig. 1A). In cells incubated in media containing 1 mM L-Arg, the addition of B1R agonist DAKD (100 nM) led to a small activation of nitric oxide release over baseline (maximum =  $90 \pm 15$  nM;  $n = 10$ ) (Fig. 1A), consistent with previous detection of a small B1R response in control HLMVEC [12].

Cytokines up-regulate the expression of iNOS and B1Rs [5, 12, 19]. HLMVEC were treated with IL-1 $\beta$  (5 ng/ml) and IFN- $\gamma$  (100 U/ml) for 16–18 hrs and then in L-Arg-free medium for 2 hrs to reduce basal iNOS activity. In these cytokine-treated HLMVEC, 1 mM L-Arg produced a much higher basal output of nitric oxide ( $200 \pm 15$  nM;  $n = 10$ ) than in control cells. This was due to iNOS as it was inhibited by the specific inhibitor 1400 W (Fig. 1B), consistent with an increase in iNOS expression in cytokine-treated HLMVEC (Fig. 1D). B1R agonist DAKD, added to cells pre-incubated in L-Arg-containing medium, caused a greater increase in nitric oxide output (maximum =  $355 \pm 25$  nM;  $n = 10$ ) compared with Arg-stimulated basal activity (Fig. 1B). This activity was inhibited by the specific B1R antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin (DALKD) (Fig. 1B). Cells stimulated with DAKD in the absence of L-Arg did not produce detectable nitric oxide (Fig. 1B),

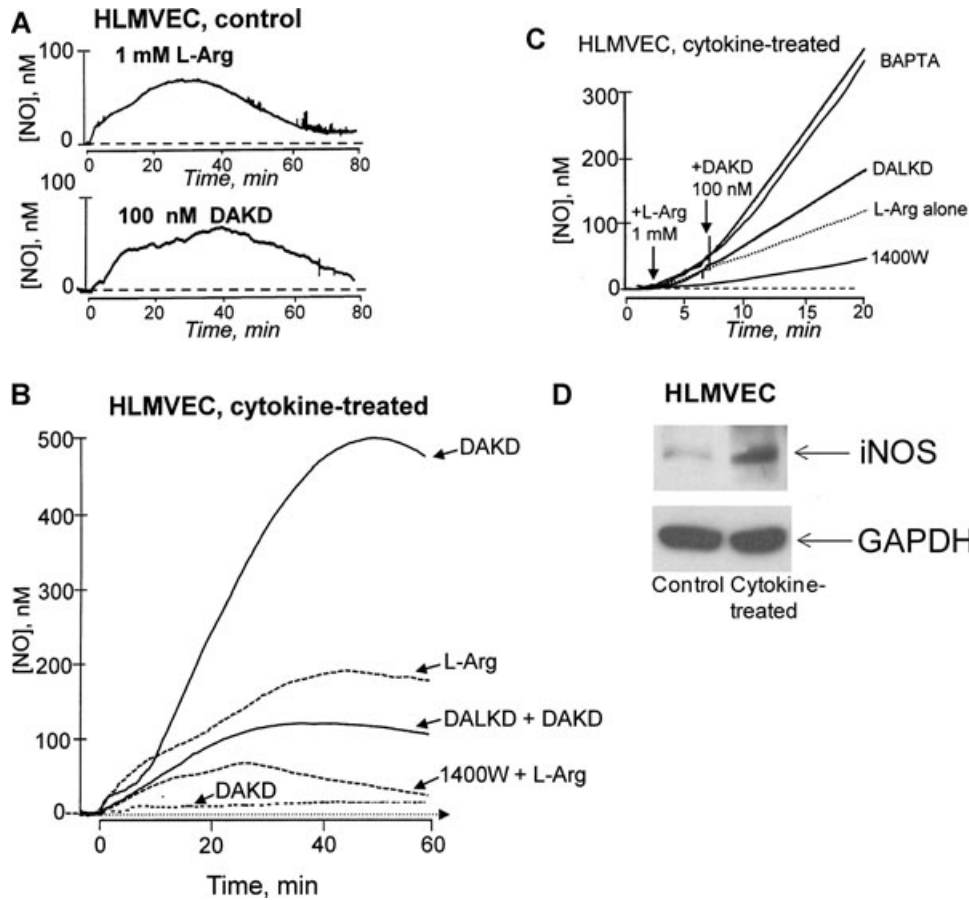
consistent with reports showing iNOS activity is dependent on extracellular Arg [5, 20].

To further demonstrate the receptor-mediated acute activation of iNOS, cytokine-treated HLMVEC were depleted of Arg for 2 hrs, then 1 mM L-Arg was added to generate basal iNOS activity (Fig. 1C). Addition of 100 nM DAKD 5 min. after adding L-Arg resulted in an increase in the rate of nitric oxide release that was blocked by B1R antagonist DALKD (Fig. 1C). The intracellular Ca<sup>++</sup>-chelating agent BAPTA-AM had no effect on L-Arg or B1R-mediated nitric oxide production (Fig. 1C), consistent with the independence of iNOS activity on increased intracellular Ca<sup>++</sup> [1, 6]. Pre-treatment of HLMVEC with iNOS specific inhibitor 1400 W inhibited basal and receptor-stimulated nitric oxide output whereas B1R antagonist DALKD (1  $\mu$ M) inhibited only the latter (Fig. 1C).

We previously found that B1R-dependent nitric oxide production in HLMVEC was not due to eNOS activation [15] as eNOS inhibitor L-N<sup>G</sup>-nitroarginine at 4  $\mu$ M concentration (which has no effect on iNOS in control studies) did not inhibit B1R-mediated nitric oxide production. Furthermore, control HLMVEC (which constitutively express eNOS) transfected with only the B1R, did not generate significant nitric oxide when stimulated with B1R agonist [15]. To further prove this point, we measured eNOS expression and activity in response to cytokine treatment. As shown in Fig. 2A, eNOS protein is readily detected in control HLMVEC, but its expression did not change after cytokine treatment. Furthermore, stimulation of eNOS activity with calcium ionophore A23187 resulted in a short burst of nitric oxide production that returned to baseline in 1 min. and did not increase after cytokine treatment (Fig. 2B and C). Finally, nNOS protein was undetectable by Western analysis in control or cytokine-treated HLMVEC and nitric oxide production was unaffected by a specific nNOS inhibitor (not shown). Taken together, the above data show that B1R-dependent nitric oxide production in cytokine-treated HLMVEC is due to activation of iNOS.

To further prove that B1Rs can activate iNOS, their cDNAs were transfected into control HLMVEC or HEK293 cells. Transfection of iNOS and B1Rs into HLMVEC increased basal, L-Arg-dependent nitric oxide production equivalent to that in cytokine-treated HLMVEC whereas B1R-stimulated nitric oxide production was even higher in transfected HLMVEC than after cytokine treatment (Fig. 3A). Mock transfection did not change B1R-dependent nitric oxide production in cytokine-treated HLMVEC (Fig. 3A).

In HEK293 cells transfected with iNOS, basal L-Arg dependent nitric oxide output was similar to that in iNOS + B1R transfected cells, while transfection of B1Rs alone gave a minor response similar to that in untransfected cells (Fig. 3B). B1R agonist did not stimulate significant nitric oxide production in untransfected HEK293 cells or cells transfected with iNOS or B1R alone, but gave a robust response in cells co-transfected with B1R and iNOS (Fig. 3B). Thus, high output nitric oxide in response to B1R agonist depends on the expression of both B1R and iNOS and transfected HEK293 cells respond similarly to cytokine-treated HLMVEC.



