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Agonist-stimulated reactive oxygen species formation regulates β2-adrenergic receptor signal transduction

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ABSTRACT

Generation of reactive oxygen species (ROS) can occur upon agonist stimulation of surface receptors to modulate downstream signaling processes. Here, we show that activation of the β2 adrenergic receptor (β2AR) by stimulation with the agonist isoproterenol leads to generation of ROS that is required for β2AR signal transduction. Specifically, we show that inhibition of NADPH oxidase with diphenyliodonium chloride, inhibition of the small GTPase Rac1 with NSC23766, and inhibition of formed ROS with the antioxidant N-acetyl-L-cysteine decreases β2AR-mediated cAMP formation, protein kinase A activation, and receptor phosphorylation and internalization, but does not impact ligand binding. The results also show that inhibition of ROS attenuates active β2AR-mediated binding of GTP to α subunits of heterotrimeric G proteins. Based on these results, we propose that agonist-dependent ROS formation is needed for β2AR signal transduction, perhaps through stabilization of active receptor conformers by redox-mediated modification of receptor and/or Gα proteins cysteine residues.

1. Introduction

G protein-coupled receptors (GPCRs) represent the largest and most diverse family of surface receptors, transducing a myriad of intracellular signaling cascades that control, among other things, cell proliferation, survival and migration [1]. Upon agonist ligand binding, GPCRs activate distinct effector enzymes through specific signaling cascades that are dependent upon heterotrimeric GTP-binding proteins (G proteins). β2-Adrenergic receptor (β2AR), the best characterized member of the GPCR family, mediates physiological responses upon binding to epinephrine and norepinephrine by coupling to heterotrimeric G proteins, leading to formation of the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) through activation of adenylyl cyclases [2]. Protein kinase A (PKA) is activated by cAMP, and mediates the cellular response to β2AR agonist ligand binding. Signal termination (i.e. desensitization) of β2AR occurs upon phosphorylation of receptor by members of the G protein-coupled receptor kinase (GRK) family (e.g. GRK2), leading to generation of high affinity binding of phosphorylated receptor and cytosolic β-arrestin proteins [3]. The binding of β-arrestins to receptor not only inhibits the further activation of G proteins, but also initiates events that lead to receptor internalization [4] and 'second wave' signaling [5].

Reactive oxygen species (ROS), which include free radicals, hydroxyl radicals, superoxide anions, as well as non-radicals such as hydrogen peroxide (H_2O_2), can play central roles in transducing intracellular signaling events [6,7]. Oxidant-generating molecules such as H_2O_2 are capable of modifying amino acid residues (e.g. methionine and cysteine), leading to modulation of protein function and

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protein-protein interactions [8]. The intracellular synthesis of ROS occurs primarily through NADPH oxidase system, activation of which requires Rac and Rap [7], members of the Ras superfamily of small G proteins. ROS generation can transpire in response to a variety of stimuli, including peptide growth factors and cytokines [9]. For example, the epidermal growth factor (EGF) receptor has been shown to be involved in acute and rapid production of ROS upon autophosphorylation following ligand binding. Here, ROS production attenuates EGF-mediated activation of ERK MAP kinases, leading to the suggestion that ROS production is an intrinsic signal transduction desensitizer [10]. Distinctly, engagement of B cell receptors with immunoglobulin in lymphoma cells promotes ROS-dependent amplification of the cell signal, leading to the conclusion that ROS is, rather, a signal transducer [11]. In the case of GPCRs, the stimulation of respective receptors with serotonin, acetylcholine, glutamate and angiotensin, for example, has also been linked to formation of ROS, although the signaling consequences that result remain unknown [9]. A study by Marques and Bicho [12] showed that β2AR-mediated signaling was inhibited by sulfhydryl reagents, and the authors concluded that ROS exerts its effects by acting on cysteine residues in the G protein and within the receptor-G protein complex.

Herein, we have examined the role of ROS on the ligand binding and function of β2AR in HEK293 cells. Initial results, consistent with those in the literature [13–15], showed that agonist stimulation of β2AR leads to increased ROS generation. To extend these observations, we tested the hypothesis that ROS effects signaling by the β2AR. Accordingly, we used inhibitors of specific steps involved in the ROS-signaling cascade to assess the effects of ROS inhibition on β2AR signal transduction. Selective pharmacological inhibition of ROS generation using the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), the Rac1 inhibitor NSC23766 (NSC), or the antioxidant *N*-aceytl-L-cysteine (NAC) all led to impairment of agonist-promoted cellular response, including attenuation of β2AR phosphorylation and internalization, as well as reduction in second messenger formation and GTP-γ-^{35}S binding to Gα proteins. These results implicate ROS as key modulators of intracellular β2AR-associated signal transduction processes.

2. Materials and methods

2.1. Cell culture and transfection

For binding and functional studies, human embryonic kidney (HEK293) cells were grown to 90% confluence in 100 mm tissue culture plates, containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM Hepes buffer, and 1.0% penicillin-streptomycin in a humidified atmosphere of air:CO_2 (95:5%) at 37 °C. If required, transient transfection using FLAG epitope-tagged wild-type β2AR cDNA was performed using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer directions, and all experiments were performed 2 days post-transfection. In all cases, treatment with NAC (1 mM), DPI (10 μM), and NSC (1 mM) was performed in serum-free DMEM for 4 h prior to stimulation with agonist, and concentration of each inhibitor is based on that reported in the literature [16,17]. Since there are relatively few reports of NSC in the literature, we conducted experiments at several concentrations below and above those used here to validate that the agent did not affect cell growth or viability within the times of treatment (4–6 h) in our experiments (data not shown).

