Cell

Regulation of β-Adrenergic Receptor Signaling by *S*-Nitrosylation of G-Protein-Coupled Receptor Kinase 2

Erin J. Whalen,[1,8] Matthew W. Foster,[1,8] Akio Matsumoto,[1,8] Kentaro Ozawa,[1] Jonathan D. Violin,[1] Loretta G. Que,[1] Chris D. Nelson,[2] Moran Benhar,[1] Janelle R. Keys,[6] Howard A. Rockman,[1,3,4] Walter J. Koch,[6] Yehia Daaka,[7] Robert J. Lefkowitz,[1,2,5,*] and Jonathan S. Stamler[1,2,*]
[1] Department of Medicine
[2] Department of Biochemistry
[3] Department of Cell Biology
[4] Department of Molecular Genetics
[5] Howard Hughes Medical Institute
Duke University Medical Center, Durham, NC 27710, USA
[6] Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107, USA
[7] Department of Pathology, Medical College of Georgia, Augusta, GA 30912, USA
[8] These authors contributed equally to this work.
*Correspondence: lefko001@receptor-biol.duke.edu (R.J.L.), staml001@mc.duke.edu (J.S.S.)
DOI 10.1016/j.cell.2007.02.046

SUMMARY

β-adrenergic receptors (β-ARs), prototypic G-protein-coupled receptors (GPCRs), play a critical role in regulating numerous physiological processes. The GPCR kinases (GRKs) curtail G-protein signaling and target receptors for internalization. Nitric oxide (NO) and/or *S*-nitrosothiols (SNOs) can prevent the loss of β-AR signaling in vivo, but the molecular details are unknown. Here we show in mice that SNOs increase β-AR expression and prevent agonist-stimulated receptor downregulation; and in cells, SNOs decrease GRK2-mediated β-AR phosphorylation and subsequent recruitment of β-arrestin to the receptor, resulting in the attenuation of receptor desensitization and internalization. In both cells and tissues, GRK2 is *S*-nitrosylated by SNOs as well as by NO synthases, and GRK2 *S*-nitrosylation increases following stimulation of multiple GPCRs with agonists. Cys340 of GRK2 is identified as a principal locus of inhibition by *S*-nitrosylation. Our studies thus reveal a central molecular mechanism through which GPCR signaling is regulated.

INTRODUCTION

G-protein-coupled receptors (GPCRs) comprise the largest known family of cell-surface receptors and are fundamentally involved in mammalian physiology (Lefkowitz and Shenoy, 2005; Pierce et al., 2002). This receptor superfamily represents the largest single target for modern drug therapy, and GPCR dysfunction/dysregulation is a major contributor to the pathophysiology of disease. The GRKs play an essential role in GPCR regulation by phosphorylating agonist-occupied receptors and promoting their desensitization, internalization, and downregulation (Lefkowitz and Shenoy, 2005; Pierce et al., 2002). β-ARs are prototypical GPCRs, playing a critical role in the regulation of cardiovascular (Rockman et al., 2002) and pulmonary function (Johnson, 1998) as well as other physiological processes, and the idea has been formalized that β-AR dysfunction may be both a cause of cardiovascular and respiratory impairment and a consequence of agonist therapy (Insel, 1996). In particular, loss of β-AR responsivity is thought to be causally linked to both heart failure (Lefkowitz et al., 2000) and asthma (Johnson, 1998) as well as to the morbidity and mortality associated with therapeutic uses of β agonists (Currie et al., 2006). Multiple lines of evidence suggest that receptor desensitization and downregulation represent important molecular mechanisms contributing to β-AR dysfunction in the clinical setting, and further point to a pathogenic role for GRKs, particularly the GRK2 isotype (a.k.a. β-adrenergic receptor kinase 1; Choi et al., 1997; Hata et al., 2004; Iaccarino et al., 1998; Lefkowitz et al., 2000; Penn et al., 1998; Perrino et al., 2005; White et al., 2000). Thus, there is great interest in elucidating the cellular mechanisms by which GRKs are regulated, including GRK2 in particular.

It is well established that bioactive products of NO synthases, principally nitric oxide (NO) and low-molecular-weight *S*-nitrosothiols (SNOs), regulate a diverse array of signal transduction pathways, acting in significant part through the covalent modification (*S*-nitrosylation) of cysteine residues that are found at active or allosteric sites of proteins (Hess et al., 2005). In a prototypic example of an

NO-signaling module, the activation of neuronal NMDA receptors is coupled to the localized production of NO, which *S*-nitrosylates both the receptor itself, downregulating receptor activity as well as associated regulatory (e.g., NSF) and effector (e.g., ras, dexras, GAPDH, and Parkin) proteins that play roles in trafficking of receptors and in transmitting the cellular signals (Chung et al., 2004; Fang et al., 2000; Hara et al., 2005; Hess et al., 2005; Huang et al., 2005; Jaffrey et al., 2001; Lipton et al., 1993). Although the molecular details are less clear, mounting evidence indicates that signaling through GPCRs might also be regulated by *S*-nitrosylation. Exposure of cells and tissues to nitrosylating agents has been shown to inhibit or potentiate the signaling of multiple GPCRs (Hess et al., 2005; Kokkola et al., 2005), and cysteine residues that confer NO responsivity have been identified in the β_2-AR (Adam et al., 1999) and AT_1 angiotensin receptors (Leclerc et al., 2006). In cultured cells, protein SNOs increase following GPCR activation (Gow et al., 2002), and *S*-nitrosylation that follows stimulation of β-ARs has been shown to modulate both G-protein signaling and receptor internalization: adenylate cyclase (Ostrom et al., 2004), the L-type calcium channel (Sun et al., 2006), and dynamin (Wang et al., 2006) have been identified as loci of NO regulation.

Interestingly, diminished responsiveness (tachyphylaxis) to β_2-AR agonists, as assessed by either vasodilation (Whalen et al., 2000) or airway relaxation (Que et al., 2005), can be reversed by administration of SNOs (Que et al., 2005; Whalen et al., 2000), and mice with a genetic alteration that impairs breakdown of SNOs are protected from tachyphylaxis (Que et al., 2005). Conversely, β_2-AR tachyphylaxis is enabled in vivo by the inhibition of NO synthase (NOS; Whalen et al., 2000), which depletes endogenous SNOs (Gow et al., 2002; Liu et al., 2004). On the basis of these data and the knowledge that asthma and heart failure are in fact characterized not only by losses of β-AR responsivity (Currie et al., 2006; Insel, 1996; Johnson, 1998; Lefkowitz et al., 2000) but also of NO bioactivity (Gaston et al., 2006; Hare and Stamler, 2005), we sought to investigate the molecular mechanism(s) by which NO or SNO might preserve β-AR function.

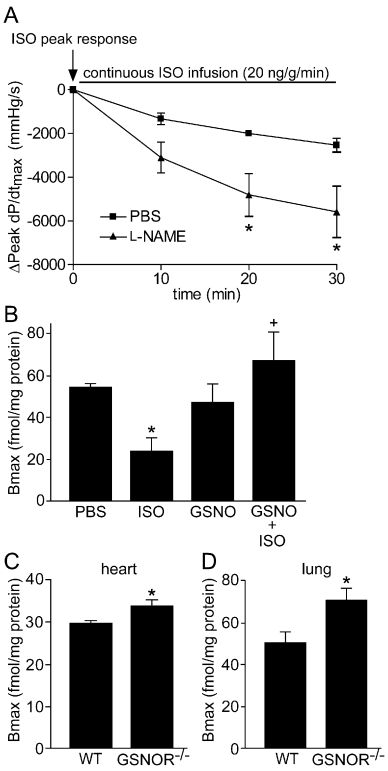

Figure 1. Effect of Endogenous and Exogenous *S*-Nitrosothiols on β-AR Signaling and Expression In Vivo

(A) Decline in ISO-stimulated (20 ng/g/min; continuous infusion) cardiac contractility (dP/dt_{max}) over time in WT mice pretreated with either PBS or L-NAME (50 nmol/g). Data (mean ± SEM) are expressed as change from peak dP/dt_{max} (for absolute dP/dt values see Figure S1). n = 6 for each group, $p < 0.001$ by two-way ANOVA, and *$p < 0.01$ versus PBS.

(B) β-AR density (Bmax; mean ± SEM) was measured (Rockman et al., 1998) in membranes derived from hearts of mice infused continuously with PBS, ISO (30 mg/kg/day), GSNO (10 mg/kg/day), or a combination of ISO and GSNO for 7 days. n = 5, *$p < 0.05$ versus PBS or GSNO-treated mice, and $^{+}p < 0.05$ versus ISO-treated mice.

(C and D) Basal β-AR density (mean ± SEM) in membranes from heart (C) and lungs (D) obtained from WT and GSNO reductase knockout ($GSNOR^{-/-}$) mice. n = 3, and *$p < 0.05$ versus WT.