**Fig. 1** B1R agonist stimulates iNOS-dependent high output nitric oxide in cytokine-treated HLMVEC. **(A)** Control HLMVEC were pre-incubated for 2 hrs in L-Arg-free medium (*top*) or maintained in medium containing 1 mM Arg (*bottom*) and then 1 mM Arg (*top*) or 100 nM DAKD (*bottom*) was added and real-time nitric oxide was measured with a porphyrinic microsensor for 80 min. **(B)** HLMVEC were treated with cytokines for 16 hrs to induce iNOS and B1R expression. Cells were either pre-incubated for 2 hrs in L-Arg-free medium (*dashed lines*) or maintained in medium containing 1 mM Arg (*solid lines*). Where indicated, cells were also pre-incubated for 20 min. with either B1R antagonist DALKD (1  $\mu$ M) or iNOS inhibitor 1400 W (4  $\mu$ M). To stimulate basal or B1R-dependent nitric oxide production, 1 mM Arg or 100 nM DAKD was added and real-time nitric oxide was measured with a porphyrinic microsensor for 80 min. **(C)** HLMVEC were pre-treated with cytokines and pre-incubated in L-Arg-free

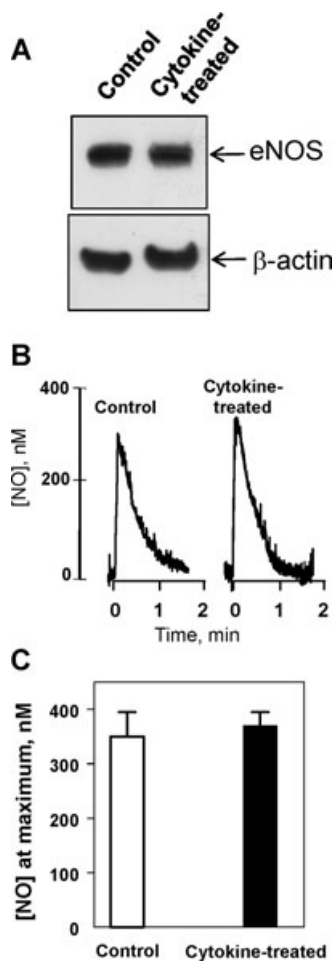
medium for 2 hrs. Some cells were pre-treated for 20 min. with 25  $\mu$ M BAPTA-AM, 1  $\mu$ M DALKD or 4  $\mu$ M 1400 W min. as indicated. 1 mM L-Arg was added first to activate basal iNOS-mediated nitric oxide, followed 5 min. later by 100 nM DAKD to stimulate B1R-dependent activation of iNOS (except tracing labelled 'L-Arg alone'). **(D)** Immunoblotting of iNOS in lysates of control or cytokine-treated HLMVEC. Data are representative of three experiments.

### B1R-mediated iNOS activation requires a pertussis toxin (PTX)-sensitive G protein

B1Rs can couple to both G $\alpha$ q/11 and G $\alpha$ i [19], but iNOS activity is not regulated by changes in intracellular Ca<sup>++</sup> [1, 6]. Indeed, intracellular calcium chelator BAPTA-AM did not inhibit the B1R-mediated response (Fig. 1C), indicating that a calcium response coupled through G $\alpha$ q/11 is not involved. To address this, cytokine-treated HLMVEC or HEK293 cells transfected with iNOS and B1R were incubated with 1.5  $\mu$ g/ml PTX for 3 hrs and then basal (L-Arg-dependent) or B1R-activated nitric oxide production was measured. PTX had no effect on basal L-Arg induced iNOS activity in HLMVEC or HEK293 cells (Fig. 4A), consistent with Western analysis showing no changes in iNOS protein level in the cells after PTX treatment (data not shown). However, pre-treatment of HLMVEC or transfected HEK293 cells with PTX caused a significant decrease in B1R agonist-induced nitric oxide production (Fig. 4B) indicating G $\alpha$ i coupling to B1Rs.

### Constitutively active G $\alpha$ i mutant mimics the effect of activation of B1R on nitric oxide release

HLMVEC were transfected with constitutively active G $\alpha$ i Q204L and then treated with cytokines to induce B1R and iNOS expression. In Arg-depleted cells, addition of 1 mM L-Arg stimulated nitric oxide production that was much greater in G $\alpha$ i Q204L transfected HLMVEC than in cytokine-treated cells and mimicked the response to B1R agonist (Fig. 5A). In contrast, B1R-activated nitric oxide production was much lower in G $\alpha$ i Q204L transfected cells, consistent with overexpression of G $\alpha$ i Q204L resulting in uncoupling of the receptor from the response. Further proof was obtained in HLMVEC and HEK293 cells transfected with B1R, iNOS and either G $\alpha$ i Q204L or G $\alpha$ q Q209L. In both cases, expression of G $\alpha$ i Q204L resulted in high output nitric oxide generation by L-Arg alone and low B1R-stimulated nitric oxide production (Fig. 5B), consistent with the results above. In contrast, cells transfected with the constitutively active G $\alpha$ q Q209L mutant did not produce high basal

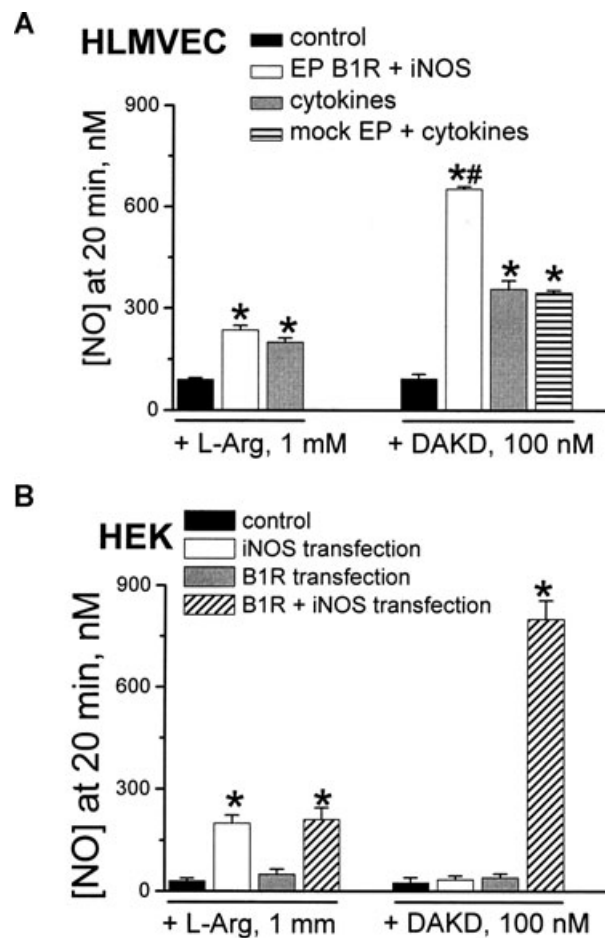


**Fig. 2** eNOS expression and  $\text{Ca}^{2+}$ -dependent activation are not affected by cytokine treatment. **(A)** Control or cytokine-treated HLMVEC were lysed and eNOS expression was assessed by Western analysis using eNOS-specific monoclonal antibody. Results are representative of two experiments done in duplicate. **(B)**  $\text{Ca}^{2+}$ -dependent eNOS activity was stimulated in control or cytokine-treated HLMVEC by adding  $10 \mu\text{M}$  calcium ionophore A23187 at time 0 and nitric oxide generation was measured with a porphyrinic electrode. Tracings shown are representative of 4 experiments. **(C)** Quantitation of maximal nitric oxide concentration achieved in experiments described in B. Results shown are mean  $\pm$  S.E.M for  $n = 4$ .

nitric oxide in response to L-Arg and B1R agonist stimulated a large increase in nitric oxide production (Fig. 5B) as seen in cytokine-treated HLMVEC or cells transfected with B1R and iNOS. Taken together, the above data show that B1R-mediated iNOS activation depends on coupling through  $\text{G}\alpha\text{i}/\text{o}$  and not  $\text{G}\alpha\text{q}/11$ .