2.2. β2AR-mediated generation of ROS

To measure intracellular ROS generation, HEK293 cells were cultured on glass coverslips to 50% confluency in serum-free medium. Cells were loaded with 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA) (Molecular Probes, Eugene, OR) for 20 min, followed by stimulation with 10 μM each of isoproterenol (ISO), phenylephrine (PE) or hydrogen peroxide (H_2O_2). Coverslips were rinsed with PBS, and cells were fixed upon addition of 4% paraformaldehyde, allowed to dry then mounted onto glass slides. For antagonist experiments, the appropriate antagonist was added 5 min before agonist addition. For ROS modulators, treatments were made for 4 h prior to addition of DCDHF-DA. DCDHF-DA fluorescence was measured using a Nikon fluorescence microscope (Southern Microscopes, Atlanta, GA) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

2.3. β2AR internalization

Agonist-induced receptor internalization was performed by biotinylation of all surface proteins, followed by specific immunoprecipitation of β2AR, exactly as described [18]. Briefly, HEK293 cells were transiently transfected with FLAG-β2AR, equally seeded into collagen-coated 60 mm dishes, and allowed to grow to 80% confluence. Cells were left untreated or treated with NAC, DPI, or NSC for 4 h, followed by stimulation with ISO for 45 min. Cells were exposed to 0.5 mg/ml sulfo-NHS-biotin (Pierce, City, State) in PBS for 30 min at 4 °C, lysed with extraction buffer (120 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, pH 7.5, 0.1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin and 2 μg/ml PMSF), and subjected to immunoprecipitation with monoclonal anti-FLAG M2 antibody (Sigma–Aldrich, St. Louis, MO) and protein A/plus protein G-conjugated sepharose beads. Immune complexes were fractionated using SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters and subjected to immunoblotting with Vectastain ABC reagent (Vector, Burlingame, CA). Immunoblots were developed by enhanced chemiluminesence (ECL) and band intensities were quantified using Alpha Innotech 2200 imager. Statistical analysis was performed by Student's *t*-test.

2.4. β2AR radioligand binding assays

The effect of ROS modulators on binding of the β-adrenergic antagonist radioligand [^{3}H]-propranolol were assessed using isolated plasma membrane fractions. Briefly, HEK293 cells were treated, or not, with NAC (1 mM), DPI (10 μM) or NSC

(1 mM) for 4 h, washed then scraped into ice-cold lysis buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.4), followed by homogenization with a Dounce homogenizer. Membranes were isolated by sequential centrifugation at 300 × *g* for 3 min and 40,000 × *g* for 30 min. Membrane aliquots (25 μg) were incubated with [^{3}H]-propranolol (ca. 1 nM) and varying concentrations of ISO (0.001–100 μM) or propranolol (0.01–1000 nM) for 1 h at 37 °C. Reactions were terminated by rapid filtration over Whatman GF/C filters followed by washing in ice-cold buffer (lysis buffer supplemented with 0.1% BSA). Filters were counted for radioactivity using liquid scintillation spectrometry (Packard 2250 Liquid Scintillation Counter, Waltham, MA), and results are expressed as the percentage of specific binding. Total binding was routinely represented by ca. 2000–2500 cpm between experiments, while non-specific binding was ca. 100–200 cpm. *F*-test was used to compare significance of the ISO treatment.

2.5. GTP-γ-^{35}S binding

ISO-stimulated binding of GTP-γ-^{35}S to heterotrimeric Gα proteins was measured as previously described [19]. Briefly, HEK293 cells were treated for 4 h, or not, with the ROS modulators and agonist in serum free medium, and washed in ice-cold PBS. Cells were scraped, homogenized and centrifuged at 40,000 × *g* for 10 min at 4 °C, followed by resuspension in ice-cold storage buffer (20 mM Hepes buffer, pH 7.4, 0.1 mM EDTA). Membranes were aliquoted at 1 mg/ml (as measured by Bradford assay) and frozen at −80 °C until used. The binding reaction consisted of membranes (80 μg) in 20 mM Hepes buffer, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 3 μM GDP and 100 pM GTP-γ-^{35}S (Perkin-Elmer Life Sciences, Boston, MA) in the presence of water (basal), 100 mM cold GTP-γ-S (Sigma–Aldrich), or ISO for 30 min at 30 °C. The reactions were terminated by rapid filtration over glass fiber filters (GF/B) using a 48-well harvester (Brandel, Gaithersburg, MD). Filters were allowed to dry overnight and counted for ^{35}S content by liquid scintillation spectrometry. Results are expressed as a percentage of maximal GTP-γ-^{35}S binding ± S.E.M. produced by ISO (10 μM) for at least two independent experiments performed in triplicate.

2.6. β2AR-mediated cAMP accumulation

For determination of intracellular cAMP content, confluent HEK293 cells in 12-well plates were treated for 4 h, or not, with ROS modulators followed by preincubation for 15 min with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) at 37 °C. Cells were then stimulated with ISO (10 μM) for 10 min, and reactions were terminated by aspirating the medium, followed by addition of ice-cold HCl (0.1 M). Intracellular cAMP content was assessed using the Assay Designs (Ann Arbor, MI) Direct cAMP formation assay, according to the manufacturer directions. Results were normalized to protein content, expressed as a percent of the maximal ISO (10 μM) induced value, and are shown as the mean ± S.E.M. for at least three independent experiments performed in triplicate. One-way ANOVA was used to assess significance between the curves.

2.7. α_{1A} AR-mediated inositol phosphate formation

Formation of [^{3}H]-inositol phosphate was measured using HEK293 cells transiently expressing α_{1A}AR (Guthrie cDNA Resource Center, Sayre, PA). Cells were equally divided into 12-well plates and grown overnight, followed by preincubation with [^{3}H]-myo-inositol in inositol-free DMEM (Perkin-Elmer, Boston, MA). Following 4 h treatment with ROS modulators, increasing concentrations of phenylephrine were added in triplicate to 12-well plates in the presence of LiCl (50 mM) and incubated at 37 °C for 45 min. Cell contents were liberated by addition of formic acid (50 mM), followed by neutralization and then separation on individual AG1-X8 200–400 formate resin anion exchange columns. Columns were washed with ammonium formate (50 mM), and ammonium formate/formic acid (1.2 M/0.1 M) was used to elute total [^{3}H]-inositol phosphates directly into scintillation vials for counting of tritium content. Results are normalized, and presented as a percent of the maximal PE (10 μM) induced value, and are shown as the mean ± S.E.M. for at least three independent experiments performed in triplicate. One-way ANOVA was used to assess significance between the curves.

