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Vascular signaling through G protein coupled receptors - new concepts

Masuko Ushio-Fukai, Ph.D.

Department of Pharmacology, Center for Lung and Vascular Biology, Center for Cardiovascular Research, University of Illinois at Chicago, Chicago, IL

Abstract

Purpose of review—G protein-coupled receptor (GPCR) signaling machinery can serve as a direct target of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2) as well as reactive nitrogen species (RNS) including nitric oxide (NO) and S-nitrosothiols (SNOs). NADPH oxidase is one of the major sources of O_2^- produced following GPCR activation in vasculature. NO is generated by three isoforms of NO synthase (NOS). This review will summarize the recent progress on GPCR signaling modulation by NADPH oxidase-derived ROS and NOS-derived SNOs.

Recent findings—ROS and RNS play an important role in GPCR signaling involved in various physiological functions such as cell growth, migration, gene expression as well as pathophysiologicals. NADPH oxidase-derived ROS activate specific redox signaling events involved in cardiovascular diseases. SNOs can modulate GPCR signaling and internalization through S-nitrosylation of the scaffolding protein β -arrestin, the G protein-coupled receptor kinases (GRKs), and dynamin, a GTPase responsible for endocytosis.

Summary—NADPH oxidase-derived ROS and NOS-derived SNOs are now recognized as important second messengers to regulate GPCR signaling, thereby contributing to various biological and pathophysiological functions. Understanding the molecular mechanism of how ROS, NO and SNOs might modulate GPCR signaling is essential for development of novel therapeutic approaches.

Keywords

G protein-coupled receptor; reactive oxygen species; NADPH oxidase; nitric oxide; S-nitrosothiols; redox signaling; S-nitrosylation; vascular smooth muscle cells; endothelial cells

Introduction

G-protein-coupled receptors (GPCRs) are fundamentally involved in mammalian physiology, while GPCR dysfunction/dysregulation is a major contributor to the pathophysiologicals of disease. Reactive oxygen species (ROS), including superoxide (O_2^-) and H_2O_2 as well as reactive nitrogen species (RNS) including nitric oxide (NO) and its biological metabolites S-nitrosothiols (SNOs) are generated following GPCR activation in vascular cells. The major

Address correspondence to: Masuko Ushio-Fukai, Ph.D., Dept. of Pharmacology, Center for Lung and Vascular Biology, Center for Cardiovascular Research, University of Illinois at Chicago, 835 S. Wolcott, M/C868, E403 MSB, Chicago, IL 60612, Phone: 312-996-7665, Fax: 312-996-1225, mfukai@uic.edu.

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source of ROS is a NADPH oxidase in the vasculature, and NO is generated by NO synthase (NOS). This review will summarize the recent information on how ROS and SNOs regulate GPCR signaling and trafficking, which is implicated in vascular biology and pathophysiology.

Role of ROS and NO/SNOs as signaling molecules

GPCRs such as β -adrenergic receptor (β -AR) and angiotensin II type1 receptor (AT_1R), transduce signals through the activation of heterotrimeric G proteins [1-4]. Evidence reveals that ROS and/or NO production is increased following GPCR activation depending on agonist and cell types, and that they function as signaling molecules. Physiological level of ROS produced in response to GPCR agonists plays an important role in normal cell signaling to mediate cell growth, migration and gene expression. Excess amount of ROS produced during hypertension, diabetes, ischemia-reperfusion injury and heart failure contributes to cell death and apoptosis. In contrast, NO and SNOs not only activate cGMP-dependent signaling pathways but also directly modify the SH residues of proteins through S-nitrosylation, which has emerged as an important posttranslational modification regulating NO-mediated signaling. Under physiological condition, NO might provide protection to cells from oxidative stress by S-nitrosylation of some critical protein thiols, preventing them from further oxidative modification by ROS. Increased oxidative stress and the formation of peroxynitrite ($ONOO^-$) via reaction of NO with O_2^- , which can irreversibly damage cells by oxidation of free thiols and nitration of tyrosine residues, are implicated in the pathogenesis of cardiovascular diseases.