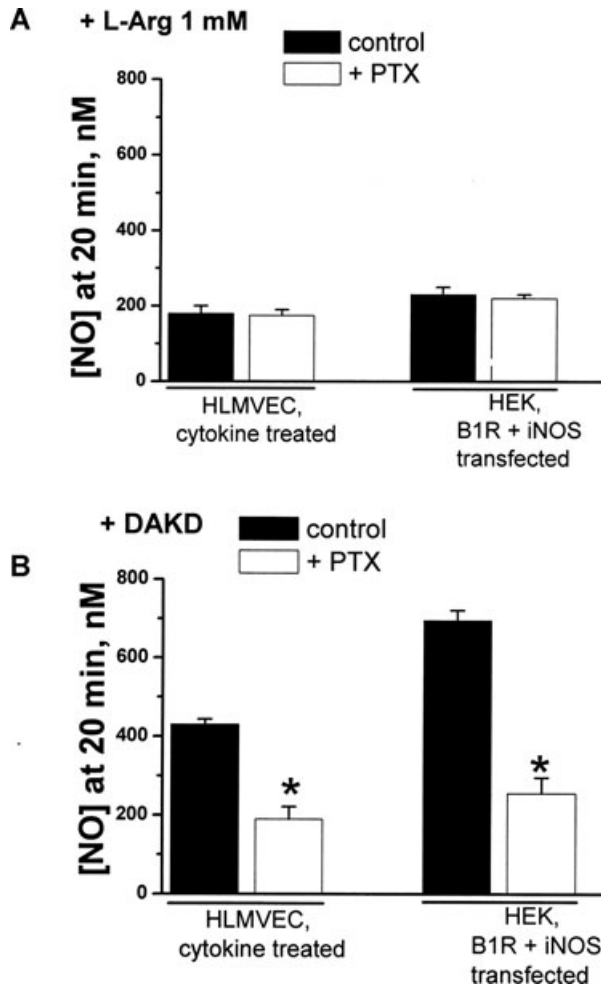
### Role of $\text{G}\beta\gamma$ in B1R-mediated iNOS activation

GPCR signalling depends on activation of effectors by both  $\text{G}\alpha$  and  $\text{G}\beta\gamma$  subunits of heterotrimeric G-proteins. To explore the role



**Fig. 3** Basal and B1R-activated nitric oxide release in cytokine treated HLMVEC or in HEK cells or HLMVEC transfected with B1R and iNOS. **(A)** HLMVEC were pre-treated with cytokines to induce iNOS and B1R expression or electroporated with iNOS and B1R cDNAs as described in 'Materials and methods'. Basal Arg-dependent nitric oxide generation was measured by incubating cells for 2 hrs in L-Arg-free media and then adding 1 mM L-Arg to initiate the response. To measure B1R-dependent iNOS activation and nitric oxide production, cells were maintained in L-Arg-containing media and B1R agonist (100 nM DAKD) was added to stimulate the response. In both cases, nitric oxide production was measured continuously with a porphyrinic electrode, and the nitric oxide concentration achieved at 20 min. used to quantitate the results. Results shown are mean values  $\pm$  S.E.M for  $n = 3$ . \* $P < 0.05$  as compared with control; # $P < 0.05$  as compared with cytokine-treated cells. **(B)** HEK293 cells were transfected with B1R or iNOS alone or with iNOS + B1R cDNAs. Basal Arg-dependent or B1R-activated nitric oxide production was measured as above. Results shown are mean values  $\pm$  S.E.M for  $n = 3$ . \* $P < 0.05$  as compared with control.

of  $\text{G}\beta\gamma$  subunits, HLMVEC or HEK293 cells were infected with adenovirus containing the C-terminus of the  $\beta$ -adrenergic receptor kinase (ct- $\beta$ ARK), an inhibitor of  $\text{G}\beta\gamma$  signalling, or empty vector control [17]. As shown in Fig. 5(C), ct- $\beta$ ARK expression resulted



**Fig. 4** B1R-mediated iNOS activation is PTX sensitive. HLMVEC were pre-treated with cytokines and HEK293 cells were transfected with iNOS and B1R as described in 'Materials and methods'. Cells were pre-treated with PTX (1.5  $\mu$ g/ml) for 3 hrs. Basal Arg-mediated (A) or B1R-activated (B) iNOS-dependent nitric oxide production was measured as described (see Fig. 3A legend and 'Materials and methods'). Data shown are mean values  $\pm$  S.E.M for  $n = 3$ . \* $P < 0.05$  compared with control.

in a significant attenuation of B1R-mediated nitric oxide production in both HLMVEC and HEK cells, compared with cells infected with adenovirus containing empty vector, but did not affect basal L-Arg-dependent nitric oxide output. To verify this, HEK293 cells transfected with iNOS and B1R were pre-treated with the membrane permeable peptide m-SIRK. This peptide promotes the dissociation of G $\beta\gamma$  from G $\alpha$  without stimulating nucleotide exchange, resulting in G $\beta\gamma$  signalling [21]. Treatment of HEK293 cells with 1  $\mu$ M m-SIRK alone induced a small output of nitric oxide (70  $\pm$  20 nM nitric oxide at 20 min.,  $n = 3$ ), likely due to activation of G $\beta\gamma$  signalling. However, pre-treatment of the cells for 15 min. with the

G $\beta\gamma$ -activating peptide m-SIRK (1  $\mu$ M) significantly reduced B1R agonist-dependent, but not L-Arg-dependent, nitric oxide production (Fig. 5D). These results are consistent with m-SIRK pre-treatment uncoupling receptors from G $\beta\gamma$  thus inhibiting the response.

### Role of Src family protein tyrosine kinases

G $\beta\gamma$  subunits are known activators of protein tyrosine kinases [22]. We investigated the role of Src in HEK293 cells co-transfected with iNOS and B1R, with or without dominant-negative (DN)-Src cDNA. B1R agonist produced significantly less nitric oxide in cells transfected with DN-Src whereas L-Arg dependent nitric oxide release was the same (Fig. 5D). The specific Src family tyrosine kinase inhibitor PP2 significantly inhibited the B1R agonist-induced nitric oxide release in both cytokine-treated HLMVEC and transfected HEK293 cells (Fig. 6A).