2.8. Agonist-stimulated phosphorylation of vasodilator-stimulated phosphoprotein (VASP)

HEK293 monolayers expressing FLAG-VASP in 60 mm dishes were pretreated, or not, with ROS modulators for 4 h followed by triplicate washes with PBS. Cells were lysed on ice with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM Na_2HPO_4, pH 7.4) for 15 min, followed by centrifugation of the lysates at 14,000 × *g* for 15 min. Supernatants were assessed for protein concentration and equal amount of protein was loaded and resolved by SDS-PAGE (10% gels). Following transfer to nitrocellulose membranes, membranes were immunoblotted with anti-phospho-VASP monoclonal antibody (Upstate, Chicago, IL). Following addition of HPR-conjugated anti-mouse secondary antibody, bands were visualized by ECL. Membranes were stripped of immunoglobulins and reprobed with anti-FLAG M2 antibody (Sigma–Aldrich) to assess equivalent expression of FLAG-VASP. Band intensities were quantified using Alpha Innotech 2200 imager. Statistical analysis was performed by Student's *t*-test.

2.9. Agonist-stimulated phosphorylation of β2AR

HEK293 monolayers expressing FLAG-β2AR in 6-well plates were pretreated, or not, with ROS modulators, for 4 h followed by washing in phosphate-free DMEM (2 ml/well). Cell labeling was performed by incubation of 0.2 mCi [^{32}Pi] in 0.5 ml phosphate-free DMEM for 45 min at 37 °C, followed by stimulation of cells with ISO (10 μM) for 10 min. Reactions were terminated upon transfer of plates onto ice and addition of ice-cold PBS. Cells were lysed on ice with RIPA buffer for 15 min, followed by centrifugation of the lysates at 14,000 × *g* for 15 min. Supernatants were assessed for protein concentration and equal amount of protein from each sample were pre-cleared using 30 μl protein A-sepharose beads for 1 h. Supernatants were subjected to β2AR immunoprecipitation

using 50 μl protein A/plus protein G-conjugated sepharose beads and 7.5 μg monoclonal anti-FLAG M2 antibody (Sigma–Aldrich). Immune complexes were washed three times with RIPA buffer, denatured in Laemmli sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (10%). Gels were dried and subjected to autoradiography. Incorporation of ^{32}P was expressed as a percentage of band intensity, compared to unstimulated control cells, and band intensities were quantified using Phosphorimager. Statistical analysis was performed by Student's *t*-test.

3. Results

3.1. *ISO mediates ROS generation through activation of β2AR*

Intracellular ROS generation following stimulation of endogenous β2AR was determined using the redox-sensitive fluorescent probe DCDHF-DA. Upon loading of cells with DCDHF-DA, acetate groups are cleaved by intracellular esterases, trapping the probe within the cell. Oxidation of intracellular DCDHF causes fluorescence of the dichlorofluorescein byproduct. Similar to previous reports [13–15], our data show that activation of endogenous β2AR by ISO significantly increases intracellular generation of ROS, causing a high level of fluorescence associated with cells, in comparison to unstimulated cells (Fig. 1A and B), and the fluorescence signal was similar to that induced by 10 μM H_2O_2 (not shown). Treatment of cells with the β-AR antagonist propranolol obliterated the ISO-mediated ROS formation (Fig. 1C), demonstrating the β2AR-specific ROS production. We utilized pharmacologic inhibitors of various stages of the ROS production machinery to assess β2AR downstream signaling events. The antioxidant cysteine protector *N*-acetyl-L-cysteine (NAC) was used to scavenge formed ROS species. Concentrations of NAC in assays was at or below the concentrations used by similar investigations [20,21]. Diphenyleneiodonium