Role of ROS in GPCR AT_1 receptor signaling

Angiotensin II (Ang II) is a pleuripotential hormone in vascular smooth muscle cells (VSMCs) and is a potent mediator of vascular hypertrophy, a hallmark of vascular wall remodeling and hypertension. These effects are mediated, in large part, through the GPCR AT_1 receptors (AT_1Rs). Many growth-related outputs of the AT_1R are dependent upon the production of NADPH oxides-derived ROS [5]. The proto-type phagocyte NADPH oxidase consists of membrane-bound gp91^{phox} (now also termed Nox2) and p22^{phox} which comprise the flavocytochrome b_{558} , together with the cytosolic p47^{phox}, p67^{phox} p40^{phox} and the small GTPase Rac. Non-phagocytic NADPH oxidase (Nox) consists of several homologs of Nox2 including Nox1, Nox3, Nox4, and Nox5, as well as the Dual oxidases (Duox) [6-9]. In VSMCs, Nox1 and Nox4 are mainly responsible for ROS generation [10]. Targets of NADPH oxidase-derived ROS include protein tyrosine kinases, protein tyrosine phosphatases (PTPs), protein kinase C, MAP kinases, Ca^{2+} channels, and transcription factors including NF- κ B, activator protein-1, and Nrf2 [11,12].

Activation of AT_1R induces ROS-dependent transactivation of EGF receptor [13], which serves as a scaffold for the assembly of signaling complexes including cSrc and downstream adaptors Shc/GRB2 [14]. Transactivation of EGF receptor is necessary for activation of ERK1/2 and Akt, which are involved in vascular hypertrophy [14]. How ROS mediate Ang II-induced EGFR transactivation? Lipid rafts are cholesterol- and sphingolipid-rich, plasma membrane domains, and 'caveolae' constitute a distinct subset of lipid rafts with cell surface flask-shaped invaginations that contain caveolin-1 (Cav1) [15]. Caveolae/lipid rafts concentrate multiple signaling molecules including GPCRs, receptor tyrosine kinases such as EGF receptor, Src family kinases, and G proteins to form signaling platforms, which is necessary for rapid and efficient activation of downstream signaling pathways [16-18]. Thus, these specialized plasma membrane microdomains play an important role in activation of specific ROS-dependent redox signaling events [19-21]. In VSMCs, NADPH oxidase Nox1 is found in Cav1-containing membranes under unstimulated conditions [22], and Ang II stimulation promotes AT_1R binding to Cav1 as well as trafficking of AT_1R from high density

non-caveolar membrane fractions into caveolae/lipid rafts [23]. This in turn promotes Rac1 translocation into caveolae/lipid rafts to increase localized ROS production [24], which is required for transactivation of EGF receptor [13]. Tyrosine phosphorylated EGF receptor and Cav1 through ROS-dependent cSrc activation subsequently appear at focal adhesions where NADPH oxidase Nox4 and paxillin localize, thereby forming redox signaling platforms. Either inhibition of NADPH oxidase or Cav1 knockdown blocks Ang II-induced increase in vascular hypertrophy [23,25,26]. These findings indicate that caveolae/lipid rafts are signaling domains in which AT₁R couples with NADPH oxidase to increase ROS locally, thereby forming active redox signaling platforms involved in EGFR transactivation and vascular hypertrophy [21] (Figure 1).

Protein tyrosine phosphorylation is controlled by the tightly regulated balance between protein tyrosine kinases and PTPs [27]. The reversible oxidative inhibition of PTPs by ROS is an important mechanism through which ROS increase tyrosine phosphorylation signaling events [27-30]. PTP activity is dependent on the reactive Cys residues (Cys-SH) with a low pKa at their active site that are readily susceptible to reversible oxidation by H₂O₂ [31]. Tabet et al. [32] recently demonstrated that Ang II inactivates SHP-2 PTPs through Nox1-derived ROS-mediated oxidation and phosphorylation. In spontaneously hypertensive rats (SHRs) Ang II-stimulated SHP-2 oxidation/inactivation is enhanced, while its phosphorylation is blunted. These SHP-2 actions are associated with augmented Akt signaling, indicating the importance of Nox1-SHP2-Akt axis for Ang II-mediated hypertension in SHRs (Figure 1). Given that several PTPs including SHP-2 are localized in caveolae/lipid rafts [33], localization of AT₁R, NADPH oxidase and its targets signaling molecules or kinases in caveolae may play an important role in specific activation of ROS-mediated GPCR signaling. Whether this ROS-dependent organized mechanism can apply to other GPCR signal transduction remains unknown.

Role of S-nitrosylation in GPCR signaling

Accumulating evidence indicates that GPCRs signaling is also regulated by S-nitrosylation of Cys residues at active or allosteric sites of proteins via NO and SNOs [34,35]. The activation of neuronal NMDA receptors produces NO, which S-nitrosylates both the receptor itself as well as associated regulatory and effector proteins that play roles in NMDA receptor trafficking and signaling [34,36-39]. Cys residues that are S-nitrosylated by NO have been identified in the β_2 -AR [40] and AT₁R [41]. Other NO-mediated S-nitrosylated proteins include, Akt, caspases, cyclooxygenase-2, eNOS, Hsp90, estrogen receptor, hypoxia-inducible factor-1, NF- κ B [42], adenylate cyclase [43], and calcium channel [44].