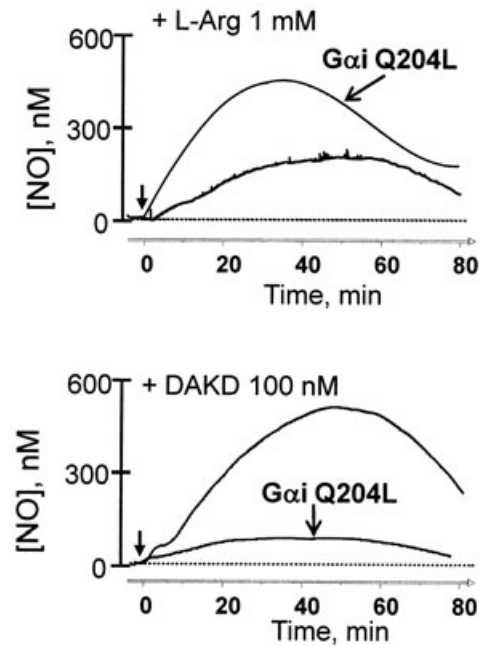
### Role of components of the ERK MAPK pathway in B1R-mediated iNOS activation

Activation of the MAPK ERK typically involves upstream sequential activation of the small GTPase Ras and the kinases Raf and MEK. To determine the role of Ras, HLMVEC were transfected with dominant negative H-Ras (DN-H-Ras) and then cytokine treated. B1R agonist generated significantly lower nitric oxide release in cells transfected with DN-H-Ras (Fig. 6A). L-Arg dependent basal nitric oxide production in these cells was not affected (data not shown) and transfection itself did not decrease B1R-mediated nitric oxide output (Fig. 3A). Similarly, in HEK293 cells expressing iNOS and B1R, DN-H-Ras transfection significantly reduced B1R-mediated nitric oxide output (Fig. 6A).

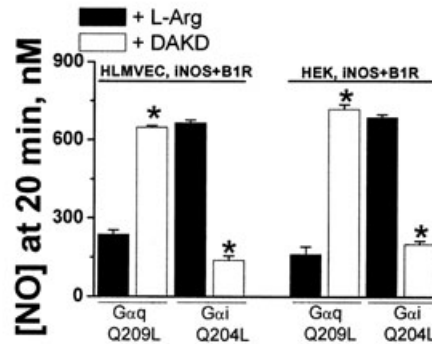
To determine the role of the MAP kinase Raf, cytokine treated HLMVEC or HEK293 cells transfected with iNOS and B1R were pre-incubated for 20 min. with 100 nM Raf kinase inhibitor (5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone). As shown in Fig. 6(A), Raf kinase inhibitor resulted in a substantial decrease in B1R-dependent nitric oxide, suggesting that Raf is an important component of the B1R signalling pathway.

One of the main downstream targets of Raf is MEK. Pre-treatment of cells with MEK inhibitor PD98059 (20  $\mu$ M for 30 min.), resulted in significant inhibition of B1R-dependent nitric oxide release in both cytokine-treated HLMVEC and transfected HEK293 cells (Fig. 6B). To confirm this, we used the cell-permeable ERK activation inhibitor peptide I Ste-MPKKKPTPIQLNP-NH<sub>2</sub> (30  $\mu$ M, 20 min.) that selectively binds to ERK and prevents its interaction with MEK. This inhibitor also significantly reduced B1R-dependent nitric oxide production in cytokine-treated HLMVEC and in transfected HEK293 cells (Fig. 6B). Basal L-Arg-dependent nitric oxide release (180  $\pm$  20 nM nitric oxide;  $n = 3$ ) was not significantly inhibited by either the ERK activation inhibitor peptide (155  $\pm$  35 nM nitric oxide;  $n = 3$ ) or PD98059 (185  $\pm$  20 nM nitric oxide;  $n = 3$ ). These data indicate that the Ras $\rightarrow$ Raf $\rightarrow$ MEK $\rightarrow$ ERK MAPK

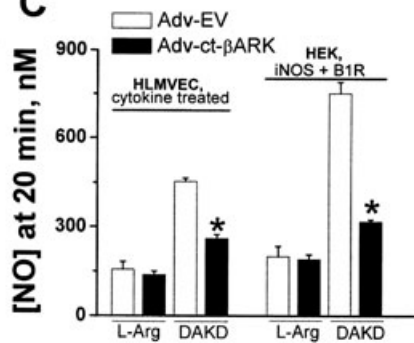
## A Cytokine-treated HLMVEC



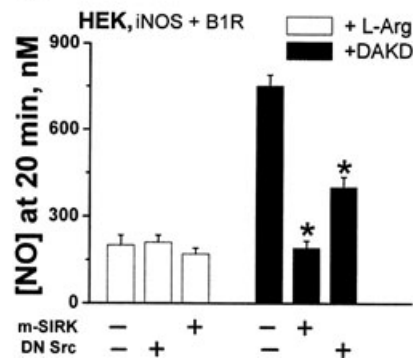
## B



## C



## D



nant negative (DN)-Src. Basal Arg-mediated or B1R-activated iNOS-dependent nitric oxide production was measured as above. For 'm-SIRK', cells were maintained in Arg-containing medium and 1  $\mu$ M m-SIRK was pre-incubated for 15 min. Data shown are mean values  $\pm$  S.E.M for  $n = 3$ ; \* $P < 0.05$  compared with nitric oxide output without m-SIRK or DN-Src.

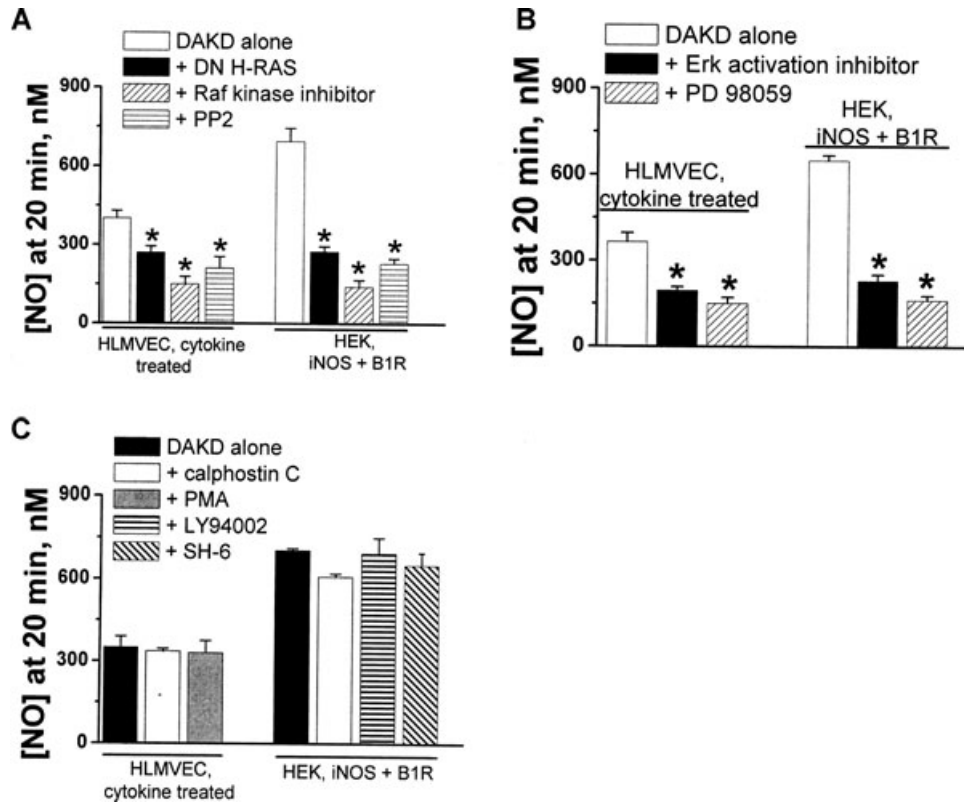
cascade is critical for B1R-dependent, but not basal, nitric oxide production by iNOS.