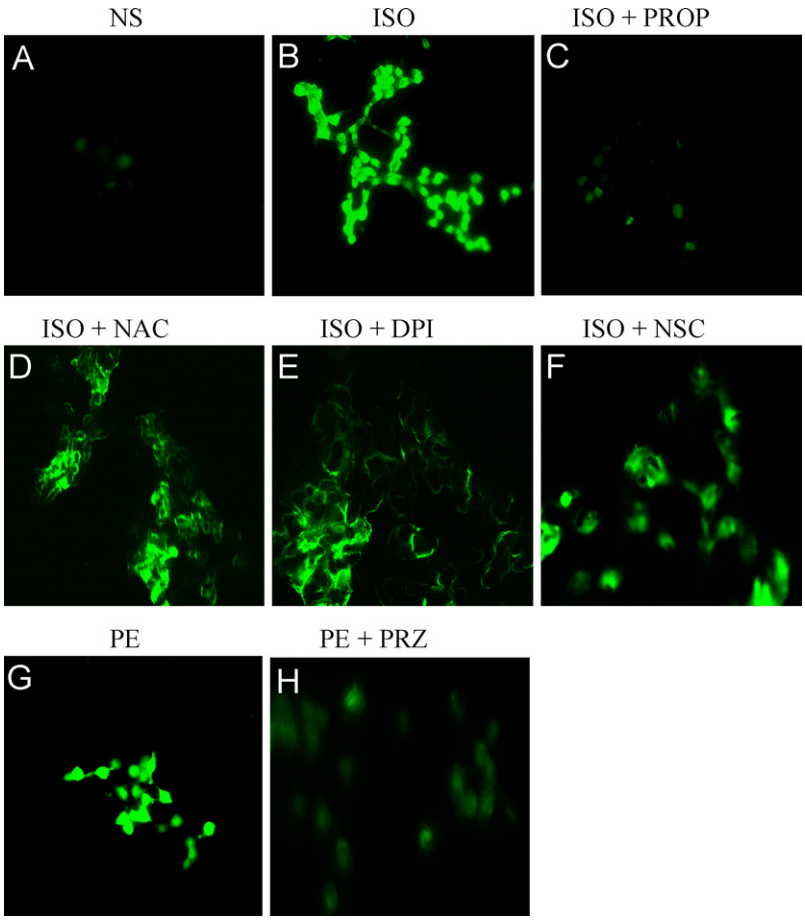

Fig. 1 – Agonist-dependent generation of ROS. Wild type HEK293 cells were loaded with the ROS-sensitive fluorescent probe DCDHF-DA (10 μM) for 20 min, washed and treated with ISO (B–F) or PE (G) for 10 min, or left non-stimulated [NS, (A)]. For drug treatment, cells were exposed to 1 mM NAC (D), 10 μM DPI (E) or 1 mM NSC (F) for 4 h prior to stimulation with ISO (10 μM) for 10 min. For antagonist experiments, cells were pretreated with β2AR antagonist (−)-propranolol (C) or α_{1A}AR antagonist prazosin (H) for 5 min prior to stimulation with ISO or PE for 10 min, respectively. Following treatment, cells were rinsed with PBS, fixed with 4% paraformaldehyde and analyzed by fluorescence microscopy with excitation wavelength of 480 nm and emission wavelength of 520 nm. Treatment with ISO (and PE) markedly increased fluorescence signal in virtually all cells examined (B and G), while pretreatment with NAC, DPI, and NSC markedly decreased ROS generation (D–F), with the fluorescence signal limited to outer membranes of cells. The results are representative of three separate experiments.

chloride (DPI), a selective flavin-containing oxidase inhibitor, was used to block NADPH oxidase, as described previously by others. NSC23766 (NSC) was used as a specific inhibitor of the small GTPase Rac1 [22], a component of the NADPH oxidase system. Following pretreatment of HEK293 cells with appropriate concentrations (based on literature surveys) of NAC, DPI, or NSC, DCDHF fluorescence was markedly decreased, and indeed, the noted fluorescence seen with the inhibitors was limited to the outer cellular areas denoting restricted ROS formation near the plasma membrane (Fig. 1D–F), and suggesting that endogenous β2AR-mediated ROS generation is attenuated by pharmacological inhibition of the NADPH complex. Next, we assessed possible generality of the GPCR-mediated ROS formation by evaluating ROS generation in HEK293 cells ectopically expressing α_{1A}AR. Our results, show that the α-AR agonist phenylephrine, stimulates ROS generation (Fig. 1G), and its effects were blocked by the α-AR antagonist prazosin (Fig. 1H), demonstrating ROS generation occurs through α_{1A}AR.

3.2. *Effects of ROS inhibition on β2AR internalization*

β2AR-mediated ROS formation involves Rac function (Fig. 1F) that is involved in actin remodeling and vesicle trafficking from the plasma membrane [23]. We tested the hypothesis that β2AR-mediated generation of ROS regulates receptor endocytosis. To assess the effects of agonist on cell surface receptor expression, we labeled FLAG-β2AR with sulfo-NHS-biotin and measured fraction of internalized biotinylated receptors, as described [18]. Stimulation of β2AR with ISO was consistent with appearance of an approximately 65 kDa protein band that represented biotinylated intracellular β2AR. ISO stimulation promoted 30–40% of the β2AR to internalize (Fig. 2A), consistent with previous reports [4,24]. Treatment of cells with NAC, DPI, or NSC prior to stimulation with ISO caused a significant reduction in β2AR internalization ($p < 0.05$ versus ISO) (Fig. 2A). These data suggest that reduction in ROS availability can impair agonist-regulated receptor internalization, and that similar to RTKs [25,26], ROS may be required for β2AR downregulation.

3.3. *Effects of ROS inhibition on β2AR phosphorylation*

Reversible phosphorylation of the β2AR is critical for ligand-controlled signaling events that regulate receptor internalization and recycling. To determine effects of ROS on β2AR phosphorylation content, we performed metabolic labeling studies using HEK293 cells pretreated, or not, with NAC, DPI, or NSC. Unstimulated cells were used as a normalized marker for basal β2AR phosphorylation, and cells stimulated with ISO showed a 2.5-fold increase in ^{32}P incorporation ($p < 0.001$, Fig. 2B). Significantly, stimulation of cells pretreated with NAC, DPI, or NSC with ISO showed marked attenuation of ^{32}P incorporation ($p < 0.01$ versus ISO for all), and pretreatment with the β2AR antagonist propranolol fully inhibited the ^{32}P incorporation (Fig. 2B). Although a mechanistic explanation pertaining to the differences in intrinsic inhibitory efficacies of NAC, DPI and NSC remains elusive, these data clearly indicate that ROS play prominent role in agonist-induced phosphorylation and internalization of β2AR, and further suggest that

