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Stereochemistry of an Agonist Determines Coupling Preference of β_2-Adrenoceptor to Different G Proteins in Cardiomyocytes

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Running title: Enantiomer-specific β_2-Adrenoceptor – G Protein Coupling

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Abbreviations: β_2-AR, β_2-adrenoceptor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; ISO, isoproterenol; PKA, protein kinase A; PTX, pertussis toxin.

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ABSTRACT

A fundamental question regarding receptor-G protein interaction is whether different agonists can lead a receptor to different intracellular signaling pathways. Our previous studies have demonstrated that while most β_2-adrenoceptor agonists activate both G_s and G_i proteins, fenoterol, a full agonist of β_2-adrenoceptor, selectively activates G_s protein. Fenoterol contains two chiral centers and may exist as four stereoisomers. We have synthesized a series of stereoisomers of fenoterol and its derivatives and characterized their receptor binding and pharmacological properties. We tested the hypothesis that the stereochemistry of an agonist determines selectivity of receptor coupling to different G protein(s). We found that the R,R-isomers of fenoterol and methoxyfenoterol exhibited more potent effects to increase cardiomyocyte contraction than their S,R-isomers. Importantly, while R,R-fenoterol and R,R-methoxyfenoterol preferentially activate G_s signaling, their S,R-isomers were able to activate both G_s and G_i proteins as evidenced by the robust pertussis toxin-sensitivities of their effects on cardiomyocyte contraction and on phosphorylation of extracellular signal-regulated kinase 1/2. The differential G protein selectivities of the fenoterol stereoisomers were further confirmed by photoaffinity labeling studies on G_s, G_{i2} and G_{i3} proteins. The inefficient G_i signaling with the R,R-isomers is not caused by the inability of the R,R-isomers to trigger the PKA-mediated phosphorylation of the β_2-adrenoceptor, since the R,R-isomers also markedly increased phosphorylation of the receptor at serine262 by PKA. We conclude that in addition to receptor subtype and phosphorylation status, the stereochemistry of a given agonist plays an important role in determining receptor-G protein selectivity and downstream signaling events.

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INTRODUCTION

Differential activation of receptors to specific signaling pathways has evolved to be a paradigm in pharmacological theory that can be translated into clinical relevance (Kenakin 2004; 2007; Mailman 2007; Urban et al. 2007; Violin and Lefkowitz, 2007). As an archetypical member of the G protein-coupled receptor (GPCR) superfamily, β_2-adrenergic receptor (β_2-AR) couples dually to G_S and G_i proteins, resulting in opposing effects on cardiac myocyte contractility and viability (Xiao et al., 1995; 1999; Zhu et al., 2001). In congestive heart failure, impaired β-AR response is often associated with increased G_i signaling (Bohm et al., 1994; Feldman et al., 1988) and selective downregulation of β_1-AR (higher β_2/β_1 ratio) (Bristow et al., 1986; 1993). Previous studies have demonstrated that disrupting G_i signaling with pertussis toxin (PTX) restores the markedly depressed β_2-AR contractile response in two rat heart failure models, and that a full β_2-AR agonist, fenoterol, which selectively activates β_2-AR-coupled G_S signaling reverses the diminished β_2-AR inotropic effect in myocytes from failing spontaneously hypertensive rat hearts in the absence of PTX (Xiao et al., 2003). *In vivo* studies have further demonstrated that prolonged use of fenoterol not only improves cardiac function, but also retards cardiac maladaptive remodeling, and that the overall beneficial effects of fenoterol with β_1-AR blockade are greater than the salutary effects of β_1-AR blockade alone in a rat chronic heart failure model-induced by myocardial infarction (Ahmet et al., 2004; 2006; 2008). These studies suggest that selective activation of the β_2-AR-coupled G_S signaling may provide a useful therapeutic target for the treatment of congestive heart failure.

While the pharmaceutical preparation of fenoterol is a racemic mixture of its R,R- and S,S-enantiomers (*rac*-fenoterol), our recent studies have shown that the R,R-enantiomer is the

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only active isoforms in receptor binding and cardiomyocyte contraction assays (Beigi et al., 2006; Jozwiak et al., 2007). A cohort of fenoterol derivatives including the R,R-, R,S-, S,R- and S,S-isomers of fenoterol were synthesized (Beigi et al., 2006; Jozwiak et al., 2007). Using some of these compounds, we attempted to examine the hypothesis that the stereochemistry of an agonist determines functional selectivity of a given receptor coupling to different G protein(s) and resultant activation of subset(s) of downstream signaling pathways.