## B1R-mediated iNOS activation is independent of PKC and phosphatidylinositol 3-kinase

Activation of ERK can occur by alternate pathways, for example in MCF-7 cells, bradykinin results in ERK activation by a PKC/Akt

dependent pathway [23]. In cytokine-treated HLMVEC, neither the PKC activator phorbol myristate acetate (1  $\mu$ M, 30 min.) nor PKC inhibitor calphostin C (1  $\mu$ M, 30 min.) had any effect on B1R agonist-induced nitric oxide output (Fig. 6C). In HEK293 cells transfected with iNOS and B1R, the PKC inhibitor also had no effect (Fig. 6C). The specific phosphatidylinositol 3-kinase inhibitor LY94002 (10  $\mu$ M, 30 min.) as well as Akt inhibitor SH-6 (25  $\mu$ M, 30 min.) also had no effect on B1R dependent nitric oxide output in transfected HEK293 cells (Fig. 6C). Thus, these

**Fig. 5** Effect of transfection with constitutively active G $\alpha$ i, ct- $\beta$ ARK or dominant-negative Src on iNOS-dependent nitric oxide production. (A) Representative tracings of real time nitric oxide measurement. HLMVEC were transfected with G $\alpha$ i Q204L (top) or G $\alpha$ q Q209L (bottom) and treated with cytokines. Basal Arg-mediated or B1R-activated iNOS-dependent nitric oxide production was measured as above (see Fig. 3A legend and 'Materials and methods'). (B) HLMVEC or HEK293 cells were transfected with B1R, iNOS, and G $\alpha$ i Q204L or G $\alpha$ q Q209L and basal Arg-mediated or B1R-activated iNOS-dependent nitric oxide production was measured as above. Data shown are mean values  $\pm$  S.E.M for  $n = 3$ ; \* $P < 0.05$  as compared with L-Arg dependent nitric oxide output. (C) HLMVEC or HEK293 cells were infected with adenovirus containing ct- $\beta$ ARK (ADV- ct- $\beta$ ARK) or empty vector (ADV-EV). HLMVEC were then treated with cytokines as above and HEK293 cells were transfected with iNOS and B1R. Basal Arg-mediated or B1R-activated iNOS-dependent nitric oxide production was measured. Data shown are mean values  $\pm$  S.E.M for  $n = 3$ ; \* $P < 0.05$  compared with nitric oxide output without ct- $\beta$ ARK transfection. (D) HEK293 cells were transfected with iNOS and B1R, or iNOS, B1R and dominant



**Fig. 6** B1R agonist-induced iNOS activation is dependent on Ras, Raf and Erk. (A) HLMVEC were cytokine treated as before and HEK293 cells were transfected with iNOS + B1R. Some cells were transfected with DN-H-Ras or pre-treated with Raf kinase inhibitor (100 nM, 20 min.) or Src family kinase inhibitor PP2 (15  $\mu$ M, 30 min.). In all samples, B1R-dependent nitric oxide release was measured in response to 100 nM DAKD as described (see Fig. 3A legend and 'Materials and methods'). (B) HLMVEC were cytokine treated and HEK293 cells were transfected with iNOS + B1R. Cells were pre-treated with ERK activation inhibitor (10  $\mu$ M, 20 min.) or MEK inhibitor PD98059 (50  $\mu$ M, 30 min.). Basal Arg-dependent or B1R-activated nitric oxide release were measured as above (see Fig. 3A legend and 'Materials and meth-

ods'). (C) Cytokine-treated HLMVEC or HEK293 cells transfected with iNOS + B1R were pre-treated with PI3K inhibitor LY94002 (10  $\mu$ M, 30 min.), PKC activator PMA (1  $\mu$ M, 30 min.), PKC inhibitor calphostin C (1  $\mu$ M, 30 min.) or Akt inhibitor SH6 (25  $\mu$ M, 30 min.) and B1R-dependent nitric oxide release in response to 100 nM DAKD was measured as above. (D) HEK293 cells were transfected with iNOS, B1R, and constitutively active mutants G $\alpha$ i Q204L or G $\alpha$ q Q209L. Some cells were pre-treated with MEK inhibitor PD98059 (50  $\mu$ M, 30 min.) and Arg-dependent nitric oxide release was measured as above. Data shown are mean values  $\pm$  S.E.M for  $n = 3$ . \* $P < 0.05$  as compared with control.

alternate pathways are not involved in B1R-dependent nitric oxide production.

### Constitutively active G $\alpha$ i Q204L signals through the ERK MAPK pathway

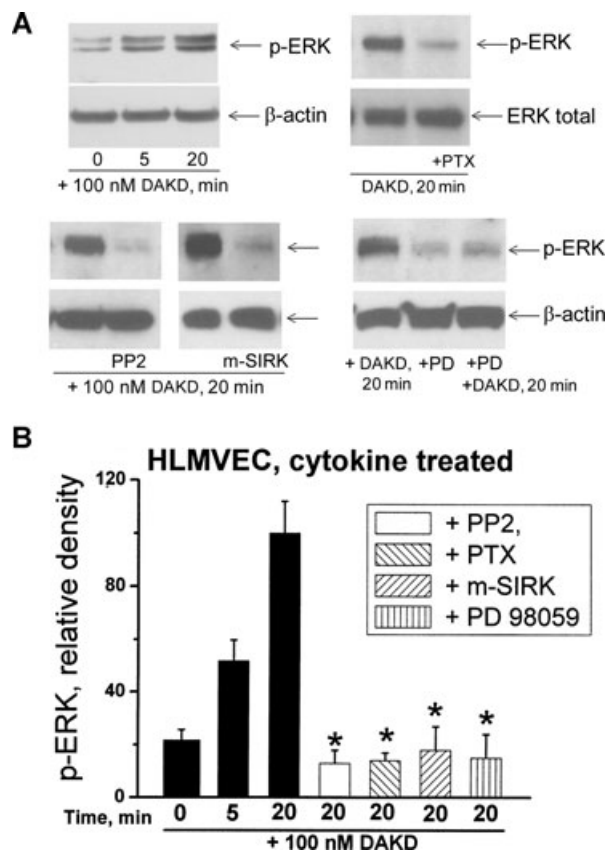
Because cells transfected with constitutively active G $\alpha$ i Q204L, generated high output nitric oxide without the need for B1R activation (Fig. 5), we investigated the role of the ERK MAPK pathway in this response. HLMVEC transfected with constitutively active G $\alpha$ i Q204L were treated with cytokines and incubated in L-Arg-free medium for 2 hrs. As before, L-Arg stimulated high output nitric oxide production in G $\alpha$ i Q204L transfected cells that was much greater (590  $\pm$  10 nM nitric oxide at 20 min.;  $n = 4$ ;  $P < 0.05$ ) than that in non-transfected cells (190  $\pm$  20 nM nitric oxide;  $n = 4$ ) or cells transfected with G $\alpha$ q Q209L (210  $\pm$  15 nM nitric oxide;  $n = 4$ ). Pre-incubation of G $\alpha$ i Q204L transfected cells with MEK inhibitor PD98059 significantly reduced L-Arg-dependent nitric oxide to 150  $\pm$  15 nM nitric oxide ( $n = 4$ ;  $P < 0.05$ ). These

findings suggest that the increase in L-Arg-dependent nitric oxide production in G $\alpha$ i Q204L transfected cells is due to activation of ERK by MEK.

### B1R-mediated ERK activation depends on G $\alpha$ i, Src and MEK

We previously found that ERK-mediated phosphorylation of iNOS is required for B1R-dependent high output nitric oxide production [15]. We investigated whether upstream molecules implicated in this study (*i.e.* G $\alpha$ i, Src and MEK) mediate their effects through ERK. Activation of the B1R in cytokine-treated HLMVEC resulted in a time-dependent increase in phospho-ERK (Fig. 7). B1R-dependent ERK activation was blocked by PTX (Fig. 7), indicating the involvement of G $\alpha$ i. Pre-treatment with the Src family tyrosine kinase inhibitor PP2 and MEK inhibitor PD98059, or pre-stimulation of  $\beta\gamma$  signalling with m-SIRK to occlude the response, also inhibited B1R-mediated ERK activation (Fig. 7).