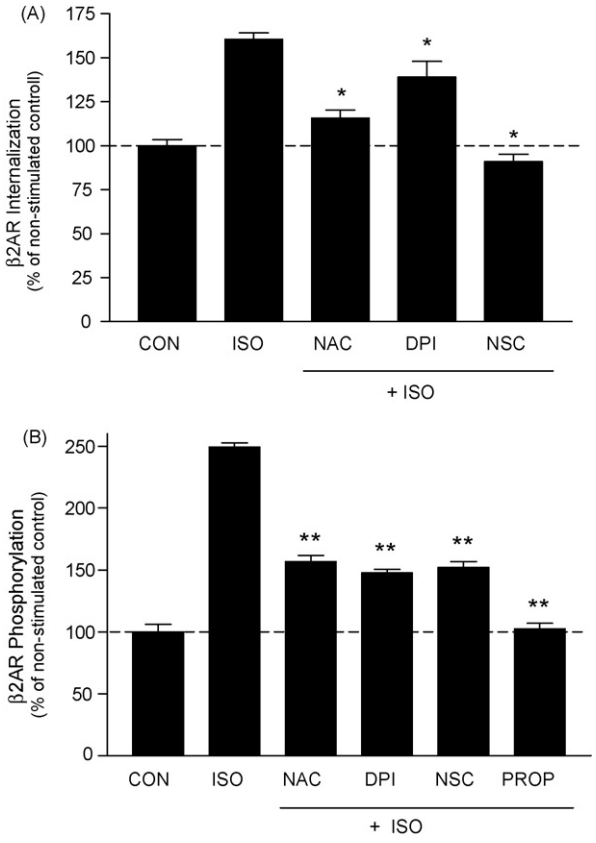

Fig. 2 – ROS regulates β2AR surface expression and phosphorylation. (A) ISO-mediated internalization of β2AR is inhibited by NAC, DPI, and NSC. HEK293 cells transiently expressing FLAG-β2AR were treated, or not, with NAC, DPI or NSC for 4 h, followed by stimulation with ISO for 45 min. Remaining surface β2ARs were biotinylated, isolated by immunoprecipitation and visualized by immunoblotting, as described. Band intensities were quantified and data are presented as percent change in biotinylated β2AR band intensity, compared to unstimulated samples (arbitrarily assigned 100%). Data shown are representative of three experiments, and quantification shows that NAC, DPI, and NSC impaired the ISO-stimulated β2AR internalization by 74, 36, and 100%, respectively. $^{*}p < 0.05$ vs. ISO alone-stimulated samples. (B) ROS regulates the ISO-stimulated phosphorylation of β2AR. Cells were pretreated with NAC, DPI, or NSC, for 4 h, loaded with ^{32}P and stimulated with ISO, as described. Receptor phosphorylation was assessed by β2AR immunoprecipitation followed by autoradiography. Band intensities of phosphorylated β2AR were quantified and data are presented as a percentage of band intensity, compared to unstimulated samples (arbitrarily assigned 100%). Quantification shows that NAC, DPI, and NSC impaired the ISO-stimulated β2AR phosphorylation by 62, 68, and 65%, respectively, while propranolol fully blocked (100% inhibition) the ISO-stimulated effect. $^{**}p < 0.01$ vs. ISO alone-stimulated samples.

ROS may be involved in regulation of cell-surface GPCR expression.

3.4. *Effects of ROS inhibition on second messenger formation*

We examined whether inhibition of ROS formation effected endogenous β2AR signal transduction processes, upstream of receptor internalization and phosphorylation. Hence, we determined the effects of NAC, DPI, and NSC on β2AR-mediated cAMP accumulation and subsequent activation of the cAMP-dependent protein kinase (PKA). Fig. 3A shows that in HEK293 cells, ISO stimulates cAMP accumulation in a dose-dependent manner with a pEC_{50} of -6.7 ± 0.23 and E_{max} of ca. 1000% above control at ISO concentration of 10 μM (ca. normalized to 100% of ISO response). Treatment with NAC had no significant effect on the EC_{50} of the ISO response ($pEC_{50} = -6.6 \pm 0.5$), but significantly decreased the E_{max} of the ISO response (70% of ISO-stimulated response at 10 μM; $p < 0.01$ versus ISO alone). Similarly, treatment with DPI or NSC had no effect on EC_{50} of the ISO response ($pEC_{50} = -6.6 \pm 0.3$ and -6.1 ± 0.5, respectively), but markedly reduced the E_{max} of the ISO response (21% and 25% of ISO-stimulated response at 10 μM, respectively; $p < 0.001$ versus ISO alone). These results show that inhibition of ROS leads to decreases in β2AR-mediated cAMP accumulation, and suggest that ROS generation may be involved in stabilizing conformations of the receptor required for ligand binding or G protein coupling.

Subsequent to its formation, cAMP binds to and activates PKA, leading to phosphorylation of downstream protein targets to manifest cellular response to agonist stimulation. Vasodilator-stimulated phosphoprotein (VASP) is a PKA substrate [27], and we used VASP phosphorylation by PKA as a measure to further document the role of ROS in ISO-regulated cellular response. Pretreatment of cells with NAC, DPI, or NSC caused marked reduction in phospho-VASP, but had no effect on total VASP levels (Fig. 3B). Quantitation of the band intensities revealed that NAC, DPI, and NSC treatment reduced VASP phosphorylation by 45 ± 11, 53 ± 11, and 67 ± 15%, respectively, versus ROS modulator-untreated cells ($p < 0.01$ for all). Treatment with propranolol, used as a positive control to inhibit β2AR signal, fully abolished VASP