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MATERIALS AND METHODS

Compounds and Reagents

A series of stereoisomers and derivatives of fenoterol, including the R,R- and S,R-isomers of fenoterol and methoxyfenoterol (see Fig.1 for structures), were synthesized into enantiomeric purity. The detail procedures of the chemical synthesis and the receptor binding affinities of the compounds have been reported (Beigi et al., 2006; Jozwiak et al., 2007). Zinterol was kindly supplied by Bristol-Myers (Evansville, IN). ICI 118,551, (-)isoproterenol (ISO), pertussis-toxin (PTX) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cardiomyocyte Isolation, Cell Culture and Adenoviral Infection

Cardiac myocytes were isolated from 2- to 4-month-old male Sprague-Dawley rats using a standard enzymatic technique, then cultured in medium 199 containing (in mmol/L): 5 creatine, 2 L-carnitine, 5 taurine, 25 HEPES, 0.01% insulin-transferrin-selenium-X and 1% penicillin-streptomycin on dishes precoated with laminin. Human embryonic kidney (HEK) 293 cells purchased from the American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were incubated at 37°C under an atmosphere of 5% CO_2. Adenovirus-mediated gene transfer was implemented by infecting the cells with 100 multiplicity of infection of adenovirus carrying the human β_2-AR gene (Zhu et al., 2001).

Cardiomyocyte Contractility

Freshly isolated cardiac myocytes were perfused with a buffer containing (in mmol/L): 137 NaCl, 4.9 KCl, 1.2 $MgCl_2$, 1 NaH_2PO_4, 1 $CaCl_2$, 20 glucose and 20 HEPES (pH 7.4), and electrically stimulated at 0.5 Hz at 23°C. Cell length was monitored by an optical edge-tracking

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method (Beigi et al., 2006; Jozwiak et al., 2007). Measurements were made under steady-state conditions before and after exposure of the myocyte to a single concentration of agonist. The specificity of the agonist towards β_2-AR was assessed by the ability of the response to be blocked by ICI 118,551 (10^{-7} mol/L), a selective β_2-antagonist. In a subset of experiments, aliquots of cells were incubated with PTX (0.75 μg/mL at 37°C for 3 hours) to block G_i signaling as described previously (Xiao et al., 1995). The complete disruption of G_i function was confirmed by the insensitivity of the β_1-adrenergic stimulatory response to carbachol.

Photoaffinity Labeling of G proteins

Photoaffinity labeling of G proteins was performed as described previously (Xiao et al., 1999) with minor modifications. In brief, rat heart membranes were prepared by differential centrifugation and stored in a buffered sucrose solution at –80°C. The labeling reaction was allowed to proceed at 25°C for 7-20 min in a mixture containing (in mmol/L): 30 HEPES (pH 7.4), 30 NaCl, 0.1 EGTA, 1 benzamidine, 1 reduced glutathione, $MgCl_2$ (5 for G_s and 1 for G_i), GDP (0.001 for G_s and 0.003 for G_i), unstimulated or agonist-prestimulated membranes (150-200 μg) and γ-[^{32}P]GTP-azidoanilide (2-3 μCi, ALT Bioscience, Lexington, KY), and stopped by cooling on ice. After removing the unbounded labels, the membranes were irradiated with UV light (254 nm, 100 W, 6 cm, 1 min), and solubilized in 2% SDS. The clarified supernatants were incubated with rabbit antisera (4-10 μL) raised against $G_{\alpha s}$ (United State Biological, Swampscott, MA), $G_{\alpha i2}$ (Abcam, Cambridge, MA) and $G_{\alpha i3}$ (Santa Cruz Biotechnology, Santa Cruz, CA) in standard radioimmunoprecipitation assay buffer overnight at 4°C and subsequently with protein-A/G agarose (Calbiochem, La Jolla, CA). Beads were washed according to standard procedures, and then boiled for 10 min in Laemmli sample buffer. The resultant

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supernatants were subjected to Urea/SDS-PAGE. Radiolabeled proteins were visualized by autoradiography.