Functionally, SNOs treatment prevents tachyphylaxis to β_2 -AR agonists-induced vasodilation [45] or airway relaxation [45,46]. Mice with a genetic alteration that impairs breakdown of SNOs are protected from tachyphylaxis [46]. Conversely, β_2 -AR tachyphylaxis is induced *in vivo* by the inhibition of NOS; [45], which depletes endogenous SNOs [47,48]. Kokkola et al. [35] reported that SNOs such as S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO) potentiate M2/M4 muscarinic responses by increasing guanine nucleotide exchange as well as number of high-affinity [³⁵S]GTP γ S binding sites for the agonist-activated receptor. In contrast, SNOs inhibit P2Y₁₂ receptor signaling in rat brain and human platelets without affecting human P2Y₁₂ receptor signaling under heterologous expression in CHO cells. This indicates that the cellular signaling partners, rather than the P2Y₁₂ receptor protein itself, are molecular target of SNOs. Furthermore, Nozik-Grayck et al. [49] reported that SNOs inhibit GPCRs 5-hydroxytryptamine- or α 1-adrenergic receptor agonist-stimulated pulmonary vasoconstriction via cGMP-independent mechanism involving S-nitrosylation of the receptor or a related protein. Given that the endogenous NOS is located in close proximity with the GPCR signaling complex which is modulated by exogenous SNOs, these findings suggest that

GPCR signaling *in vivo* is likely to be subject to receptor-specific modulation by NO and SNOs.

S-nitrosylation of GRK2, dynamin, and β -arrestin in GPCR β -adrenergic receptor signaling

Transduction through GPCRs is regulated by agonist-induced desensitization, in which receptors are functionally uncoupled from G protein activation, and by internalization, in which receptors undergo endocytosis followed by recycling or degradation [1,2]. The G protein-coupled receptor kinases (GRKs) phosphorylate agonist-occupied GPCRs and promote their desensitization and internalization [3,50]. Receptor phosphorylation promotes the recruitment of the scaffolding protein β -arrestins to the activated receptor, where they prevent further activation of heterotrimeric G proteins, thereby desensitizing the receptor while promoting clathrin-mediated receptor endocytosis [1-3]. NO directly regulates desensitization and internalization of GPCR by modulating the function of GPCR trafficking machinery through S-nitrosylation [34,35,40,41,51,52]. Activation of the β -AR results in dynamic S-nitrosylation of both dynamin [53] [52] and GRK2 [54], which serves as critical components of GPCR trafficking.

Wang et al. [52] reported that the GTPase dynamin, which regulates endocytic vesicle budding from the plasma membrane and interacts with NOS, is S-nitrosylated at a single Cys607 after stimulation of the β_2 -AR. S-nitrosylation increases dynamin self-assembly and GTPase activity and facilitates its redistribution to the membrane. In NO-generating cells, expression of mutant dynamin C607A inhibits β_2 -AR internalization, while NO enhances internalization of β_2 -AR. Thus, NO regulates endocytic vesicle budding by S-nitrosylation of dynamin. Whalen et al. [54] reported how NO and/or SNOs prevent the loss of β -AR signaling *in vivo*. In mice, 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 inhibiting receptor desensitization and internalization. GRK2 S-nitrosylation of Cys340 increases following stimulation of multiple GPCRs with agonists (Figure 2). These findings suggest novel mechanism through which GPCR signaling is regulated by NO and SNOs.

Ozawa et al. [55] recently reported that isoproterenol stimulates the S-nitrosylation of β -arrestin 2 by eNOS-derived SNOs. In human umbilical vein endothelial cells, S-nitrosylation of β -arrestin 2 promotes its dissociation from eNOS and its association with clathrin HC/ β -adaptin, thereby facilitating internalization of the β_2 -AR into the clathrin-based endocytotic pathway, which in turn denitrosylates β -arrestin 2. S-nitrosylation seems to play an important role in regulating the β -arrestin-dependent trafficking of GPCRs including the AT_1R that are functionally coupled to eNOS [56]. S-nitrosylation is the only known facilitatory modification of β -arrestins, because phosphorylation of β -arrestin 1 or 2 inhibits clathrin-mediated β_2 -AR internalization [57-59]. Selective S-nitrosylation of β -arrestin 2 versus β -arrestin 1 provides for differential regulation of these widely distributed isoforms. Of note, stimulus-induced dephosphorylation of Ser412 in β -arrestin 1 is prerequisite for clathrin binding and receptor endocytosis [57,58]. In contrast, β -arrestin 2 does not contain an equivalent C-terminal Ser residue, but has a highly conserved Cys410. The dephosphorylation of Ser412 of β -arrestin 1 promotes clathrin binding, and this effect is recapitulated by S-nitrosylation of Cys410 within β -arrestin 2. Thus, S-nitrosylation of β -arrestin may co-operate with phosphorylation and is important mechanism by which NO influences GPCR function via regulating protein-protein interaction of β -arrestin 2 [52,54](Figure 2).