RESULTS

NO/SNOs Modulate β-AR Function and Homeostasis In Vivo

In light of previous findings showing that NO/SNOs protect against tachyphylaxis to β-agonists in the vasculature (Whalen et al., 2000) and lungs (Que et al., 2005), we sought to determine whether a similar effect could be observed in the heart. Specifically, we examined the effect of NOS inhibition on the inotropic response to sustained high-dose infusions of the β-AR agonist, isoproterenol (ISO), using a previously established model of tachyphylaxis (Matkovich et al., 2006). NOS inhibition with L-N^G-nitroarginine methyl ester (L-NAME; 50 nmol/g) was found to markedly accelerate a decline in contractility during maintained catecholamine (ISO) stimulation (dP/dt_{max}; Figure 1A) without impairing basal contractility or initial ISO-stimulated contractility (ISO-stimulated dP/dt was in fact higher with L-NAME, reflecting an increase in mean arterial pressure; Figure S1; Table S1). NOS-derived NO bioactivity thus preserves β-AR signaling in the heart. As an additional measure of NO/SNO's effect on β-AR responsivity, we examined whether SNOs can modulate β-AR expression in the heart; the β-AR is known to be downregulated by prolonged infusion of ISO (Zhao and Muntz, 1993). Notably, coinfusion of the endogenous SNO, *S*-nitrosoglutathione (GSNO), completely prevented the ISO-stimulated downregulation of the β-AR without affecting basal β-AR expression (Figure 1B). To ascertain whether endogenous SNOs might similarly influence β-AR

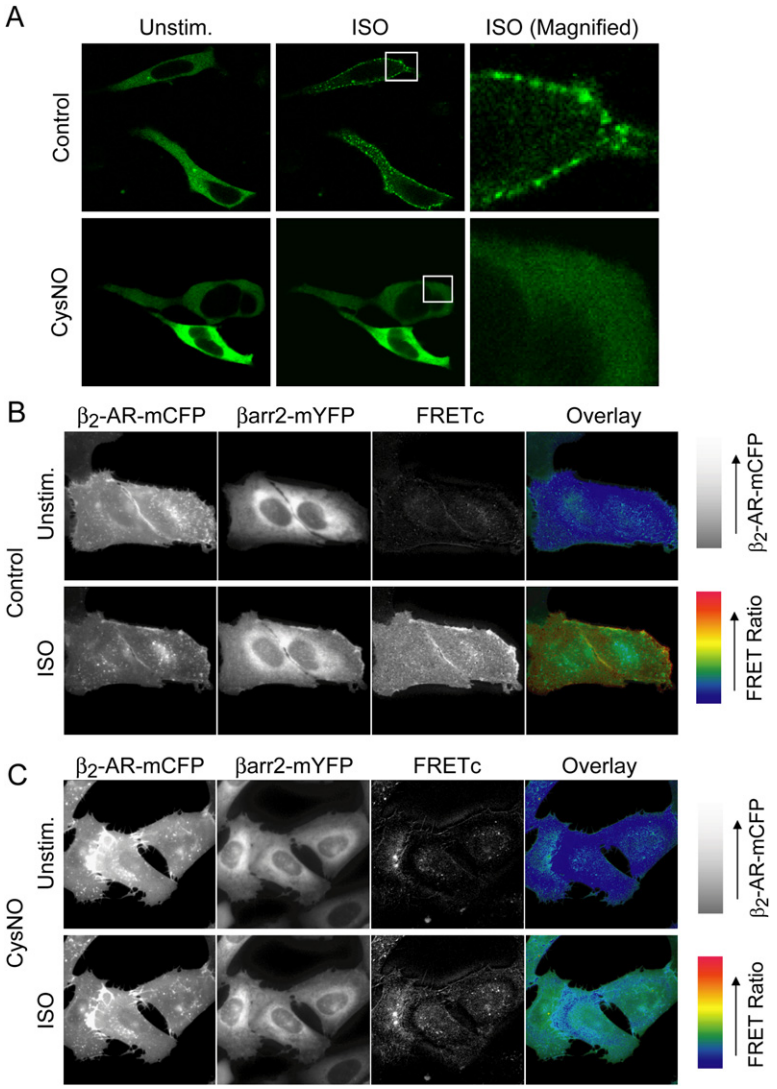

Figure 2. CysNO Inhibits β-Arrestin Association with the β_2-AR

(A) CysNO (10 μM; 5 min pretreatment) markedly decreases ISO-stimulated (10 μM; 10 min) recruitment of β-arrestin2-GFP to the plasma membrane of HEK293 cells (shown as punctae; Barak et al., 1997) transiently overexpressing the β_2-AR. For clarity, magnified confocal image shows punctate pattern of β-arrestin at the membrane.

(B) Agonist-induced interaction between β-arrestin and β_2-AR and subsequent internalization of the receptor in U2-OS cells coexpressing both β_2-AR-mCFP and β-arrestin2-mYFP. β_2-AR is localized to plasmalemma and β-arrestin to cytosol prior to stimulation (top row); under nonstimulated conditions, β_2-AR:β-arrestin do not interact as assayed by FRET corrected for spectral overlap (FRETc). Ten minutes after addition of 1 μM ISO (bottom row), β-arrestin-mYFP has translocated to plasmalemmal punctae, and β_2-AR-mCFP has internalized. Robust β_2-AR:β-arrestin interaction is detected by FRET at the plasma membrane but not in vesicles containing internalized receptor. Data are also displayed as an intensity-modulated pseudocolor display, where intensity corresponds to β_2-AR-mCFP fluorescence, and color ranges from low FRET ratio (blue) to high FRET ratio (red).

(C) Inhibition by CysNO of agonist-induced β-arrestin translocation and β_2-AR:β-arrestin interaction. CysNO (50 μM pretreatment for 10 min followed by washout) visibly reduced ISO-stimulated β-arrestin2-mYFP translocation (punctae on membrane) and β_2-AR-mCFP:β-arrestin2-mYFP (FRETc intensity). Images are representative of four independent experiments.

homeostasis, we measured β-AR surface expression in the hearts and lungs of wild-type (WT) mice as well as in GSNO reductase null ($GSNOR^{-/-}$) mice that are deficient in the breakdown of GSNO (Liu et al., 2004). $GSNOR^{-/-}$ mice exhibited significantly higher β-AR expression in both the heart (Figure 1C) and lungs (Figure 1D) compared to WT mice. These data, in addition to previously published reports (Que et al., 2005; Whalen et al., 2000), demonstrate that both β-AR signaling and cell-surface expression can be regulated in vivo by SNOs.

Effect of SNOs on Recruitment of β-Arrestin to the β-AR

GRK-mediated phosphorylation of the agonist-stimulated receptor constitutes a central locus of control over β-AR signaling and internalization. Receptor phosphorylation promotes the recruitment of β-arrestins to the receptor, where they prevent further activation of heterotrimeric G proteins, thereby desensitizing the receptor while promoting clathrin-mediated receptor endocytosis (Pierce et al., 2002). Desensitization and downregulation of β-ARs in the heart, which is exemplified in Figures 1A and 1B, is in large part mediated by GRK2 (Matkovich et al., 2006; Tilley and Rockman, 2006). Inhibition of β-AR phosphorylation might therefore explain a major part of the effect of SNOs, and it should be noted in this regard that the effects elicited by SNOs (Figure 1) are very similar to those reported for GRK2-ct, an inhibitor of GRK2 (Koch et al., 1995; Rockman et al., 1998).

To begin to explore this possibility, we first expressed GFP-labeled β-arrestin2 in HEK293 cells and examined the effect of *S*-nitrosocysteine (CysNO), a cell-permeable SNO (and endogenous byproduct of GSNO; Lipton et al., 2001), on the ISO-induced recruitment of β-arrestin2 to the β_2-AR, which is highly dependent on GRK activity. As visualized by confocal microscopy (Figure 2A), recruitment of