Western Blot Analysis

Solubilized proteins were analyzed in a denaturing polyacrylamide gel, electro-transferred to a polyvinylidine difluoride membrane, and immunoblotted with the appropriate primary antibody, followed by incubation with a peroxidase-conjugated secondary antibody. Films were exposed to enhanced chemiluminescence (GE Healthcare, Buckinghamshire, United Kingdom) reaction. The intensities of the gel bands were quantified using the NIH ImageJ software. Extracellular signal-regulated kinase (ERK) 1/2 activation was detected using the phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000, Cell Signaling Technology, Danvers, MA). Total ERK1/2 was detected by reprobing the membrane with the p44/42 MAP kinase antibody (1:1000, Cell Signaling). Phosphorylation of the PKA site serine262 on β_2-AR was detected using an anti-pSer262 monoclonal antibody at 1 μg/ml (generously provided by Dr. Richard B. Clark of The University of Texas, Houston, Medical School, Tran et al., 2004). Total β_2-AR was detected with the anti-C-tail antibody (1:500, Santa Cruz) recognizing the carboxy-terminal of β_2-AR.

Statistical Analysis

Results are expressed as mean ± standard error. Student's *t*-test was performed to compare the means between two groups and one-way ANOVA for multiple group comparison followed by post-hoc analysis with Dunnett's test. For each treatment group in the contractility study, the concentration-response curves were prepared using the Prism software, followed by statistical analysis with curve-fitting using the three parameter logistic fixed bottom model with

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constrains (bottom=100; top<360; Hill-slope<1.8), and calculation of the $LogEC_{50}$±SE and the *P* values. A value of $P<0.05$ was considered statistically significant.

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RESULTS

In our previous studies, we have characterized the binding affinities to β-ARs for the 26 stereospecific fenoterol derivatives (Jozwiak et al., 2007). The previously reported binding affinities to β_1- and β_2-AR for the R,R- and S,R-isomers of fenoterol and methoxyfenoterol could be found in Supplementary Table 1. Here, the specificity of these compounds was further assessed pharmacologically using cardiomyocyte contractility. Preliminary experiments had shown that near maximal contractile response could be achieved by the R,R-isomers at 0.5 μmol/L and by the S,R-isomers at 10 μmol/L. The selectivity of these compounds towards β_2-AR was assessed by the inhibitory effect of ICI 118,551 (10^{-7} mol/L). As shown in Fig. 2, all of these compounds could produce contractile responses that were blocked by ICI 118,551, suggesting that they are selective to β_2-AR. These results are consistent with the binding affinity data.

The full concentration-response profiles for the fenoterol compounds stimulated contraction with or without PTX treatment are shown in Fig. 3. All four stereoisomers of fenoterol and methoxyfenoterol caused a concentration-dependent increase in the contraction amplitude of adult rat cardiomyocytes with similar maximal responses (~3-fold increase in cell contractility) (Fig. 3), while the S,S-isomers had no detectable effect (data not shown) consistent with our previous report (Beigi et al., 2006). Disruption of G_i signaling by PTX had only minor effects on the contractility profiles of the R,R-isomers (Fig. 3A,B), as indicated by the insignificant changes in the EC_{50}s (in terms of Log [M]: -7.11±0.17 for the control versus -7.44±0.07 for the PTX-treated group in R,R-fenoterol, P=0.085; and -6.76±0.15 versus -6.84±0.10 in R,R-methoxyfenoterol, P=0.579). To the contrary, PTX treatment caused a clear leftward shift of the concentration-response curves of the S,R-isomers (Fig.3 C,D), and

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significantly decreased the EC_{50}s (from -5.63±0.21 to -6.08±0.03 for S,R-fenoterol, $P<0.01$; and from -5.50±0.03 to -5.96±0.09 for S,R-methoxyfenoterol, $P<0.05$). These results suggest that the R,R-isomers evoke G_S-selective β_2-AR signaling, while the S,R-isomers allow the receptor to activate both G_S and G_i pathways.