β -arrestin-regulated β -AR trafficking is directed primarily through clathrin- rather than caveolar-based pathways [1,2]. However, eNOS colocalizes with β -AR primarily in caveolae

through association with the scaffolding protein Cav1. Since eNOS associates with both dynamin and β -arrestin, and indirectly with GRK2 through Akt [52,54,60], S-nitrosylation of these three receptor trafficking elements appear to be mediated by discrete pools of eNOS. Thus, coordinated changes in the S-nitrosylation of multiple, functionally interrelated receptor trafficking machinery subserves dynamic regulation by NO [34]. Whether S-nitrosylation/denitrosylation may influence the class characteristics of GPCRs by altering their affinity for β -arrestin 2, or whether S-nitrosylation differentially regulates signaling dependent upon β -arrestin isoforms [61,62] are subject of future investigation.

Role of ROS and S-nitrosylation in GPCR signaling in vascular pathophysiologies

In VSMCs, production of ROS and the consequent activation of ROS-mediated GPCR signaling are involved in cell growth, migration, collagen deposition, and altered MMP activity [12]. In endothelial cells, NO/SNO-mediated S-nitrosylation of GPCR machinery plays an important role in normal GPCR trafficking and signaling. NO might provide protection to cells from oxidative stress by S-nitrosylation of some critical protein thiols, preventing them from further oxidative modification by ROS. Oxidative stress stimulates activation of transcription factors NF- κ B and AP-1 and pro-inflammatory genes, chemokine production and recruitment of inflammatory cells (monocytes and macrophages), which are involved in vascular inflammation and injury [63]. Further excess amount of ROS impairs endothelium-dependent relaxation, increases GPCR-mediated contractility and alters vascular tone. These effects may be regulated indirectly by reducing NO bioavailability by reaction of O_2^- and NO to produce ONOO $^-$. Thus, excess ROS production and loss of S-nitrosylation will be potential factor in vascular dysfunction that involves altered GPCR trafficking and signaling linked to hypertension, atherosclerosis, heart failure and diabetes.

Conclusion

NADPH oxidase-derived ROS and NOS-derived SNOs are now recognized as important second messengers to regulate GPCR signaling, thereby contributing to various physiological and pathophysiological functions. SNOs and protein S-nitrosylation may serve multiple roles to mitigate oxidation of proteins induced by NADPH oxidase-derived ROS produced following GPCR activation. Dysregulation of GPCR signaling and trafficking due to oxidative stress may contribute to various pathophysiologies in hypertension, atherosclerosis, heart failure and diabetes. Thus, further understand the molecular mechanisms of ROS- and NO-dependent GPCR signaling should provide new therapeutic targets for intervention in cardiovascular diseases.

Acknowledgments

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Figure 1. Caveolae-dependent AT₁R-mediated redox signaling in VSMCs

Ang II stimulation promotes AT₁ receptor trafficking into caveolin1-enriched membrane fractions where Nox1 is found. This causes localized ROS production and ROS-cSrc dependent transactivation of the EGFR and its egress from caveolae. Tyrosine phosphorylated EGFR and caveolin-1 subsequently appear at focal adhesions where Nox4 and paxillin localize, thereby forming redox signaling platforms. These events are essential for activation of specific redox signaling pathways such as Akt involved in vascular hypertrophy. Note that SHP-2 is target of Nox1-derived ROS, which is involved in Akt activation, thereby contributing to AT₁R-mediated hypertension.

Figure 2. Regulation of agonist-induced β_2 -AR trafficking by S-nitrosylation of β -arrestin 2, GRK2 and dynamin

A, β -arrestin2 (β -Arr2) serves as a scaffold to functionally colocalize with eNOS and β -ARs. Ligand (isoproterenol) stimulation results in the activation of eNOS and the S-nitrosylation of β -arrestin2. S-nitrosylation of β -arrestin2 promotes its dissociation from eNOS and its association with clathrin HC/ β -adaptin, which facilitates routing of the β_2 -AR into the clathrin-based endocytotic pathway, and β -arrestin2 is subsequently denitrosylated. **B**, Ligand-coupled inhibition of GRK2 by S-nitrosylation suppresses agonist-stimulated β -AR phosphorylation, β -arrestin recruitment, and receptor desensitization and downregulation. (Reprinted with permission from Ozawa et al. [55])