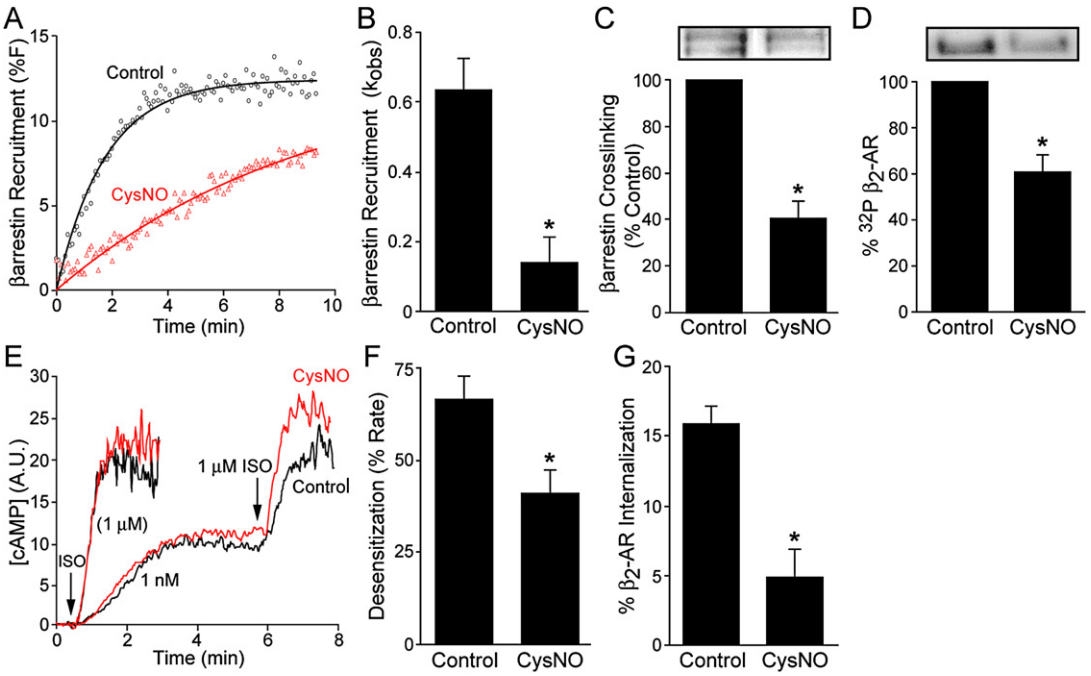

Figure 3. Inhibition by SNOs of GRK-Dependent Functions

(A) Inhibitory effect of CysNO on ISO-stimulated β-arrestin recruitment. U2-OS cells expressing β_2-AR-mCFP and β-arrestin2-mYFP were stimulated with 1 μM ISO (with or without a 10 min pretreatment with 50 μM CysNO followed by washout as in Figures 2B and 2C), and the FRET ratio was fit to a monoexponential curve.

(B) Quantification of β-arrestin binding and its inhibition by CysNO. The rate of ISO-stimulated FRET increase (k_{obs}) was determined from curves fit as in (A), providing an estimation of GRK function (Violin et al., 2006). n = 4, and *p < 0.001 by ANOVA.

(C) CysNO (50 μM; 10 min pretreatment followed by washout) decreases ISO-stimulated (10 μM; 30 s) association of endogenous β-arrestins with stably expressed β_2-AR in U2OS cells, as revealed by immunoprecipitation of crosslinked complexes. n = 3, and *p < 0.05 versus control. Grouping of images from within the same gel is indicated by dividing lines (white).

(D) CysNO inhibits whole-cell receptor phosphorylation. U2-OS cells stably expressing FLAG-β_2-AR were pretreated with either PBS or 50 μM CysNO and stimulated with 10 μM ISO for 2 min. n = 3, and *p < 0.05 versus control.

(E and F) CysNO prevents β_2-AR desensitization. U2-OS cells expressing the cAMP sensor ICUE2 were stimulated with 1 μM ISO with or without a 5 min pretreatment with 1 nM ISO. The reduction in rate of cAMP accumulation resulting from ISO pretreatment is reported as percent desensitization. Addition of CysNO 10 min prior to the experiment (50 μM; followed by washout) inhibited desensitization (i.e., increased cAMP) versus control (*p < 0.02; n = 5). CysNO (red) had no effect on ISO-stimulated (1 μM) cAMP accumulation in the absence of ISO pretreatment.

(G) CysNO inhibits β_2-AR internalization. Internalization was measured by flow cytometry in U2-OS cells stably expressing FLAG-β_2-AR after stimulation with 10 μM ISO for 10 min. Pretreatment of cells with 50 μM CysNO was for 10 min. n = 6, and *p < 0.05 versus control.

Data in (B), (C), (D), (F) and (G) are means ± SEM.

β-arrestin2 was markedly inhibited by pretreatment with CysNO (1–10 μM). Additionally, in U2-OS cells we utilized a fluorescent resonance energy transfer (FRET) assay, which tracks the interaction between CFP-β_2-AR and YFP-β-arrestin2 as the latter is recruited to the plasma membrane following ISO-stimulated, GRK2/3-dependent phosphorylation of the β_2-AR-mCFP (Violin et al., 2006). Quantitation in U2-OS cells of the rate and amplitude of ISO-stimulated β-arrestin association with the β_2-AR may thus provide an estimate, in real time, of the activity of the closely related GRK isoforms GRK2 and GRK3, which stands in opposition to HEK293 cells, where the β_2-AR is phosphorylated primarily by GRK6 (Violin et al., 2006). Notably, CysNO pretreatment (50 μM, followed by washout) produced a marked decrease in the observed FRET signal (FRETc; Figure 2B versus Figure 2C) and, accordingly, in the measured rate (k_{obs}) of ISO-stimulated recruitment of β-arrestin2 to the β_2-AR (Figures 3A and 3B), which is an effect comparable to that recently reported with siRNA for GRK2/3 in these cells (Violin et al., 2006). Confirmation that CysNO inhibits ISO-stimulated recruitment to the receptor of both endogenous β-arrestins (Figure 3C) and YFP-β-arrestin2 (Figure S2) was obtained in crosslinking studies. CysNO, like siRNA for GRK2/3 (Violin et al., 2006), did not affect the maximum amount of β-arrestin recruitment (Figure 3A); these findings are consistent with emerging evidence that the β-AR may be regulated by multiple GRKs and/or with the possibility that β-arrestin binding to the β-AR can be induced by changes in receptor conformation (consequent upon agonist stimulation) that are independent of phosphorylation (Shenoy et al., 2006; Violin et al., 2006).

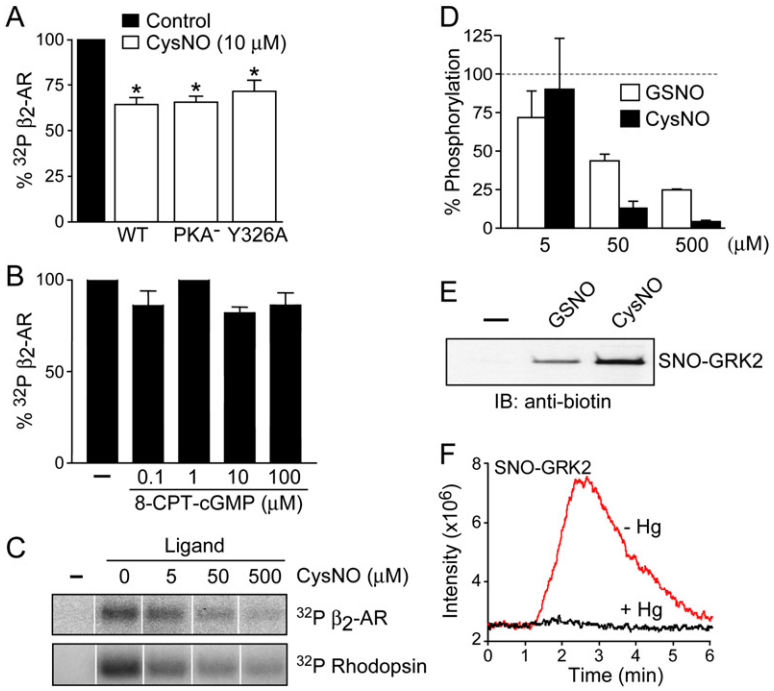

Figure 4. Regulatory *S*-Nitrosylation of GRK2

(A) Inhibition of whole-cell receptor phosphorylation by CysNO. WT, PKA$^-$, and Y326A β_2-AR phosphorylation in HEK293 cells treated with 10 μM CysNO. Mean ± SEM, n = 3. *p < 0.05 versus control.

(B) cGMP has no effect on whole-cell phosphorylation of the WT β_2-AR. HEK293 cells were treated with increasing doses of the membrane-permeable cGMP analog, 8-chlorophenylthio-cGMP (for 10 min) and then stimulated with ISO. Data show mean ± SEM, and n = 3.

(C and D) In vitro phosphorylation of either purified β_2-AR or rhodopsin (C) or peptide substrate (D) by GRK2 is inhibited by *S*-nitrosothiols. Negative controls in (C) are without GRK2. Grouping of images from within the same gel is indicated by dividing lines (white). Data in (C) are representative of three experiments and, in (D), are the mean of two experiments (error bars represent the range of the data).

(E) *S*-nitrosylation of GRK2 by GSNO and CysNO (50 μM each), as measured by biotin switch (representative of two experiments), correlates with the degree of GRK2 inhibition in (D).

(F) *S*-nitrosylation of GRK2 by CysNO (50 μM for 15 min) yields ~1:1 SNO to GRK2 stoichiometry (mean, n = 3) as quantified by photolysis chemiluminescence, where SNO is the difference ± Hg (with reference to GSNO standard).