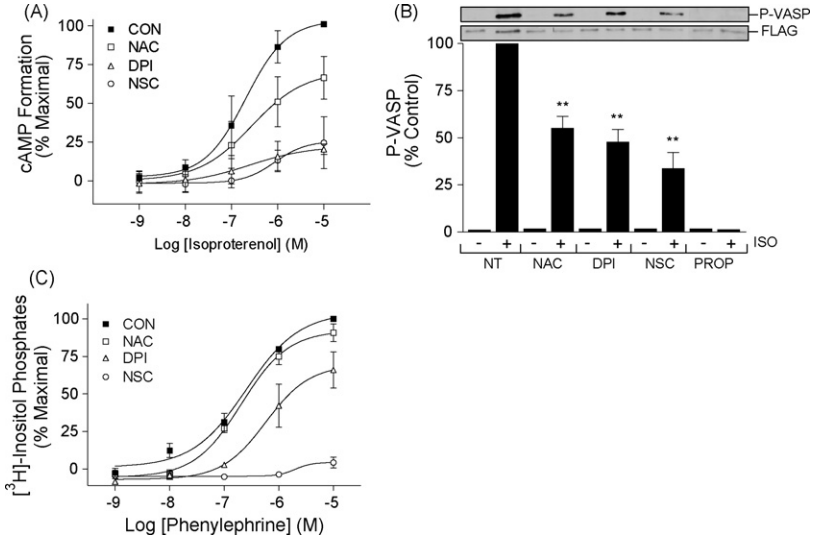

Fig. 3 – Effect of ROS on second messenger generation. (A) Inhibition of ROS attenuates ISO-stimulated cAMP formation. Wild type HEK293 cells were pretreated with 1 mM NAC, 10 μM DPI, or 1 mM NSC for 4 h, and stimulated with increasing concentrations of ISO, or left unstimulated, in the presence of 1 mM IBMX. Cyclic AMP formation was quantified, as described, and data are expressed as a percentage of the maximal (at 10 μM) ISO response in ROS modifier-untreated cells. Treatment with NAC, DPI or NSC had no effect ($p > 0.05$) on EC_{50} of the ISO dose–response curve, but significantly inhibited the E_{max} of the response by 32, 78, and 75%, respectively. $^{**}p < 0.01$ for all curves vs. untreated samples. (B) Inhibition of ROS attenuates ISO-stimulated phosphorylation of the PKA substrate VASP. Cells were not-treated (NT) or pretreated with 1 mM NAC, 10 μM DPI, or 1 mM NSC for 4 h in 60 mm dishes, and stimulated (+), or not (−), with ISO (1 μM) for additional 15 min. VASP phosphorylation was assessed by immunoblotting with anti-phospho-VASP antibody. Membranes were stripped of immunoglobulins and re-probed with anti-FLAG antibody to assess equivalent FLAG-β2AR expression between the lanes. Band intensities were quantified and data are expressed as percentage of the maximal stimulation seen with ISO alone-treated cells. NAC, DPI and NSC inhibited the ISO-mediated phosphorylated VASP, in comparison to untreated samples, by 45, 53, and 67%, respectively. $^{**}p < 0.01$. (C) Inhibition of ROS attenuates PE-stimulated inositol phosphate formation. Cells transiently expressing the α_{1A}AR were incubated overnight with the PLC substrate [^{3}H]-*myo*-inositol and treated, or not, with NAC (1 mM), DPI (10 μM), or NSC (1 mM) for 4 h. Cells were stimulated with increasing concentration of PE for 45 min, and total inositol phosphates were quantified by ion-exchange chromatography, as described. Data are expressed as a percentage of the maximal (at 10 μM) PE alone response. ROS modifier treatment had no effect on EC_{50} of the PE dose–response curve, but significantly inhibited the E_{max} of the response by 12, 36, and 96%, respectively. $^{*}p < 0.05$ for DPI and NSC vs. untreated samples.

phosphorylation (Fig. 3B). These results confirm that ROS generation is critical in endogenous β2AR signal transduction processes.

We wished to determine whether ROS-dependent downstream signaling seen here was limited to the β2AR, or if other adrenergic receptors have similar properties. Accordingly, we examined ROS generation and second messenger formation in response to α_{1A}AR activation. The α_{1A}AR, like the β2AR, is a member of the monoamine subclass of rhodopsin-like GPCRs, but is the farthest removed, phylogenetically, from the β2AR in this class. Stimulation of HEK293 cells transiently expressing α_{1A}AR with phenylephrine leads to ROS accumulation (Fig. 1G and H), as well as a dose-dependent formation of intracellular inositol phosphates ($pEC_{50} = -6.6 \pm 0.1$) with an E_{max} of approximately 900% above control (Fig. 3C). This response was right-shifted in the presence of the α_{1A}AR competitive antagonist prazosin (100 nM), validating the α_{1A}AR-mediated mechanism (data not shown). Treatment with NAC had no effect on the EC_{50} of the response (-6.6 ± 0.1), but modestly (although significantly) decreased the E_{max} (90% of PE-stimulated response at 10 μM; $p < 0.05$). Similar to the results seen with ISO and β2AR-mediated cAMP accumulation (Fig. 3A), treatment with DPI had no significant effect on EC_{50} of the PE-mediated formation of inositol phosphates ($pEC_{50} = -6.2 \pm 0.3$), but markedly reduced the E_{max} of the PE response (69% of PE-stimulated response at 10 μM; $p < 0.001$ versus PE alone) (Fig. 3C). Further, treatment with NSC completely abolished the dose response curve yielding an indeterminate EC_{50}, and decreasing the E_{max} to only 5% of the PE response at 10 μM (Fig. 3C). Taken together, these data suggest that ROS-dependent signal transduction is conserved, at least amongst the adrenergic subfamily of rhodopsin-like GPCRs.

3.5. *Effects of ROS inhibition on ligand binding*

To begin to identify locus of ROS action in the β2AR signaling cascade, we performed competition binding experiments to monitor displacement of the high affinity β2AR antagonist radioligand [^{3}H]-propranolol in HEK293 cells in the absence, or presence, of NAC, DPI, or NSC. Displacement of [^{3}H]-propranolol by ISO was unaffected by treatment with NAC, DPI, or NSC (Fig. 4A–C). Specifically, the EC_{50} of the ISO-mediated displacement (pEC_{50} -5.7 ± 0.1) was not significantly altered upon treatment with NAC (Fig. 4A), DPI (Fig. 4B), or NSC (Fig. 4C): pEC_{50} being -5.8 ± 0.1, -5.5 ± 0.2, and -5.7 ± 0.01, respectively. Furthermore, displacement with ISO exhibited a shallow Hill slope ($n_H = 0.76 \pm 0.03$) typical of agonist binding to a GPCR, in accordance with the ternary complex model [28]. Treatment with NAC, DPI, or NSC had no significant effect on the slope of the ISO displacement curves, yielding Hill slopes of 0.73 ± 0.1, 0.81 ± 0.03, and 0.64 ± 0.2, respectively. We also assessed the displacement of [^{3}H]-propranolol with "cold" propranolol to delineate any differences ROS inhibition would have in preferential coupling of GPCRs. The EC_{50} of the propranolol-mediated displacement ($pEC_{50} = -8.5 \pm 0.1$) was not significantly altered upon treatment with NAC (Fig. 4D), DPI (Fig. 4E), or NSC (Fig. 4F): pEC_{50} being -8.7 ± 0.1, -8.4 ± 0.1, and -8.6 ± 0.1, respectively. Furthermore, displacement with propranolol exhibited a steep