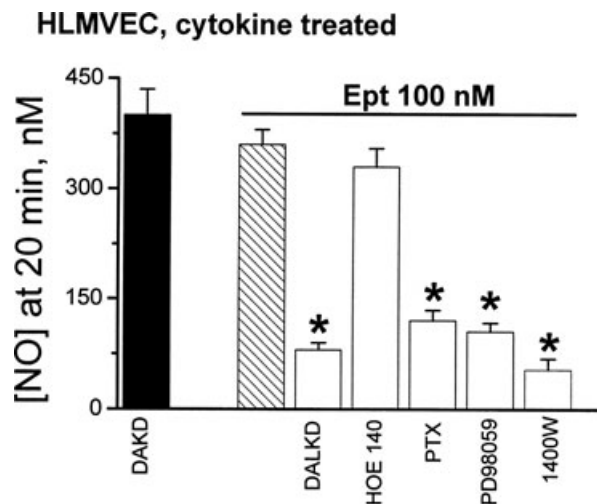




**Fig. 7** B1R-mediated ERK activation depends on  $G_{\alpha i}$ , Src and MEK. (A) Representative Western blots of phospho-ERK (p-ERK) in cytokine-treated HLMVEC which were pre-incubated without or with PP2 (15  $\mu$ M, 30 min.), PTX (1.5  $\mu$ g/ml, 3 hrs), m-SIRK (1  $\mu$ M, 30 min.), or PD98059 (50  $\mu$ M, 30 min.) and stimulated with 100 nM DAKD for the indicated time. Cells were lysed and analyzed by Western blotting with polyclonal antibody to phospho-ERK. Blots were stripped and re-probed with monoclonal antibody to  $\beta$ -actin or total ERK as protein loading controls. (B) Graph shows quantitation of Western blots by densitometry, normalized for protein loading ( $\beta$ -actin or total ERK staining). Bars indicate mean  $\pm$  S.E.M. ( $n = 3$  each). \* $P < 0.05$  versus DAKD treatment alone (20 min.).

### Angiotensin converting enzyme inhibitor generates iNOS-dependent high output nitric oxide through the B1R- $G_{\alpha i}$ -MAPK pathway

ACE inhibitors can act as direct B1R agonists [24] to generate iNOS-derived high output nitric oxide in cytokine-treated HLMVEC [13, 14]. ACE inhibitors also stimulate B1R-dependent high-output nitric oxide production in bovine pulmonary artery endothelial cells although the signalling pathways activated appear to differ from those activated by peptide agonist [13]. In cytokine-treated HLMVEC, 100 nM ACE inhibitor enalaprilat stimulated high output nitric oxide production equivalent to that generated by 100 nM



**Fig. 8** ACE inhibitor generates iNOS-dependent high output nitric oxide through the B1R- $G_{\alpha i}$ -MAPK pathway. HLMVEC were pre-treated with cytokines and pre-incubated with or without B1R antagonist DALKD (1  $\mu$ M, 30 min.), B2R antagonist HOE140 (1  $\mu$ M, 30 min.), PTX (1.75  $\mu$ g/ml, 3 hrs), PD98059 (50  $\mu$ M, 30 min.) or 1400 W (4  $\mu$ M, 30 min.). B1R peptide agonist DAKD (100 nM) or ACE inhibitor enalaprilat (100 nM) were added and nitric oxide production was recorded continuously for 20 min. Data show mean values  $\pm$  S.E.M for  $n = 3$ . \* $P < 0.05$  as compared with enalaprilat alone.

B1R peptide agonist DAKD (Fig. 8). The nitric oxide output was B1R and iNOS-dependent as it was blocked by the specific B1R antagonist DALKD or by iNOS-specific inhibitor 1400 W, but not by B2 receptor antagonist HOE140 (Fig. 8). As with the peptide agonist, the signalling pathway stimulated by ACE inhibitor depended on activation of  $G_{\alpha i}$  and MEK/ERK as it was inhibited by PTX or PD98059 (Fig. 8). Thus, ACE inhibitor induces nitric oxide release in HLMVEC by triggering the same B1R-dependent signalling pathway as B1R peptide ligand DAKD does.

## Discussion

Kinin peptides generated by the kallikrein-kinin system regulate renal and cardiovascular functions by activating two G-protein coupled receptors, named B1 and B2 [25]. B2R signal transduction has been the focus of many studies, but B1R signalling is less well characterized. Both receptors couple to  $G_{\alpha q/11}$  and  $G_{\alpha i/o}$ , but have unique peptide agonists and are regulated differentially at the level of receptor expression and desensitization [19]. The B2R is constitutively expressed in many cell types but expression of the B1R is induced in response to injury or inflammatory mediators [19]. Bradykinin or kallidin released from the kininogen precursor are specific agonists of the B2R but not the B1R, which is instead activated by des-Arg<sup>9</sup>-bradykinin or DAKD, metabolites generated

by carboxypeptidase M and carboxypeptidase N, [26]. We recently showed that the membrane carboxypeptidase M binds to B1Rs on the cell surface to form a signalling complex required for efficient B1R signalling in response to bradykinin or kallidin [27].

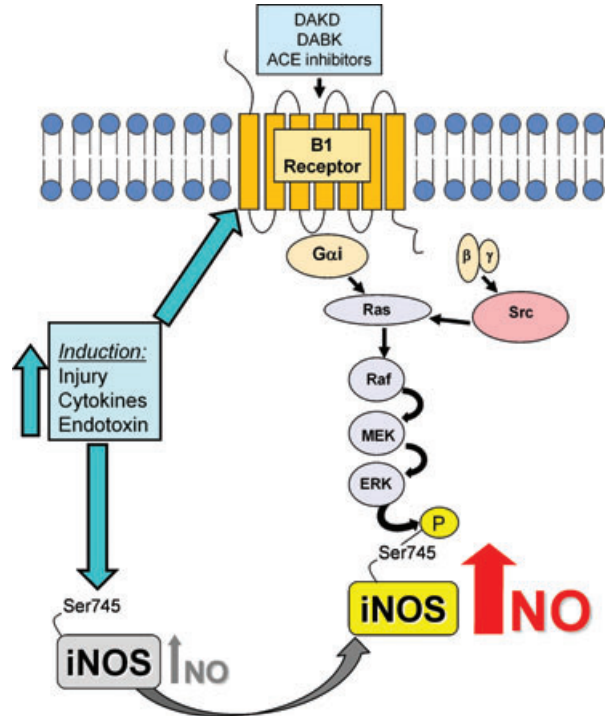
The parallel but distinct B1R and B2R signalling pathways extend to activation of nitric oxide synthases. The B2R activates the constitutively expressed eNOS [28], but we found that the B1R specifically activates iNOS in human endothelial cells [13, 15], whose expression is induced under the same inflammatory conditions as the B1R [5, 19]. In other studies on endothelial cells or transfected HEK cells, we found no evidence for activation of eNOS by B1Rs [15] or activation of iNOS by B2Rs (Zhang Y, Lowry J, Brovkovich V, Skidgel, RA, unpublished).

iNOS was considered to be regulated primarily at the level of expression [1, 4–6], but the present studies showing GPCR signalling can acutely activate iNOS-mediated nitric oxide production reveal a new paradigm for regulation of this NOS isoform. B1R-mediated activation of iNOS results from  $G_{\alpha i}$  and  $G_{\beta\gamma}$ -dependent activation of Src, Ras, Raf, MEK and ERK (Fig. 9). ERK-dependent phosphorylation of iNOS on Ser<sup>745</sup> is responsible for activation of high output nitric oxide as we showed previously [15]. Thus, iNOS activity can be more finely regulated than previously appreciated.