SNOs Modulate Multiple GRK2-Dependent Processes

In further support of these findings, we determined in U2-OS cells that CysNO pretreatment (followed by washout) decreased cellular responses that are dependent on GRK2 activity: (1) CysNO decreased ISO-stimulated whole-cell phosphorylation of the β_2-AR (Figure 3D); (2) CysNO reduced ISO-stimulated receptor desensitization (Figures 3E and 3F), assayed using a FRET-based cAMP reporter with which desensitization is measured as the extent to which prestimulation with ISO attenuates the rise in cAMP induced by a subsequent ISO challenge (DiPilato et al., 2004; Experimental Procedures). Thus, CysNO enhanced the rate of ISO-stimulated cAMP production in cells that had been prestimulated with ISO (Figure 3E) while having no effect on the initial ISO-stimulated increase in cAMP, recapitulating the effects of siRNA for either GRK2/3 or β-arrestin (data not shown); and (3) CysNO decreased ISO-stimulated receptor internalization (Figure 3G) as quantified by flow cytometry.

S-Nitrosylation Inhibits GRK2-Mediated Phosphorylation

HEK293 cells have long been used as a model system to explore the molecular mechanisms of β-AR regulation (Lefkowitz et al., 2002) and, more recently, to identify the effects of NO on several classes of receptors (Huang et al., 2005; Wang et al., 2006). Consistent with our results in U2-OS cells, preincubation of HEK293 cells with CysNO (10 min) significantly inhibited agonist-induced whole-cell phosphorylation of the β_2-AR (Figure 4A). In addition, CysNO inhibited the phosphorylation of a mutant β_2-AR from which PKA phosphorylation sites had been removed (Figure 4A; Hausdorff et al., 1989), and, in cells overexpressing GRK2, CysNO inhibited the phosphorylation of a Y326A mutant β_2-AR that can only be phosphorylated by overexpressed GRK (Ferguson et al., 1995; Figure 4A). CysNO effects may be either cGMP-dependent or -independent, with the latter mainly being elicited by *S*-nitrosylation (Hess et al., 2005). The cell-permeable cGMP analog, 8-chlorophenylthio-cGMP, had no effect on agonist-induced phosphorylation (Figure 4B). Collectively, these data are consistent with the possibility that CysNO may inhibit GRK2 activity through a cGMP-independent mechanism.

To establish that SNOs can directly inhibit GRK2 by *S*-nitrosylation, we incubated purified recombinant GRK2 with several substrates (β_2-AR, rhodopsin, or synthetic peptide) in the presence or absence of CysNO and assayed for both phosphorylation of substrates and SNO content of GRK2. CysNO caused a dose-dependent inhibition of agonist-induced phosphorylation of both β_2-AR and rhodopsin (Figure 4C), and a direct effect on GRK2 was verified by pretreatment with CysNO (followed by desalting) prior to assay (Figure S3). In addition, GSNO or CysNO inhibited phosphorylation of a synthetic, noncysteinyl peptide substrate (RRREEEEESAAA; Onorato et al., 1991; Figure 4D) that is not reactive to NO. *S*-nitrosylation

of GRK2 was demonstrated in these experiments by biotin-switch assay (Jaffrey et al., 2001), in which the Cys-bound NO (S-NO) is removed with ascorbate and replaced with biotin to enable affinity purification and quantification (see Supplemental Data for a complete description; Figure 4E), and the amount of *S*-nitrosylation was shown to be proportional to the degree of GRK2 inhibition. Precise quantification of *S*-nitrosylated GRK2 (SNO-GRK2), as measured by photolysis chemiluminescence, showed that the SNO to GRK2 stoichiometry was ~1:1 (Figure 4F) under conditions where substrate phosphorylation was inhibited by >80% (Figures 4C and 4D).

Endogenous GRK2 *S*-Nitrosylation

In support of the relevance of these data to the physiological situation, we determined that exposure of HEK293 cells expressing GRK2 to CysNO resulted in the in situ *S*-nitrosylation of GRK2 (vide infra) and that GRK2 was constitutively *S*-nitrosylated in HEK293 cells that stably express either nNOS (HEK-nNOS) or eNOS (HEK-eNOS; Figure 5A), as measured by both biotin switch (Figure 5A) and photolysis-chemiluminescence (not shown). The identification of endogenous SNO in the biotin-switch assay was validated by the elimination of (ascorbate-dependent) signals by UV irradiation applied prior to assay (which cleaves the S-NO bond; Stamler et al., 1992; Figure 5A). Similarly, GRK2 was found to be endogenously *S*-nitrosylated in cultured human umbilical vein endothelial cells (HUVEC), and, moreover, the activation (by ATP, bradykinin, and isoproterenol) of multiple endogenously expressed GPCRs, which are known to be coupled to eNOS in these cells (Ferro et al., 1999; Gosink and Forsberg, 1993), led to an increase in GRK2 *S*-nitrosylation (Figure 5B). Additionally, we analyzed the *S*-nitrosylation state of native GRK2 in mouse tissues: *S*-nitrosylated GRK2 was readily detected at basal conditions in the lungs of WT mice, whereas the levels were significantly depressed in eNOS$^{-/-}$ mice (Figure 5C). Conversely, SNO-GRK2 levels were greater in the lungs of GSNOR$^{-/-}$ mice than in WT mice (Figure 5D); tissue levels of SNO-GRK2 (Figure 5D) thus paralleled changes in basal β-AR expression (Figure 1D).

Mapping the Site(s) of *S*-Nitrosylation in GRK2

We developed several complementary strategies to map the site(s) of *S*-nitrosylation among the 15 cysteines of GRK2. First, SNO-GRK2 was subjected to limited proteolysis followed by the biotin-switch assay, resulting in cleavage of full-length GRK2 into smaller biotinylated fragments (Figure 6A). Sequencing by MALDI-TOF/TOF mass spectrometry showed that the *S*-nitrosylation was mostly identified with a 50 kDa N-terminal fragment, which spanned the catalytic domain and contained 12 cysteines (data not shown). To further pinpoint the sites of *S*-nitrosylation contained within the N-terminal fragment, SNOs in GRK2 were replaced with biotin (biotin switch), and peptides generated by trypsinization were purified by avidin chromatography and analyzed by ESI-MS/MS. Six biotinylated cysteine-containing peptides, corresponding to sites of *S*-nitrosylation, were identified (Figures 6B and 6C), including two Cys within the catalytic domain (Cys340 and Cys439). Although the relative abundance of each peptide could not be determined with this

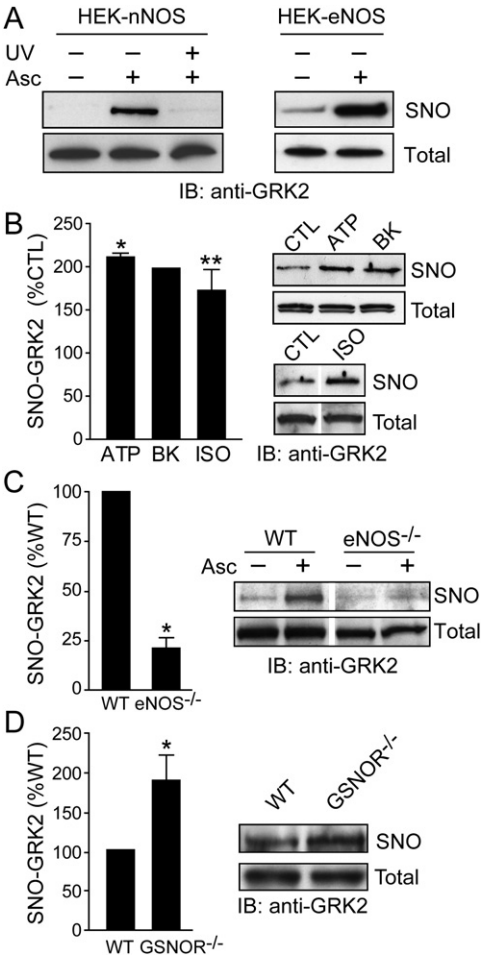

Figure 5. Dynamic *S*-Nitrosylation of GRK2 by Endogenous NO/SNO in Cells and Tissues

(A) GRK2 is constitutively *S*-nitrosylated in both HEK-nNOS and HEK-eNOS cells. SNO signal (ascorbate-dependent biotin labeling) is eliminated by UV pretreatment of cell lysates (data are representative of three experiments).

(B) Dynamic *S*-nitrosylation of endogenous GRK2 in endothelial cells. Endothelial cells were treated with 5 μM ATP, 100 nM bradykinin, or 10 μM ISO for 5 min. ATP: n = 5, *p < 0.001 versus control (CTL); bradykinin: n = 2, average; ISO: n = 3, **p < 0.05 versus control. Representative biotin-switch blots for *S*-nitrosylated GRK2 are shown. Grouping of images from within the same gel here and in (C) is indicated by dividing lines (white).

(C and D) Constitutive *S*-nitrosylation of GRK2 in mouse lungs. SNO-GRK2 in lungs of WT versus eNOS$^{-/-}$ (C) and GSNOR$^{-/-}$ (D) mice was measured by biotin-switch assay; total GRK2 levels are unchanged between groups. n = 3, and *p < 0.05 versus WT. Representative blots show *S*-nitrosylated and total GRK2.

Data in (B), (C), and (D) are mean ± SEM.