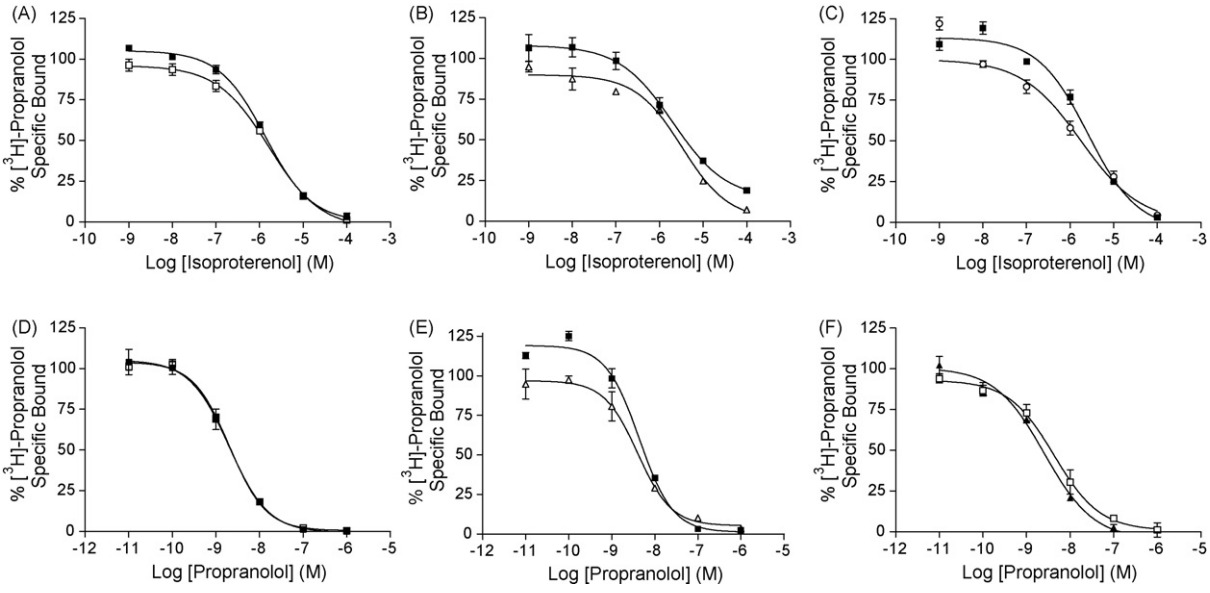

Fig. 4 – Effect of ROS on ligand binding to β2AR. Competition binding curves of ISO vs. [^{3}H]-propranolol (A–C) and propranolol vs. [^{3}H]-propranolol (D–F) are not significantly affected by treatment with NAC (A and D), DPI (B and E) or NSC (C and F). Cells were treated with NAC (1 mM), DPI (10 μM), or NSC (1 mM) for 4 h and membranes were prepared by differential centrifugation. Competition binding experiments were performed using varying concentrations of ISO (0.001–100 μM), or propranolol (0.01–1000 nM), for 1 h at 37 °C to displace [^{3}H]-propranolol (ca. 1 nM). Reactions were terminated after 60 min incubations by rapid filtration over glass fiber filters (GF/B) and trapped radiation was assessed by scintillation counting. Data are expressed as the percentage of specific-bound [^{3}H]-propranolol. Maximal displacements, EC_{50} and Hill slopes for curves of ROS modifier-treated samples (open squares) were not significantly different from untreated curves (filled squares).

Hill slope ($n_H = 0.96 \pm 0.02$) typical of antagonist binding to pre-coupled or un-coupled GPCRs. Treatment with NAC, DPI, or NSC had no significant effect on the slope of these displacement curves, yielding Hill slopes of 1.03 ± 0.02, 1.11 ± 0.14, 0.8 ± 0.15, respectively. Results were fitted to one and two-site binding models with no significant differences in EC50 or Hill slopes. These data suggest that ROS generation is not involved in stabilizing conformations or alterations of the ligand binding domain of the β2AR.

3.6. *Effects of ROS inhibition on agonist-stimulated GTP-γ-^{35}S binding*

Our results show that ROS inhibition diminishes second messenger formation and subsequent effector activation, but does not effect receptor ligand binding. Therefore, we hypothesized that the effects of ROS may lie at the interphase of receptor and G protein coupling. We assessed the effects of ROS inhibition on ISO-mediated binding of GTP-γ-^{35}S to β2AR-coupled heterotrimeric G proteins. Results in Fig. 5 show that the binding of GTP-γ-^{35}S is increased in a dose-dependent manner upon stimulation with ISO (maximal ISO-mediated stimulation was ~150–175% of basal), compared to untreated cells ($pEC_{50} = -5.98 \pm 0.32$). Remarkably, we could not detect any significant ISO-stimulated GTP-γ-^{35}S signal, over basal, in cells pretreated with either NAC, DPI or NSC. Furthermore, treatment of cells with inhibitors had no statistically significant effect on basal GTP-γ-^{35}S binding (data not shown), suggesting that ROS may not be involved in agonist-independent (constitutive) receptor activation. These data also suggest that ROS may be involved in maintaining β2AR-G protein coupling conformations suitable to activate the G protein and to drive downstream signal transduction processes.

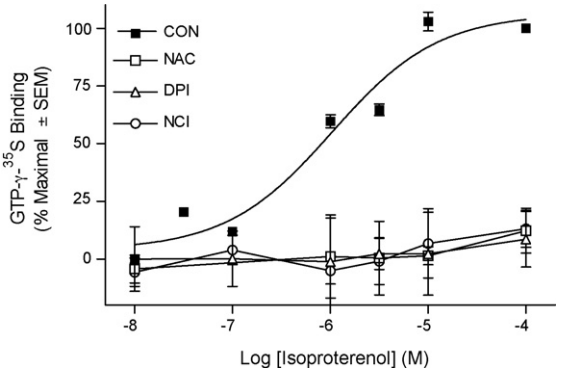

Fig. 5 – Effect of ROS on ISO-stimulated binding of GTP-γ-^{35}S to HEK293 cell membranes. Cells were treated with NAC (1 mM), DPI (10 μM), or NSC (1 mM) for 4 h, and membranes were prepared by differential centrifugation. Reactions were initiated upon addition of membranes (80 μg) to reaction buffer containing the indicated concentration of ISO and 100 pM of GTP-γ-^{35}S. Reactions were terminated after 30 min by rapid filtration over glass fiber filters (GF/B), and trapped radiation assessed by scintillation counting. Data are expressed as a percentage of the maximal (at 100 μM) ISO response in untreated cells. ISO produced a does-dependent response with an EC_{50} of 1.05 ± 0.32 μM. In the ROS-modulator treated cells, there was no significant binding of GTP-γ-^{35}S seen over basal.