Activation of MAPKs by GPCRs can occur through several pathways, including those coupled through  $G_{\alpha i}$  or  $G_{\alpha q/11}$  [29]. B2Rs couple through  $G_{\alpha q/11}$  and/or  $G_{\alpha i}$  to activate MAPKs *via* several pathways (depending on cell type), but PKC activation is usually required [30]. Effective B2R activation of ERK in transfected HEK cells required the cooperative signalling of both  $G_{\alpha i}$ -mediated Ras activation and  $G_{\alpha q}$ -mediated stimulation of PLC $\beta$ →PKC→Raf [30]. In contrast, our results suggest that B1R activation of ERK is independent of  $G_{\alpha q}$ , Ca<sup>2+</sup> and PKC but instead requires both  $G_{\alpha i}$  and  $\beta\gamma$  (Fig. 9).

Activation of ERK by  $G_{\alpha i}$  is usually mediated through the release of  $\beta\gamma$  subunits and activation of Src, consistent with our results. However, the constitutively active  $G_{\alpha i}$  Q204L mutant stimulated high output nitric oxide in our studies, indicating an additional role for direct ERK activation through the  $G_{\alpha i}$  subunit. Although the mechanism is unknown, it has been reported that  $G_{\alpha i}$  can directly activate Ras or stimulate the ERK MAPK pathway indirectly by activating Rap1GAP1 to relieve Rap1 inhibition of C-Raf or by inhibiting adenylyl cyclase and cAMP formation to reduce PKA-mediated inhibition of C-Raf [29]. GPCR activation of ERK can also occur *via* transactivation of the EGF receptor [29]. However, in other studies we have shown that B1R activation does not transactivate the EGF receptor and that EGF-mediated ERK activation does not lead to activation of iNOS-derived nitric oxide production (Kuhr FK, Zhang Y, Brovkovich V and Skidgel RA, unpublished).

MAPK activation usually results in its translocation to the nucleus to regulate transcription. However, B1R-mediated activation of iNOS would require retention of activated ERK in the cytosol where it can phosphorylate iNOS. GPCR-dependent ERK activation can be mediated by  $\beta$ -arrestin, which scaffolds members of the MAPK pathway to enhance efficiency of activation, resulting in a slower and more sustained activation and retention



**Fig. 9** Schematic diagram of the signalling pathway activated by B1R agonists leading to iNOS-derived high output nitric oxide. Expression of iNOS and the B1R are induced under inflammatory conditions. iNOS has constitutive activity, which can be further enhanced by B1R agonists DABK, DAKD or ACE inhibitors *via* B1R-dependent activation of the ERK/MAP kinase pathway as shown. This results in phosphorylation of iNOS at Ser<sup>745</sup>, leading to generation of high output nitric oxide in endothelial cells.

of active ERK in the cytosol [31]. Consistent with this model, we recently found that sustained, B1R-dependent ERK and iNOS activation is  $\beta$ -arrestin 2 dependent (Kuhr FK, Zhang Y, Brovkovich V, Skidgel RA, unpublished).

B1R and iNOS expression are both induced by inflammatory mediators as is the expression of carboxypeptidase M, the membrane enzyme critical for generating and delivering B1R agonist to the receptor [27]. Thus, the B1R–iNOS pathway may play important roles in inflammation where B1R signalling can have both deleterious and beneficial effects [19, 32, 33]. For example, B1R knockout protected mice from LPS-induced hypotension, reduced pain in response to thermal or chemical stimuli as well as neuropathic pain, and reduced intestinal ischemia/reperfusion injury and lethality [33]. However, B1R signalling is also beneficial, for example in protecting kidneys from ischemia/reperfusion injury [34], promoting vasodilation, angiogenesis and neovascularization during wound healing [33], and reducing lethality in a porcine model of endotoxic shock [35].

The B1R may also participate in the protective effects of ACE inhibitors in heart disease and diabetes [36–38]. One way ACE

inhibitors might do this is by acting as direct agonists of the B1R as reported previously [24, 38]. ACE inhibitor stimulation of B1Rs depends on a consensus HEXXH zinc binding motif in the second extracellular loop that is not required for peptide agonist binding or activation [19, 24, 38]. In bovine pulmonary artery endothelial cells that constitutively express B1Rs, ACE inhibitor enalaprilat and B1R peptide agonist DAKD both stimulate B1R-mediated nitric oxide production *via* eNOS and iNOS, but the signalling pathways differ between the ACE inhibitor and peptide agonist [13]. The present data show that ACE inhibitor activates iNOS-mediated nitric oxide production in cytokine-treated HLMVEC *via* the same signalling pathway found with peptide agonist DAKD. This could reflect species differences or a change in signalling induced by cytokine treatment. We found that B1R-dependent high-output nitric oxide in cytokine-treated HLMVEC inhibited protein kinase C $\epsilon$  (PKC $\epsilon$ ) [14] which could also have beneficial effects on the failing heart [39].

Taken together, our data show that iNOS activity can be regulated post-translationally and dynamically by a GPCR-activated signalling pathway. The reason this went undetected for so long could be due to two factors. First, traditional methods for measuring iNOS activity entail treating cells or animals with agents that up-regulate iNOS expression followed by collection of blood, tissue or cell culture medium after 8–48 hrs and assaying for the nitric oxide metabolites nitrite and nitrate. Distinguishing basal *versus* receptor-dependent nitric oxide production would not be possible under these conditions. In contrast, our measurement of the kinetics of real-time nitric oxide output can easily differentiate these processes. Second, most work on iNOS has been conducted with rodent macrophages or macrophage cell lines where iNOS

expression can be induced to very high levels, leading to a high background basal activity. Thus, under these conditions, receptor stimulation would activate only a small fraction of the total iNOS present (*e.g.* receptor expression could be rate limiting), making it difficult to detect. Indeed, we have detected B1R-mediated activation of iNOS in the mouse macrophage-like cell line, RAW264.7, but the relative increase in overall nitric oxide production is much less than seen in HLMVEC (Brovkovych V, Skidgel, RA, unpublished). In human cells, iNOS is induced to only relatively low levels in response to inflammatory mediators due to epigenetic silencing [40]. Thus, GPCR-dependent activation of iNOS provides a mechanism for generating high output nitric oxide in human cells without the need for high expression levels.

B1R-dependent activation of high output nitric oxide in endothelial cells could play important roles in regulating vascular permeability. Low output nitric oxide generally promotes barrier function whereas high output nitric oxide, especially when combined with oxidants, is damaging and increases permeability. Indeed, a recent study showed that endothelial-specific expression of B1R in transgenic rats markedly enhanced their susceptibility to endotoxic shock [41].

## Acknowledgements

We thank Dr. Tiffany Sharma and Debra Salvi of the Molecular Resources Core for amplifying and preparing plasmids for use in these studies. This work was supported by the National Institutes of Health Grants HL60678, DK 41431 and HL36473.