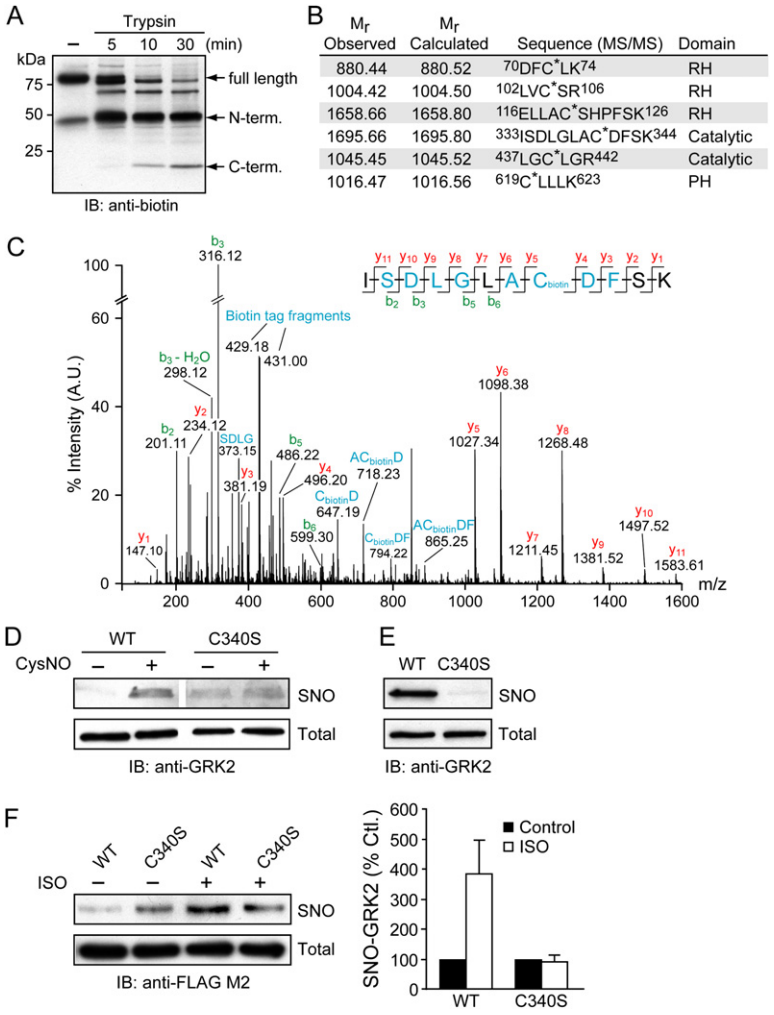

Figure 6. Identification of *S*-Nitrosylation Sites in GRK2

(A) Initial mapping of SNO sites. Limited proteolysis (for the indicated times) of CysNO-treated GRK2 followed by the biotin-switch assay reveals biotin incorporation into N- and C-terminal protein fragments (arrows). Data are representative of three independent experiments.

(B) SNO-containing peptides identified by ESI-MS/MS. Biotinylated cysteines (C*; produced by biotin switch; see Experimental Procedures) represent sites of *S*-nitrosylation. Target cysteines are located within the RH (regulator of G-protein-signaling homology), Catalytic, and PH (pleckstrin homology) domains.

(C) Representative ESI-MS/MS spectrum derived from *S*-nitrosylated GRK2. SNO-GRK2 was subjected to a biotin switch, followed by trypsinization and purification of biotinylated peptides by avidin chromatography. The tryptic peptide containing Cys340 is identified; the peptide sequence is shown in the inset. Labels are as follows: b-ions (green), y-ions (red), and internal acyl fragments (blue).

(D) GRK2 is *S*-nitrosylated at Cys340 by CysNO (50 μM; 5 min). HEK cells were transfected with WT or C340S GRK2. Grouping of images from within the same gel is indicated by a dividing line.

(E) *S*-nitrosylation of GRK2 at Cys340 by endogenously derived NO. HEK-nNOS cells were transfected with WT or C340S GRK2.

(F) ISO-stimulated (10 μM; 5 min) *S*-nitrosylation of GRK2 at Cys340. HUVEC cells were transfected with WT or C340S GRK2 (Data are mean ± SEM).

S-nitrosylation in (D), (E), and (F) was measured by biotin switch. Images are representative of three independent experiments.

approach, quantitative analysis by photolysis chemiluminescence suggested that most NO in fact resided with one or few peptides (SNO:GRK2 ≈1), and it was noted that Cys340 conforms to an acid-base motif (Greco et al., 2006; Stamler et al., 1997) that is a characteristic site of *S*-nitrosylation by GSNO or CysNO (Greco et al., 2006; Hess et al., 2005). Notably in HEK293 cells overexpressing GRK2, mutation of C340 to serine (C340S) greatly attenuated *S*-nitrosylation by exogenous CysNO (Figure 6D), as well as by NO derived from nNOS (Figure 6E). Moreover, in HUVEC cells, robust agonist-dependent *S*-nitrosylation of GRK2 was eliminated by C340 mutation (Figure 6F). While these data do not exclude NO effects on additional Cys, they point strongly to Cys340 as a primary regulatory site.

Identification of Cys340 as a Site of Regulatory *S*-Nitrosylation

We examined the effect of CysNO on phosphorylation of the Y326A β_2-AR by WT C340S or C439S GRK2 proteins expressed in HEK293 cells (Y326A β_2-AR was employed because it can only be phosphorylated by overexpressed GRKs; Figure 7A). Using a β_2-AR antibody specific for GRK phosphorylation sites (pSer355,356; Tran et al., 2004), we determined that CysNO inhibited β_2-AR phosphorylation by either WT or C439S GRK2 but had no effect on receptor phosphorylation by C340S GRK2, thus identifying Cys340 with CysNO responsivity (Figure 7B). Further, the effects of CysNO on β_2-AR phosphorylation by GRK2 (WT and C340S) translated directly to an effect on receptor internalization (Figure 7C): HEK293 cells stably expressing β_2-AR and transiently transfected with WT GRK2 showed a significant decrease in agonist-stimulated β_2-AR internalization after CysNO pretreatment, whereas cells expressing C340S GRK2 actually showed a slight increase in agonist stimulated β_2-AR internalization (Figure 7C). Likewise, in HEK293 cells stably expressing NOS, β_2-AR internalization promoted by WT GRK2 was significantly diminished relative to that by C340S GRK2 (Figure 7D), presumably reflecting the much higher level of endogenously *S*-nitrosylated WT GRK2 versus C340S GRK2 (Figure 6E). Notably, the effects of both exogenous CysNO (Figures

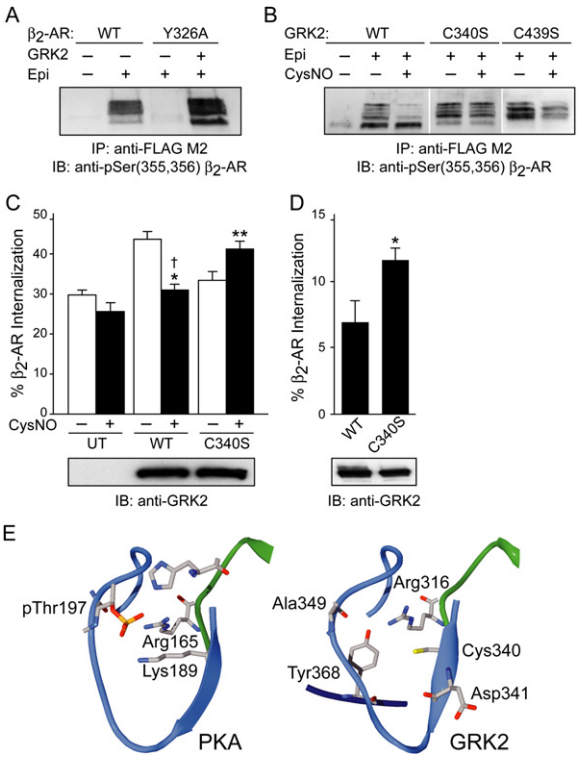

Figure 7. Identification of Regulatory Locus of *S*-Nitrosylation in GRK2

(A) Phosphorylation of the Y326A β_2-AR requires cotransfection of GRK2. HEK cells transfected with Y326A β_2-AR were or were not cotransfected with WT GRK2 and stimulated with agonist (epinephrine). Western blot is representative of three independent experiments.

(B) CysNO inhibits phosphorylation of the β_2-AR by WT and C439S GRK2 but not by C340S GRK2. Agonist-stimulated phosphorylation of the Y326A β_2-AR (at Ser355, Ser356) by WT, C439S GRK2, or C340S GRK2 in the presence or absence of CysNO (50 μM) is shown. β_2-AR phosphorylation was determined in HEK293 cells 20 min after addition of epinephrine (1 μM). Data are representative of three independent experiments. Within gel grouping of images is indicated by dividing lines (white).