In HEK293 cells, treatment with agonists triggers the phosphorylation of ERK1/2, which peaks at 5 min (Daaka et al., 1997). Based on the concentration-response relationships obtained above for fenoterol and its derivatives induced cardiomyocyte contraction, preliminary experiments were conducted to determine the concentrations of the compounds to be used for agonist-induced ERK1/2 activation in HEK 293 cells. We found that treatment of cells with a nonselective β-AR agonist, ISO (10^{-6} mol/L), resulted in a 5-fold increase in phosphorylation of ERK1/2 over the control and that treatment of cells with PTX reduced ERK1/2 activation to about 2-fold of the control level (Fig. 4A,C), which is similar to the previous notion (Daaka et al., 1997). Interestingly, the S,R-compounds at 10^{-6} mol/L, a concentration with minimal effects on myocyte contraction (Fig. 3C,D), were able to induce a full activation of ERK1/2, an effect comparable to that induced by ISO treatment (Fig. 4C,D) and also completely inhibitable by ICI 118,551 (data not shown). The R,R-isomers at this concentration (10^{-6} mol/L) also increased ERK1/2 activation to a similar extent (Fig. 4C,D). It is noteworthy that treatment of cells with PTX largely abrogated ERK1/2 activation induced by the S,R-isomers, but had minimal effects on ERK1/2 activation induced by the R,R-isomers (Fig. 4).

The lack of PTX-sensitivity in the myocyte contractile responses and in ERK1/2 activation in cells stimulated with R,R-fenoterol and R,R-methoxyfenoterol suggests that the R,R-isomers selectively stimulate β_2-AR-coupled G_S signaling. To directly assay G protein activation, we measured photoaffinity labeling of the α subunits of G proteins with the

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photoreactive GTP analog γ-[^{32}P]GTP-azidoanilide in response to the fenoterol derivatives. Subsequent immunoprecipitation with specific antisera was carried out to determine the amount of the activated G protein(s). The upper panel in Fig. 5A shows that at the same concentration (10^{-6} mol/L), fenoterol compounds and the nonspecific β-AR agonist, ISO, increased the incorporation of γ-[^{32}P]GTP-azidoanilide in $G_{\alpha s}$. Both the short and long isoforms of $G_{\alpha s}$ (which have approximate molecular weights of 45 and 47 kDa, respectively) are activated similarly in response to the stimuli. Moreover, the $G_{\alpha s}$ labeling induced by R,R-fenoterol is significantly greater than that induced by S,R-fenoterol (P<0.05, Fig. 5B). As a positive control, zinterol (10^{-5} mol/L), a selective β_2-AR partial agonist, was able to activate both $G_{\alpha i2}$ and $G_{\alpha i3}$ (Fig. 5A lower panels), consistent with our previous notion (Xiao et al., 1999). Interestingly, the fenoterol compounds (10^{-6} mol/L) exhibited diverse effects on G_i proteins (Fig. 5A lower panels). Specifically, activation of $G_{\alpha i2}$, the predominant G_i protein in the heart, was observed only if β_2-AR was stimulated with S,R-fenoterol, but not by R,R-fenoterol, R,R-methoxyfenoterol or S,R-methoxyfenoterol (Fig. 5C). The activation effects of S,R-fenoterol and R,R-fenoterol on $G_{\alpha i2}$ are also statistically different (P<0.01, Fig. 5C). In addition, activation of $G_{\alpha i3}$ in response to S,R-isomers was significantly greater than that induced by R,R-isomers of the fenoterol derivatives (Fig. 5D, P<0.01 for fenoterol and P<0.05 for methoxyfenoterol).

It has been previously proposed that PKA-mediated phosphorylation of β_2-AR is necessary and sufficient for the switch of the receptor coupling from G_s to G_i (Daaka et al., 1997). To determine whether PKA-mediated phosphorylation of β_2-AR is affected by the chirality of the fenoterol compounds, cultured rat cardiomyocytes overexpressing human β_2-AR

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were stimulated with ISO (10^{-6} mol/L) and the four fenoterol compounds (10^{-6} mol/L), and the extend of PKA-mediated receptor phosphorylation was then detected by Western blotting using the anti-pSer262 antibody (Tran et al., 2004). The levels of the total β_2-AR in the cell lysates were also determined in parallel using an anti-C-tail antibody. As expected, treatment with ISO increased the PKA-mediated phosphorylation of β_2-AR by 9 folds after adjusted for the amount of total β_2-AR (Fig. 6), a result confirming those obtained from another study (Iyer et al., 2006). The effects of the S,R-isomers on receptor phosphorylation at Ser262 are comparable to that of ISO in magnitude. Interestingly, the R,R-isomers also evoked about 12-fold increase in phosphorylation of the receptor at Ser262. The differences in the potencies of these agonists are not significant though. These results suggest that the degree of β_2-AR-G_i coupling in response to the fenoterol isomers is not proportional to the phosphorylation status of β_2-AR by PKA. Thus, PKA-mediated phosphorylation of β_2-AR at Ser262 is insufficient for β_2-AR-G_i coupling.