4. Discussion

Engagement of surface receptors leads to activation of multiple signaling cascades that collectively determine the cellular response. It is becoming evident that ROS play an integral part to dictate the overall cellular response to external stimuli [9–11,29]. Indeed, agonist binding to certain tyrosine kinase [30,31] and G protein-coupled [32,33] receptors has now been shown to regulate production of ROS that, in turn, determine the nature and duration of the cell signal. Although the mechanisms of such receptor-mediated ROS generation remain elusive, our results, consistent with those in the literature [13–15], demonstrate that stimulation of β2AR with the agonist isoproterenol leads to formation of ROS, and that ROS play key role in receptor coupling to G proteins, as well as expression of receptor on the plasma membrane.

Traditionally, signal transduction by the β2AR has focused largely on activation of Gs proteins and their downstream effectors, including cAMP-generating adenylyl cyclase and β-arrestin proteins. Cyclic AMP and its effectors, as well as the β-arrestin proteins have been viewed as chief mediators of the cell response to β2AR activation [5]. The current results present an added dimension of β2AR signaling, namely, the generation of ROS that seem to be required for 'classical' receptor-mediated signaling events. We show that the depletion of intracellular ROS impairs active receptor-induced accumulation of second messenger cAMP (and α_{1A}AR-mediated inositol phosphates). In support of the idea that receptor-driven ROS formation facilitates downstream signaling, a recent study showed that exposure of cells to β2AR inverse-agonist carvedilol dose-dependently inhibited ROS production [34]. The authors suggested that inhibitory effects of ROS generation by carvedilol may explain benefits seen with this drug in the clinical treatment of heart disease. Similarly, in a recent paper, Gomes et al. [35] describe that many β2AR antagonists are potent scavengers of both ROS and reactive nitrogen species, further strengthening the β2AR-ROS signaling relationship.

Here, we have used NAC, DPI, and NSC as pharmacological tools to study the effects of inhibition of β2AR-mediated ROS generation on receptor signal transduction. NAC is a potent thiolic antioxidant that possesses the ability to scavenge free oxygen radicals through direct chemical interaction with radical species and/or ROS-dependent by-products [36]. By virtue of these properties, NAC has been extensively used for prophylaxis and therapy in a wide array of clinical conditions. The ability of NAC to be a relatively non-selective scavenger of radical species makes it useful as a pharmacological inhibitor of hypochlorous acid, hydroxyl radical, and hydrogen peroxide, but not superoxide radical [16]. Meanwhile, DPI is a potent and selective inhibitor of the NADPH oxidase that binds specifically to the 45 kDa phox-like subunit of the enzyme and reduces superoxide formation at low to mid micromolar concentrations [17]. NSC23766, obtained from the drug library of the National Cancer Institute, is a rationally-synthesized inhibitor of Rac1, an integral component of the NADPH oxidase

ROS generating enzyme. NSC binds to the guanine-nucleotide exchange factor (GEF) recognition groove of Rac1, and inhibits the guanine turnover of Rac1, thereby facilitating oxidase inhibition [22].

Mechanistically, β2AR agonists have been shown to stimulate alterations in the redox states of sulfhydryl groups within the receptor binding pocket (and other sites), implicating modifications of β2AR cysteine residues by ROS [12]. Indeed, previous studies demonstrated that β2AR agonists act as electron donors [37] and that formation of high affinity β2AR states (e.g. pre-coupled to G protein) is putatively controlled by redox [38]. In this regards, it is noteworthy that β2AR proteins contain numerous cysteine residues (15–20 depending on species), and this fact is of special significance given the propensity of ROS to affect cysteine residues. The critical nature of GPCR cysteine residues in regards to the formation of intramolecular disulfide bridges, stabilization of receptor conformations, and downstream signaling cannot be overstated [39–42]. Interestingly, mutation of Cys 184 has been documented to engender functional uncoupling of the receptor from G protein, and demonstrates a decreased ability to form the high affinity ternary complex [39]. Moreover, mutation of Cys 106 and Cys 184 has been shown to dramatically decrease membrane β2AR expression as well as affinity of the β2AR ligand iodocyanopindolol, a characteristic suggestive of a critical disulfide bond within the ligand binding domain [42]. Previous results also show that intramolecular disulfide bridges are essential for agonist binding [43] as well as agonist-dependent functional activation of β2AR [44]. Importantly, Vauquelin et al. demonstrated that β-1AR can be deactivated by treatment with dithiothreitol (DTT), leading to a decline in high affinity ligand binding sites, while subsequent removal of the reducing agent leads to a recovery of binding [45]. Additionally, these authors demonstrate that the β-receptor ligands can stereoselectively protect against DTT-mediated inactivation, an observation that suggests the presence of a disulfide bond within the receptor binding pocket [45]. Taken together, these data, along with the fact that NAC serves as an antioxidant by protecting cysteine sulfhydryl groups from oxidation, raise the possibility that ROS play a role in oxidation of cysteine residues involved in intra- or intermolecular disulfide bridges which stabilize active conformations of β2AR.

Our results show no affect on β2AR ligand binding, suggesting that if ROS modifies receptor conformation, this change is not sensed by ligand. The results also suggest that ROS acts on the G protein, rather than the receptor. In support of this conclusion, Marques and Bicho [12] suggest that catechol-like agonists (such as ISO) facilitate ROS generation via a mechanism that either reduces a disulfide bond or exposes an existing sulfhydryl group within the β2AR-G protein complex. Furthermore, experiments utilizing sulfhydryl reagents and guanine nucleotides showed the apparent confinement of the ternary complex through alkylation of sulfhydryl groups, presumably on the G protein itself [12].

In conclusion, we show here that with the exception of ligand binding, ISO-mediated ROS generation affects the highly orchestrated molecular steps involved in activated β2AR signal transduction. It is tempting to speculate that the effects on β2AR signaling seen here occur through modifications of cysteine-linked disulfide bridges by ROS, however, the precise nature of this interaction remains elusive and is being actively investigated in our laboratory.

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