(C) Inhibition by CysNO of GRK2-dependent receptor internalization in cells expressing WT GRK2 (WT) but not C340S GRK2 (C340S). Receptor internalization (mean ± SEM) was quantified by flow cytometry in HEK293 cells stably coexpressing FLAG-β_2-AR after stimulation with 10 μM ISO for 20 min (as in C). n = 8, *p < 0.001 CysNO versus control, WT GRK2-transfected cells, and **p < 0.05 CysNO versus control, C340S GRK2-transfected cells; †p < 0.001 WT GRK2 plus CysNO versus C340S GRK2 plus CysNO. The relative expression of WT and C340S GRK2 were equivalent. UT indicates untransfected cells at 20 min post ISO.

(D) Inhibition of GRK2-dependent receptor internalization by endogenous NO is dependent on Cys340. Receptor internalization (mean ± SEM) was quantified in HEK-eNOS cells transiently overexpressing the β_2-AR and either WT or C340S GRK2 after stimulation with 10 μM ISO for 5 min. C340S GRK2 expression was equivalent to WT. n = 12, and *p < 0.001, C340S GRK2 versus WT GRK2.

(E) Activation loops of GRK2 and cyclic AMP-dependent protein kinase (PKA), a prototypic AGC kinase. In PKA (PDB:1CMK), the phosphate of phosphoThr197 (within the activation loop [light blue]) maintains an electrostatic interaction with an Arg165 (of the catalytic loop [green]) that is required for ATP binding and phosphoryltransfer. The catalytic and activation loop structures are preserved in GRK2 (PDB:1OMW), where Arg316 might be stabilized through interactions with Tyr368 and Cys340.

3A, 3E, and 7C) and endogenous NO (Figure 7D) on GRK2-dependent receptor internalization were most readily demonstrated at early time points following ISO stimulation (5–10 min versus 20–30 min), corresponding to the exponential phase of β-arrestin recruitment (Figure 3A) and receptor internalization (Wang et al., 2006), during which inhibition of β-arrestin recruitment by NO is most pronounced (Figure 3A).

DISCUSSION

A Novel Regulatory Locus

The dampening of G-protein signaling and coordinate targeting of receptors for internalization that occurs following their stimulation by agonists is a central aspect of GPCR regulation. In the prototypic case of the β-AR, the uncoupling of receptor from cognate G protein (Gs) suppresses second messenger signaling (cAMP/PKA) and promotes the β-arrestin-dependent recycling of receptors via the trafficking machinery (Lefkowitz and Shenoy, 2005; Pierce et al., 2002). β-arrestin binding to the β-AR is initiated by GRKs, and most of our understanding of these phosphorylation events is based on studies of GRK2. Excessive GRK2 activity can lead to downregulation of receptors that impairs second messenger signaling (Lefkowitz and Shenoy, 2005; Pierce et al., 2002). The question arises as to how GPCR phosphorylation events are regulated to prevent unwanted receptor desensitization and downregulation. Here we establish that agonist-induced phosphorylation of the β-AR by GRK2, as well as the subsequent recruitment of β-arrestin to curtail signaling (desensitization) and promote receptor internalization, are regulated by *S*-nitrosylation. GRK2 *S*-nitrosylation puts a brake on GRK2 activity that potentiates second messenger (cAMP) signaling and mitigates agonist-dependent receptor internalization.

Our data show that Cys340 is a primary target of endogenously derived and exogenously supplied NO or SNO, and that *S*-nitrosylation at this site inhibits GRK2 activity. Inhibitory *S*-nitrosylation of GRK2 provides a potential explanation for prior reports that NO/SNO can facilitate GPCR signaling and prevent tachyphylaxis in vivo (Que et al., 2005; Whalen et al., 2000). It is interesting to note that Cys340 (and surrounding residues that conform to a canonical *S*-nitrosylation motif (Greco et al., 2006; Hess et al., 2005; Stamler et al., 1997), is located on a β-sheet flanking the "activation loop" of the catalytic domain (see Figure 7F); in PKA and other AGC kinases, the loop contains a phospho-Thr residue (not present in GRK2) that is required for kinase activity through an interaction with Arg165 (PKA numbering; Adams et al., 1995). *S*-nitrosylation of Cys340 may thus perturb the structure of the activation loop or orientation of Arg316. Primary sequence analysis indicates that Cys340 is conserved in the

homologous GRK3, but not other GRKs, or related AGC kinases, suggesting that the widely distributed GRKs 2 and 3 are subject to a unique mode of regulation. It is important to note that GRK2 has additional functions, and mass spectrometric and cellular results (Figure 6B and 6F, and data not shown) have indicated that GRK2 *S*-nitrosylation by exogenous NO donors and endogenously generated NO may, under the appropriate conditions, be induced at additional Cys residues. Thus, there is the possibility of as yet undiscovered, distinct effects on GRK activity and function by nitrosylation at these additional sites.

Confluence of NO and GPCR Systems

nNOS, eNOS (and to some degree iNOS) are widely associated with plasma membranes, overlapping the localization of GPCRs (Brenman et al., 1996; Dudzinski et al., 2006; Felley-Bosco et al., 2000; Fulton et al., 2001). NOSs not only bind directly to certain GPCRs (Ju et al., 1998), but also co-segregate with the receptors in signaling modules (Prabhakar et al., 1998) through interactions with caveolin (Dudzinski et al., 2006; Felley-Bosco et al., 2000; Garcia-Cardena et al., 1996), PSD-95 (Brenman et al., 1996; Hess et al., 2005; Hu et al., 2000), and other membrane proteins (Brenman et al., 1996; Cao et al., 2001; Wang et al., 2006). Stimulation of multiple classes of GPCRs, including the β_2-AR, P2Y receptor, cholinergic receptor and bradykinin receptor, induces Ca^{2+} influx and activation of eNOS (Dudzinski et al., 2006; Ferro et al., 1999; Kaiser et al., 2002). In addition, eNOS can be activated downstream of β_2-AR by PKA- and Akt-dependent mechanisms (Ferro et al., 1999). Interestingly, eNOS may associate with GRK2 through a mutual interaction with several elements, including Akt, calmodulin, caveolin and Hsp90 (Hansen et al., 2006; Liu et al., 2005). NO production within the confines of protein complexes allows for the spatiotemporal exactitude of *S*-nitrosylation requisite in signal propagation (Hess et al., 2005; Stamler et al., 2001). However, while multiple signaling elements are known to be *S*-nitrosylated (or denitrosylated) downstream of activated GPCRs, including adenylate cyclase, L-type calcium channels and TRP channels (Hess et al., 2005; Ostrom et al., 2004; Sun et al., 2006; Yoshida et al., 2006), the finding that NO can regulate β-AR phosphorylation provides a unique locus through which *S*-nitrosylation exerts conjoint control over receptor signaling and internalization.

It has been previously reported that NO produced upon β_2-AR stimulation activates the large GTPase dynamin and thereby facilitates receptor internalization (Wang et al., 2006). Opposing effects of *S*-nitrosylation on GRK2-mediated receptor desensitization (−) and dynamin-mediated receptor internalization (+) (Wang et al., 2006), suggest that *S*-nitrosylation of those components is differentially regulated, perhaps as a result of their association with different pools of NOS (unlike the binding of GRK2 with eNOS-associated proteins, dynamin associates directly with eNOS; Wang et al., 2006). Thus NO derived from different populations of NOS may operate in concert to attenuate desensitization and internalization on the one hand while facilitating receptor trafficking on the other. Taken together, these and other findings reveal multiple targets of NO/ SNO, which comprise both constitutively and dynamically *S*-nitrosylated proteins that may serve to control GPCR-regulated functions both under basal conditions and following stimulation by agonists.

Perspective

On the basis of our findings, we suggest that the dynamic *S*-nitrosylation of GRK2, observed in cells and tissues, exerts an inhibitory influence on GPCR phosphorylation that functions to prevent receptor desensitization and downregulation. Diseases such as heart failure and asthma are characterized by both a deficiency of NO bioactivity and dysfunction of β-AR signaling, including downregulation and desensitization of receptors (Currie et al., 2006; Gaston et al., 2006; Hare and Stamler, 2005; Insel, 1996; Johnson, 1998; Lefkowitz et al., 2000), and much evidence points to GRK2 as a culprit (Choi et al., 1997; Hata et al., 2004; Iaccarino et al., 1998; Lefkowitz et al., 2000; Penn et al., 1998; Perrino et al., 2005; White et al., 2000). Further, β-AR desensitization can be recapitulated in healthy tissues by inhibition of NOS, and function is restored in vivo by infusion of SNOs (Whalen et al., 2000; Figure 1), and we now also show that both endogenous and exogenous SNOs can mitigate β-AR desensitization and downregulation. Taken together, these findings support the idea that inhibition of GRK2 by supplementation of SNOs might restore receptor signaling in disease states and thus provide a novel therapeutic modality. More generally, our data identify a novel role for NO in regulation of GPCR function and point to the possibility that deficiencies in NO bioactivity, which characterize many disease states, including hypertension, diabetes, atherosclerosis, cystic fibrosis, neurodegenerative conditions, and aging, may entail dysregulation of GPCR-based signaling.