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DISCUSSION

As a prototypical G_S-coupled GPCR, β-AR stimulation activates the well-established G_S-adenylyl cyclase-cAMP-PKA signaling cascade, which increases cardiac contractility via PKA-mediated phosphorylation of a panel of proteins involved in cardiac excitation-contraction coupling. Whereas β_1-AR couples only to the G_S signaling pathway, β_2-AR couples dually to G_S and G_i proteins (Xiao et al., 1995, 1999). As a result, stimulation of the β_2-AR by most β_2-AR agonists results in increased cardiomyocyte contractility that can be augmented by the inhibition of G_i signaling by PTX (Xiao et al., 1995, 2003). A major finding of the present study is that the positive inotropic effects and activation of ERK1/2 induced by R,R-fenoterol and R,R-methoxyfenoterol are insensitive to PTX treatment, suggesting the R,R-isomers selectively direct β_2-AR to the G_S bypassing the G_i coupling. Interestingly, alteration of their stereochemistry from the R,R- to the S,R-configuration is able to change this property. Apart from reducing the potencies, the contractile responses become sensitive to PTX treatment (Fig. 3C,D).

β_2-AR Activates ERK1/2 Signaling Via G_i-dependent and independent Mechanisms

The β_2-AR-mediated activation of ERK1/2 in HEK 293 cells expressing endogenous β_2-AR has previously been shown to be G_i-dependent (Daaka et al., 1997), although alternative G_i-independent mechanisms (Friedman et al., 2002; Schmitt and Stork, 2000) have been proposed. Recently, some G protein–independent mechanisms have also been described (Shenoy et al., 2006; Sun et al., 2007). We have demonstrated here that activation of ERK1/2 by R,R-fenoterol and R,R-methoxyfenoterol is insensitive to PTX treatment (Fig. 4), suggesting the involvement of a G_i-independent mechanism. On the other hand, activation of ERK1/2 by S,R-

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fenoterol and S,R-methoxyfenoterol is PTX-sensitive (Fig.4), suggesting the G_i-dependence of these effects. The lack of PTX-sensitivity in their effects on ERK1/2 activation further suggests that R,R-fenoterol and R,R-methoxyfenoterol selectively activate the β_2-AR-coupled G_S pathway, while S,R-fenoterol and S,R-methoxyfenoterol can activate both the G_S and G_i proteins, as manifested by the robust PTX-sensitivities in their responses.

Substitution and Chirality Confers the Fenoterol Compounds Different Effectiveness in Activating G_S and G_i Proteins

In the present study, we compared different fenoterol compounds on their effectiveness in activating G_S and G_i proteins by means of direct labeling of the G proteins on isolated heart membranes with a radioactive GTP analog (Fig. 5). We found that substituting the hydroxyl group with the methoxy group has a smaller impact to the G-protein selectivity of R,R-fenoterol than changing its configuration to the S,R-form in terms of G_S and G_{i2} stimulation (Fig. 5B,C). This is consistent with the functional data on cardiomyocyte contraction (Fig. 3) and ERK1/2 activation (Fig. 4). Conversely, substituting the hydroxyl group with the methoxy group increase selectivity of fenoterol to G_{i3}, while maintaining the differential coupling efficiency between the R,R- and S,R-isomers (Fig. 5D). Thus, the different effectiveness of R,R- and S,R-fenoterol in activating G_S and G_i proteins echoes the different PTX-sensitivities of their functional effects. The distinction of the G protein activation effects and their correlation with functional data for the methoxyfenoterol isomers are less obvious as compared to the fenoterol isomers (Fig. 5B-D), suggesting that substitution can also affect the coupling and functional selectivity of the agonist.