## References

- Papapetropoulos A, Rudic RD, Sessa WC. Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovasc Res.* 1999; 43: 509–20.
- Dudzinski DM, Igarashi J, Greif D, *et al.* The regulation and pharmacology of endothelial nitric oxide synthases. *Annu Rev Pharmacol Toxicol.* 2006; 46: 235–76.
- Maniatis NA, Brovkovych V, Allen SE, *et al.* Novel mechanism of endothelial nitric oxide synthase activation mediated by caveolae internalization in endothelial cells. *Circ Res.* 2006; 99: 870–7.
- Kone BC, Kunczewicz T, Zhang W, *et al.* Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol.* 2003; 285: F178–90.
- Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem.* 2003; 384: 1343–64.
- Forstermann U, Gath I, Schwarz P, *et al.* Isoforms of nitric oxide synthase. Properties, cellular distribution and expression control. *Biochem Pharmacol.* 1995; 50: 1321–32.
- Mitchell DA, Erwin PA, Michel T, *et al.* S-Nitrosation and regulation of inducible nitric oxide synthase. *Biochemistry.* 2005; 44: 4636–47.
- Yoshida M, Xia Y. Heat shock protein 90 as an endogenous protein enhancer of inducible nitric-oxide synthase. *J Biol Chem.* 2003; 278: 36953–8.
- Kolodziejaska KE, Burns AR, Moore RH, *et al.* Regulation of inducible nitric oxide synthase by aggresome formation. *Proc Natl Acad Sci USA.* 2005; 102: 4854–9.
- Hausel P, Latado H, Courjault-Gautier F, *et al.* Src-mediated phosphorylation regulates subcellular distribution and activity of human inducible nitric oxide synthase. *Oncogene.* 2006; 25: 198–206.
- Tyryshkin A, Gorgun FM, Abdel Fattah E, *et al.* SRC kinase-mediated phosphorylation stabilizes iNOS in normal and in cancer cells. *J Biol Chem.* 2009; doi: 10.1074/jbc.M109.055038.
- Sangsree S, Brovkovych V, Minshall RD, *et al.* Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B1 receptor agonists. *Am J Physiol Heart Circ Physiol.* 2003; 284: H1959–68.
- Ignjatovic T, Stanislavljevic S, Brovkovych V, *et al.* Kinin B1 receptors stimulate nitric oxide production in endothelial cells: signaling pathways activated by angiotensin I-converting enzyme inhibitors and peptide ligands. *Mol Pharmacol.* 2004; 66: 1310–6.

14. **Stanisavljevic S, Ignjatovic T, Deddish PA, et al.** Angiotensin I-converting enzyme inhibitors block protein kinase C(epsilon) by activating bradykinin b1 receptors in human endothelial cells. *J Pharmacol Exp Ther.* 2006; 316: 1153–8.
15. **Zhang Y, Brovkovich V, Brovkovich S, et al.** Dynamic receptor-dependent activation of inducible nitric-oxide synthase by ERK-mediated phosphorylation of Ser745. *J Biol Chem.* 2007; 282: 32453–61.
16. **Marjanovic JA, Stojanovic A, Brovkovich VM, et al.** Signaling-mediated functional activation of inducible nitric oxide synthase and its role in stimulating platelet activation. *J Biol Chem.* 2008; 283: 28827–34.
17. **Shajahan AN, Tiruppathi C, Smrcka AV, et al.** Gβγ activation of Src induces caveolae-mediated endocytosis in endothelial cells. *J Biol Chem.* 2004; 279: 48055–62.
18. **Brovkovich V, Stolarczyk E, Oman J, et al.** Direct electrochemical measurement of nitric oxide in vascular endothelium. *J Pharm Biomed Anal.* 1999; 19: 135–43.
19. **Leeb-Lundberg LM, Marceau F, Muller-Esterl W, et al.** International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev.* 2005; 57: 27–77.
20. **Closs EI, Scheld JS, Sharafi M, et al.** Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol.* 2000; 57: 68–74.
21. **Goubaeva F, Ghosh M, Malik S, et al.** Stimulation of cellular signaling and G protein subunit dissociation by G protein betagamma subunit-binding peptides. *J Biol Chem.* 2003; 278: 19634–41.
22. **van Biesen T, Hawes BE, Luttrell DK, et al.** Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. *Nature.* 1995; 376: 781–4.
23. **Greco S, Storelli C, Marsigliante S.** Protein kinase C (PKC)-delta/-epsilon mediate the PKC/Akt-dependent phosphorylation of extracellular signal-regulated kinases 1 and 2 in MCF-7 cells stimulated by bradykinin. *J Endocrinol.* 2006; 188: 79–89.
24. **Ignjatovic T, Tan F, Brovkovich V, et al.** Novel mode of action of angiotensin I converting enzyme inhibitors: direct activation of bradykinin B1 receptor. *J Biol Chem.* 2002; 277: 16847–52.
25. **Bhoola KD, Figueroa CD, Worthy K.** Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev.* 1992; 44: 1–80.
26. **Skidgel RA, Erdös EG.** Cellular carboxypeptidases. *Immunol Rev.* 1998; 161: 129–41.
27. **Zhang X, Tan F, Zhang Y, et al.** Carboxypeptidase M and kinin B1 receptors interact to facilitate efficient B1 signaling from B2 agonists. *J Biol Chem.* 2008; 283: 7994–8004.
28. **Venema RC.** Post-translational mechanisms of endothelial nitric oxide synthase regulation by bradykinin. *Int Immunopharmacol.* 2002; 2: 1755–62.
29. **Goldsmith ZG, Dhanasekaran DN.** G protein regulation of MAPK networks. *Oncogene.* 2007; 26: 3122–42.
30. **Blaukat A.** Structure and signalling pathways of kinin receptors. *Andrologia.* 2003; 35: 17–23.
31. **Lefkowitz RJ, Shenoy SK.** Transduction of receptor signals by (beta)-arrestins. *Science.* 2005; 308: 512–7.
32. **Campos MM, Leal PC, Yunes RA, et al.** Non-peptide antagonists for kinin B1 receptors: new insights into their therapeutic potential for the management of inflammation and pain. *Trends Pharmacol Sci.* 2006; 27: 646–51.
33. **Pesquero JB, Bader M.** Genetically altered animal models in the kallikrein-kinin system. *Biol Chem.* 2006; 387: 119–26.
34. **Kakoki M, McGarrath RW, Kim HS, et al.** Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury. *Proc Natl Acad Sci USA.* 2007; 104: 7576–81.
35. **Siebeck M, Spannagl E, Schorr M, et al.** Effect of combined B1 and B2 kinin receptor blockade in porcine endotoxin shock. *Immunopharmacology.* 1996; 33: 81–4.
36. **Xu J, Carretero OA, Shesely EG, et al.** The kinin B1 receptor contributes to the cardioprotective effect of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in mice. *Exp Physiol.* 2009; 94: 322–9.
37. **Couture R, Girolami J-P.** Putative roles of kinin receptors in the therapeutic effects of angiotensin 1-converting enzyme inhibitors in diabetes mellitus. *Eur J Pharmacol.* 2004; 500: 467–85.
38. **Skidgel RA, Stanisavljevic S, Erdös EG.** Kinin- and angiotensin-converting enzyme (ACE) inhibitor-mediated nitric oxide production in endothelial cells. *Biol Chem.* 2006; 387: 159–65.
39. **Goldspink PH, Montgomery DE, Walker LA, et al.** Protein kinase Cepsilon overexpression alters myofilament properties and composition during the progression of heart failure. *Circ Res.* 2004; 95: 424–32.
40. **Chan GC, Fish JE, Mawji IA, et al.** Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. *J Immunol.* 2005; 175: 3846–61.
41. **Merino VF, Todiras M, Campos LA, et al.** Increased susceptibility to endotoxic shock in transgenic rats with endothelial overexpression of kinin B(1) receptors. *J Mol Med.* 2008; 86: 791–8.