EXPERIMENTAL PROCEDURES

Detailed Experimental Procedures with References are included in the Supplemental Data. An abbreviated summary is provided.

Hemodynamic Studies

Isoflurane-anesthetized mice underwent cardiac catheterization. A Millar catheter was used to measure hemodynamics, and drugs were infused through the jugular vein.

GSNO Infusion and Radioligand Binding

Mice were implanted with micro-osmotic pumps (Alzet) for the chronic administration of drugs. Heart and lung membranes were prepared, and ^{125}I-cyanopindolol binding was performed.

β-Arrestin Recruitment

HEK293 and U2-osteosarcoma (U2-OS) cells were transfected with either the FLAG-β_2-AR and β-arrestin2-GFP or the β_2-AR-mCFP and β-arrestin2-mYFP, and agonist-stimulated increases in the association of β-arrestin2 with the receptor were measured using confocal

microscopy, FRET, or chemical crosslinking (coimmunoprecipitation) methodologies.

Real-Time cAMP Assay

U2-OS cells were stably transfected with an enhanced version of the cAMP reporter ICUE (DiPilato et al., 2004), which was kindly provided by Jin Zhang. β-AR desensitization was measured using a restimulation protocol.

Phosphorylation Assays

Whole-cell phosphorylation assays were performed using both ^{32}P orthophosphate labeling and an anti-pSer(355,356) β_2-AR antibody. In vitro phosphorylation assays were performed with purified bovine GRK2 and various substrates (purified β_2-AR, rhodopsin, and a synthetic peptide).

Receptor Internalization

Agonist-induced internalization of Flag-β_2-AR was determined by flow cytometry.

Identification of SNO-GRK2

S-nitrosylated GRK2 from mouse lungs, mammalian cells, and sf9 cells was measured using a number of established techniques, including photolysis-chemiluminescence, biotin switch, and mass spectrometry. The sites of GRK2 *S*-nitrosylation were identified by MALDI-TOF/TOF and LC-MS/MS mass spectrometry (coupled with biotin switch).

Statistical Analysis

Cardiac contractility in mice and effects of CysNO treatment on β-arrestin binding to the β_2-AR over time were analyzed by ANOVA (with post hoc Bonferonni). Other comparisons were made with the unpaired Student's t test.

Supplemental Data

Supplemental Data include one table, four figures, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/129/3/511/DC1/.

ACKNOWLEDGMENTS

This work was supported by: P01-HL075443 (Project 1 to H.A.R., Project 2 to W.J.K., and Project 3 to J.S.S. and R.J.L.); an award to J.S.S. from the Sandler Program for Asthma Research; U19-ES012496 (J.S.S.), RO1 HL61690 (W.J.K.), RO1 HL16037 (R.J.L.), and RO1 HL70631 (R.J.L.); and the Howard Hughes Medical Institute (R.J.L.). The authors are grateful to: Karrie Comatas, Lan Mao, and Barbara Williams for technical assistance; Keshava Rajagopal for careful reading of the manuscript; Maria Warren and Carol Parker for help with the acquisition and analysis of ESI-MS/MS spectra; Jin Zhang for kindly providing the ICUE2 plasmid; and Douglas Hess for advice. Dr. Stamler has a financial interest in Nitrox LLC.

Received: September 29, 2006
Revised: January 12, 2007
Accepted: February 26, 2007
Published: May 3, 2007

REFERENCES

Adam, L., Bouvier, M., and Jones, T.L. (1999). Nitric oxide modulates beta(2)-adrenergic receptor palmitoylation and signaling. J. Biol. Chem. *274*, 26337–26343.

Adams, J.A., McGlone, M.L., Gibson, R., and Taylor, S.S. (1995). Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. Biochemistry *34*, 2447–2454.

Barak, L.S., Ferguson, S.S., Zhang, J., and Caron, M.G. (1997). A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J. Biol. Chem. *272*, 27497–27500.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., et al. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell *84*, 757–767.

Cao, S., Yao, J., McCabe, T.J., Yao, Q., Katusic, Z.S., Sessa, W.C., and Shah, V. (2001). Direct interaction between endothelial nitric-oxide synthase and dynamin-2. Implications for nitric-oxide synthase function. J. Biol. Chem. *276*, 14249–14256.

Choi, D.J., Koch, W.J., Hunter, J.J., and Rockman, H.A. (1997). Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase. J. Biol. Chem. *272*, 17223–17229.

Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L., and Dawson, T.M. (2004). S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science *304*, 1328–1331.

Currie, G.P., Lee, D.K., and Lipworth, B.J. (2006). Long-acting beta2-agonists in asthma: not so SMART? Drug Saf. *29*, 647–656.

DiPilato, L.M., Cheng, X., and Zhang, J. (2004). Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. Proc. Natl. Acad. Sci. USA *101*, 16513–16518.

Dudzinski, D.M., Igarashi, J., Greif, D., and Michel, T. (2006). The regulation and pharmacology of endothelial nitric oxide synthase. Annu. Rev. Pharmacol. Toxicol. *46*, 235–276.

Fang, M., Jaffrey, S.R., Sawa, A., Ye, K., Luo, X., and Snyder, S.H. (2000). Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron *28*, 183–193.

Felley-Bosco, E., Bender, F.C., Courjault-Gautier, F., Bron, C., and Quest, A.F. (2000). Caveolin-1 down-regulates inducible nitric oxide synthase via the proteasome pathway in human colon carcinoma cells. Proc. Natl. Acad. Sci. USA *97*, 14334–14339.

Ferguson, S.S., Menard, L., Barak, L.S., Koch, W.J., Colapietro, A.M., and Caron, M.G. (1995). Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1. J. Biol. Chem. *270*, 24782–24789.

Ferro, A., Queen, L.R., Priest, R.M., Xu, B., Ritter, J.M., Poston, L., and Ward, J.P. (1999). Activation of nitric oxide synthase by beta 2-adrenoceptors in human umbilical vein endothelium in vitro. Br. J. Pharmacol. *126*, 1872–1880.

Fulton, D., Gratton, J.P., and Sessa, W.C. (2001). Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? J. Pharmacol. Exp. Ther. *299*, 818–824.

Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J.E., and Sessa, W.C. (1996). Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. Proc. Natl. Acad. Sci. USA *93*, 6448–6453.

Gaston, B., Singel, D., Doctor, A., and Stamler, J.S. (2006). S-Nitrosothiol signaling in respiratory biology. Am. J. Respir. Crit. Care Med. *173*, 1186–1193.

Gosink, E.C., and Forsberg, E.J. (1993). Effects of ATP and bradykinin on endothelial cell Ca2+ homeostasis and formation of cGMP and prostacyclin. Am. J. Physiol. *265*, C1620–C1629.

Gow, A.J., Chen, Q., Hess, D.T., Day, B.J., Ischiropoulos, H., and Stamler, J.S. (2002). Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. J. Biol. Chem. *277*, 9637–9640.

Greco, T.M., Hodara, R., Parastatidis, I., Heijnen, H.F., Dennehy, M.K., Liebler, D.C., and Ischiropoulos, H. (2006). Identification of

S-nitrosylation motifs by site-specific mapping of the S-nitrosocysteine proteome in human vascular smooth muscle cells. Proc. Natl. Acad. Sci. USA *103*, 7420–7425.

Hansen, J.L., Theilade, J., Aplin, M., and Sheikh, S.P. (2006). Role of G-protein-coupled receptor kinase 2 in the heart–do regulatory mechanisms open novel therapeutic perspectives? Trends Cardiovasc. Med. *16*, 169–177.

Hara, M.R., Agrawal, N., Kim, S.F., Cascio, M.B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J.H., Tankou, S.K., Hester, L.D., et al. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat. Cell Biol. *7*, 665–674.

Hare, J.M., and Stamler, J.S. (2005). NO/redox disequilibrium in the failing heart and cardiovascular system. J. Clin. Invest. *115*, 509–517.

Hata, J.A., Williams, M.L., and Koch, W.J. (2004). Genetic manipulation of myocardial beta-adrenergic receptor activation and desensitization. J. Mol. Cell. Cardiol. *37*, 11–21.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G., and Lefkowitz, R.J. (1989). Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitization. J. Biol. Chem. *264*, 12657–12665.

Hess, D.T., Matsumoto, A., Kim, S.O., Marshall, H.E., and Stamler, J.S. (2005). Protein S-nitrosylation: purview and parameters. Nat. Rev. Mol. Cell Biol. *6*, 150–166.

Hu, L.A., Tang, Y., Miller, W.E., Cong, M., Lau, A.G., Lefkowitz, R.J., and Hall, R.A. (2000). beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with N-methyl-D-aspartate receptors. J. Biol. Chem. *275*, 38659–38666.

Huang, Y., Man, H.Y., Sekine-Aizawa, Y., Han, Y., Juluri, K., Luo, H., Cheah, J., Lowenstein, C., Huganir, R.L., and Snyder, S.H. (2005). S-nitrosylation of N-ethylmaleimide sensitive factor mediates surface expression of AMPA receptors. Neuron *46*, 533–540.