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PKA-mediated Phosphorylation of β_2-AR Is Insufficient to Cause Receptor Coupling to G_i Protein

Desensitization of β_2-AR is triggered by the phosphorylation of the receptor by a combination of actions of PKA and G protein coupled receptor kinases. In particular, phosphorylation of β_2-AR at the PKA sites is suggested to switch the receptor coupling from G_S to G_i (Daaka et al., 1997). The subsequent binding of β-arrestin-2 to the phosphorylated receptor uncouples G_S from β_2-AR and triggers receptor internalization. Here, we have demonstrated that R,R-fenoterol and R,R-methoxyfenoterol induce robust phosphorylation of β_2-AR at its PKA site located at the third intracellular loop of the receptor and this action is similar to that induced by their S,R-isomers or the nonselective β-AR agonist, ISO, (Fig. 6). Importantly, R,R-isomer-induced β_2-AR phosphorylation by PKA is not accompanied by activation of G_{i2} proteins (Fig. 5C), the predominant G_i proteins in the heart. In addition, R,R-isomer-induced β_2-AR phosphorylation is associated with significantly less potent effects on G_{i3} as compared to those induced by S,R-isomers (Fig. 5D). Taken together, our pharmacological and biochemical data suggest that the degree of PKA-mediated phosphorylation of β_2AR is not proportional to their ability to induce G_i coupling. Thus, PKA-dependent phosphorylation of β_2-AR is insufficient to trigger the receptor coupling to G_i proteins in the physiologically relevant setting, adult rat cardiac myocytes.

Molecular Nature of R,R-Fenoterol-activated G_S Signaling

Ligand-induced different G protein coupling has been described in various GPCRs (Akam et al., 2001; Beyermann et al., 2007; Cordeaux et al., 2001; 2004; Gazi et al., 2003). The

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present study is the first to suggest the role of chirality in the functional selectivity of an agonist. Differential activation of receptors to specific signaling pathways might be explained by the fact that agonists can stabilize a receptor into multiple conformational states (Gether et al., 1995; Ghanouni et al., 2001; Granier et al., 2007; Swaminath et al., 2004) and each of which can trigger a distinct pluridimensional functional outcomes, including G protein coupling, receptor phosphorylation, receptor dimerization/oligomerization, receptor desensitization, receptor internalization and/or β-arrestin–dependent ERK activation (see recent reviews by Kenatin, 2007; Kobilka and Deupi, 2007 and Urban et al., 2007). Further studies using these compounds to characterize the receptor conformation that confers selectivity to G protein coupling is highly warranted. The data thus obtained could be applied on the development of assay systems for future drug-screening.

Potential Clinical Implications of G_S-Signaling Selective β_2AR Activation

Since enhanced G_i signaling is involved in the dysfunction of both β_1-AR and β_2-AR in the failing heart (He et al., 2005; Sato et al., 2004; Xiao and Balke, 2004; Zhu et al., 2005), selective activation of the β_2-AR-G_S coupling may provide an effective means to improve contractile function of the failing heart without β_1-AR detrimental effects (for review see Zheng et al., 2005). We have previously reported the contractility stimulatory effects of a number of β_2-agonists on rat cardiomyocytes (Xiao et al., 2003). While most β_2-agonists increased myocyte contraction in a PTX-sensitive manner, the contractility stimulatory effect of *rac*-fenoterol was insensitive to PTX (Xiao et al., 2003). We have concluded that *rac*-fenoterol selectively activates the β_2-AR-G_S pathway, and further postulated that this special property of *rac*-fenoterol might have potential therapeutic implications in heart failure. This idea is

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supported by recent *in vivo* studies in an ischemic rat heart failure model (Ahmet et al., 2004; 2006; 2008). Since S,S-fenoterol is inactive (Beigi et al., 2006), the pharmacological effects observed for *rac*-fenoterol are exerted by R,R-fenoterol. In the present study, we have provided multiple lines of evidence to demonstrate that R,R-fenoterol and its derivative R,R-methoxyfenoterol selectively activate the β_2-AR-coupled G_S signaling and largely spare the β_2-AR-coupled G_i pathway. Importantly, the selectivity for G_S activation is determined by the chirality of the compounds because this selectivity is lost in the S,R-isomers of these fenoterol compounds. This finding may have broad reaching implications in GPCR biology and signaling pathway-targeted drug development.

We conclude that, in addition to receptor subtype and phosphorylation status, the stereochemistry of an agonist plays a role in the selectivity of the receptor-G protein interaction and downstream signaling. Moreover, different stereoisomers of an agonist can selectively activate different post-receptor events, presumably by stabilizing the receptor in multiple conformational states, which possess distinctly different G protein selectivity.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Chemical structures of fenoterol and methoxyfenoterol.