Iaccarino, G., Tomhave, E.D., Lefkowitz, R.J., and Koch, W.J. (1998). Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by beta-adrenergic receptor stimulation and blockade. Circulation *98*, 1783–1789.

Insel, P.A. (1996). Seminars in medicine of the Beth Israel Hospital, Boston. Adrenergic receptors–evolving concepts and clinical implications. N. Engl. J. Med. *334*, 580–585.

Jaffrey, S.R., Erdjument-Bromage, H., Ferris, C.D., Tempst, P., and Snyder, S.H. (2001). Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat. Cell Biol. *3*, 193–197.

Johnson, M. (1998). The beta-adrenoceptor. Am. J. Respir. Crit. Care Med. *158*, S146–S153.

Ju, H., Venema, V.J., Marrero, M.B., and Venema, R.C. (1998). Inhibitory interactions of the bradykinin B2 receptor with endothelial nitric-oxide synthase. J. Biol. Chem. *273*, 24025–24029.

Kaiser, R.A., Oxhorn, B.C., Andrews, G., and Buxton, I.L. (2002). Functional compartmentation of endothelial P2Y receptor signaling. Circ. Res. *91*, 292–299.

Koch, W.J., Rockman, H.A., Samama, P., Hamilton, R.A., Bond, R.A., Milano, C.A., and Lefkowitz, R.J. (1995). Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. Science *268*, 1350–1353.

Kokkola, T., Savinainen, J.R., Monkkonen, K.S., Retamal, M.D., and Laitinen, J.T. (2005). S-nitrosothiols modulate G protein-coupled receptor signaling in a reversible and highly receptor-specific manner. BMC Cell Biol. *6*, 21.

Leclerc, P.C., Lanctot, P.M., Auger-Messier, M., Escher, E., Leduc, R., and Guillemette, G. (2006). S-nitrosylation of cysteine 289 of the AT(1) receptor decreases its binding affinity for angiotensin II. Br. J. Pharmacol. *148*, 306–313.

Lefkowitz, R.J., and Shenoy, S.K. (2005). Transduction of receptor signals by beta-arrestins. Science *308*, 512–517.

Lefkowitz, R.J., Rockman, H.A., and Koch, W.J. (2000). Catecholamines, cardiac beta-adrenergic receptors, and heart failure. Circulation *101*, 1634–1637.

Lefkowitz, R.J., Pierce, K.L., and Luttrell, L.M. (2002). Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. Mol. Pharmacol. *62*, 971–974.

Lipton, A.J., Johnson, M.A., Macdonald, T., Lieberman, M.W., Gozal, D., and Gaston, B. (2001). S-nitrosothiols signal the ventilatory response to hypoxia. Nature *413*, 171–174.

Lipton, S.A., Choi, Y.B., Pan, Z.H., Lei, S.Z., Chen, H.S., Sucher, N.J., Loscalzo, J., Singel, D.J., and Stamler, J.S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature *364*, 626–632.

Liu, L., Yan, Y., Zeng, M., Zhang, J., Hanes, M.A., Ahearn, G., McMahon, T.J., Dickfeld, T., Marshall, H.E., Que, L.G., and Stamler, J.S. (2004). Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. Cell *116*, 617–628.

Liu, S., Premont, R.T., Kontos, C.D., Zhu, S., and Rockey, D.C. (2005). A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. Nat. Med. *11*, 952–958.

Matkovich, S.J., Diwan, A., Klanke, J.L., Hammer, D.J., Marreez, Y., Odley, A.M., Brunskill, E.W., Koch, W.J., Schwartz, R.J., and Dorn, G.W., 2nd. (2006). Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. Circ. Res. *99*, 996–1003.

Onorato, J.J., Palczewski, K., Regan, J.W., Caron, M.G., Lefkowitz, R.J., and Benovic, J.L. (1991). Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase. Biochemistry *30*, 5118–5125.

Ostrom, R.S., Bundey, R.A., and Insel, P.A. (2004). Nitric oxide inhibition of adenylyl cyclase type 6 activity is dependent upon lipid rafts and caveolin signaling complexes. J. Biol. Chem. *279*, 19846–19853.

Penn, R.B., Panettieri, R.A., Jr., and Benovic, J.L. (1998). Mechanisms of acute desensitization of the beta2AR-adenylyl cyclase pathway in human airway smooth muscle. Am. J. Respir. Cell Mol. Biol. *19*, 338–348.

Perrino, C., Naga Prasad, S.V., Schroder, J.N., Hata, J.A., Milano, C., and Rockman, H.A. (2005). Restoration of beta-adrenergic receptor signaling and contractile function in heart failure by disruption of the betaARK1/phosphoinositide 3-kinase complex. Circulation *111*, 2579–2587.

Pierce, K.L., Premont, R.T., and Lefkowitz, R.J. (2002). Seven-transmembrane receptors. Nat. Rev. Mol. Cell Biol. *3*, 639–650.

Prabhakar, P., Thatte, H.S., Goetz, R.M., Cho, M.R., Golan, D.E., and Michel, T. (1998). Receptor-regulated translocation of endothelial nitric-oxide synthase. J. Biol. Chem. *273*, 27383–27388.

Que, L.G., Liu, L., Yan, Y., Whitehead, G.S., Gavett, S.H., Schwartz, D.A., and Stamler, J.S. (2005). Protection from experimental asthma by an endogenous bronchodilator. Science *308*, 1618–1621.

Rockman, H.A., Chien, K.R., Choi, D.J., Iaccarino, G., Hunter, J.J., Ross, J., Jr., Lefkowitz, R.J., and Koch, W.J. (1998). Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. Proc. Natl. Acad. Sci. USA *95*, 7000–7005.

Rockman, H.A., Koch, W.J., and Lefkowitz, R.J. (2002). Seven-transmembrane-spanning receptors and heart function. Nature *415*, 206–212.

Shenoy, S.K., Drake, M.T., Nelson, C.D., Houtz, D.A., Xiao, K., Madabushi, S., Reiter, E., Premont, R.T., Lichtarge, O., and Lefkowitz, R.J.

(2006). beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J. Biol. Chem. *281*, 1261–1273.

Stamler, J.S., Jaraki, O., Osborne, J., Simon, D.I., Keaney, J., Vita, J., Singel, D., Valeri, C.R., and Loscalzo, J. (1992). Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. Proc. Natl. Acad. Sci. USA *89*, 7674–7677.

Stamler, J.S., Lamas, S., and Fang, F.C. (2001). Nitrosylation. the prototypic redox-based signaling mechanism. Cell *106*, 675–683.

Stamler, J.S., Toone, E.J., Lipton, S.A., and Sucher, N.J. (1997). (S)NO signals: translocation, regulation, and a consensus motif. Neuron *18*, 691–696.

Sun, J., Picht, E., Ginsburg, K.S., Bers, D.M., Steenbergen, C., and Murphy, E. (2006). Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca2+ channel alpha1 subunit and reduced ischemia/reperfusion injury. Circ. Res. *98*, 403–411.

Tilley, D.G., and Rockman, H.A. (2006). Role of beta-adrenergic receptor signaling and desensitization in heart failure: new concepts and prospects for treatment. Expert Rev. Cardiovasc. Ther. *4*, 417–432.

Tran, T.M., Friedman, J., Qunaibi, E., Baameur, F., Moore, R.H., and Clark, R.B. (2004). Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the beta2-adrenergic receptor using phosphoserine-specific antibodies. Mol. Pharmacol. *65*, 196–206.

Violin, J.D., Ren, X.R., and Lefkowitz, R.J. (2006). G-protein-coupled receptor kinase specificity for beta-arrestin recruitment to the beta2-adrenergic receptor revealed by fluorescence resonance energy transfer. J. Biol. Chem. *281*, 20577–20588.

Wang, G., Moniri, N.H., Ozawa, K., Stamler, J.S., and Daaka, Y. (2006). Nitric oxide regulates endocytosis by S-nitrosylation of dynamin. Proc. Natl. Acad. Sci. USA *103*, 1295–1300.

Whalen, E.J., Johnson, A.K., and Lewis, S.J. (2000). Beta-adrenoceptor dysfunction after inhibition of NO synthesis. Hypertension *36*, 376–382.

White, D.C., Hata, J.A., Shah, A.S., Glower, D.D., Lefkowitz, R.J., and Koch, W.J. (2000). Preservation of myocardial beta-adrenergic receptor signaling delays the development of heart failure after myocardial infarction. Proc. Natl. Acad. Sci. USA *97*, 5428–5433.

Yoshida, T., Inoue, R., Morii, T., Takahashi, N., Yamamoto, S., Hara, Y., Tominaga, M., Shimizu, S., Sato, Y., and Mori, Y. (2006). Nitric oxide activates TRP channels by cysteine S-nitrosylation. Nat. Chem. Biol. *2*, 596–607.

Zhao, M., and Muntz, K.H. (1993). Differential downregulation of beta 2-adrenergic receptors in tissue compartments of rat heart is not altered by sympathetic denervation. Circ. Res. *73*, 943–951.