A. R,R-fenoterol, **B**. S,R-methoxyfenoterol. Chiral centers are marked with asterisks.

Figure 2: β_2-AR selectivity of the fenoterol compounds in myocyte contraction.

Single ventricular myocytes from rats were electrically stimulated. The contraction amplitude of the cell in response to the fenoterol compound: R,R-fenoterol (RR1, 5×10^{-7} mol/L) (**A**), S,R-fenoterol (SR1, 10^{-5} mol/L) (**B**), R,R-methoxyfenoterol (RR2, 5×10^{-7} mol/L) (**C**), and S,R-methoxyfenoterol (SR2, 10^{-5} mol/L) (**D**) followed by ICI 118,551 (ICI, 10^{-7} mol/L) were recorded. The top part for each panel depicts a typical chart recording, and the averaged data (n=6-9 independent observations on cells from 4-6 hearts for each data point) are shown at the bottom. *** $P<0.001$ as compared with the corresponding agonist-stimulated contraction amplitude under the steady state without ICI 118,551.

Figure 3: Effect of PTX treatment on the contractile responses to the fenoterol compounds.

Concentration-response profiles of cardiomyocyte contractility subjected to R,R-fenoterol (**A**), R,R-methoxyfenoterol (**B**), S,R-fenoterol (**C**) and S,R-methoxyfenoterol (**D**) with and without PTX treatment. Contractile response to the agonist is expressed as a percentage of the basal contractility (n=9-14 cells from 7-9 hearts for each data point). Basal contraction amplitude is 5.46±0.14% (n=138 cells). PTX did not alter basal contraction (5.33±0.14%, n=130 cells).

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Figure 4: Chirality determines the PTX-sensitivities of fenoterol derivatives in activating ERK1/2.

HEK 293 cells were grown to confluence in growth medium in 6-well plates before deprivation of the serum for 7 h. Treatment with PTX (0.5 μg/ml) was implemented during serum starvation. The cultured cells were then stimulated with agonists for 5 min followed by cell-lysis. After adjusting the protein concentration of the resultant cell lysates, the extent of ERK1/2 phosphorylation was analyzed by Western blotting. **A** and **B**, Representative Western blots of phospho-ERK1/2 and total ERK1/2 (as protein loading control) after stimulation with ISO (10^{-6} mol/L) or the fenoterol compounds (10^{-6} mol/L) with or without PTX as indicated. **C** and **D**, Averaged data from 3-4 independent experiments. The data are presented as fold increase over the –PTX control. $^{\#}$ $P<0.01$ as compared with the –PTX control; * $P<0.05$, ** $P<0.01$ as compared with the –PTX group in the same agonist treatment group.

Figure 5: Differential activation of G_s and G_i proteins by fenoterol stereoisomers.

Rat heart membranes were labeled with γ-[^{32}P]GTP-azidoanilide in the presence of ISO (10^{-6} mol/L), zinterol (10^{-5} mol/L), or fenoterol compounds (10^{-6} mol/L). The radiolabeled G protein alpha subunits $G_{\alpha s}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ were immunoprecipitated with the corresponding subunit-specific rabbit polyclonal antibodies and then subjected to electrophoresis. **A**, representative autoradiographs of the resolved G proteins. **B-D**, Averaged data of the densitometric analysis of the labeled G protein bands from 3 experiments. Data are presented as percentages of the control. * $P<0.05$, ** $P<0.01$ as compared with the control; † $P<0.05$, ‡ $P<0.01$ as compared with the corresponding diastereomer.

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Figure 6: Fenoterol compounds trigger phosphorylation of β_2-AR at the PKA site Ser262.

Rat cardiac myocytes were cultured in 35 mm dishes and infected with adenovirus to overexpress human β_2-AR. After 24 h, the myocytes were stimulated with ISO (10^{-6} mol/L) and the fenoterol compounds (10^{-6} mol/L) for 10 min, followed by cell lysis. The resultant cell lysates were subjected to Western blot analysis using the anti-pSer262 β_2-AR antibody to detect for the phosphorylation of β_2-AR at the PKA site, and the anti-C-tail β_2-AR antibody to detect for total β_2-AR. **A**, Representative Western blots of the phosphorylated Ser262 β_2-AR and the total β_2-AR. **B**, Averaged data from 3-4 independent experiments. The data are presented as fold increase over basal phosphorylation of β_2-AR after normalization with the amount of total β_2-AR.

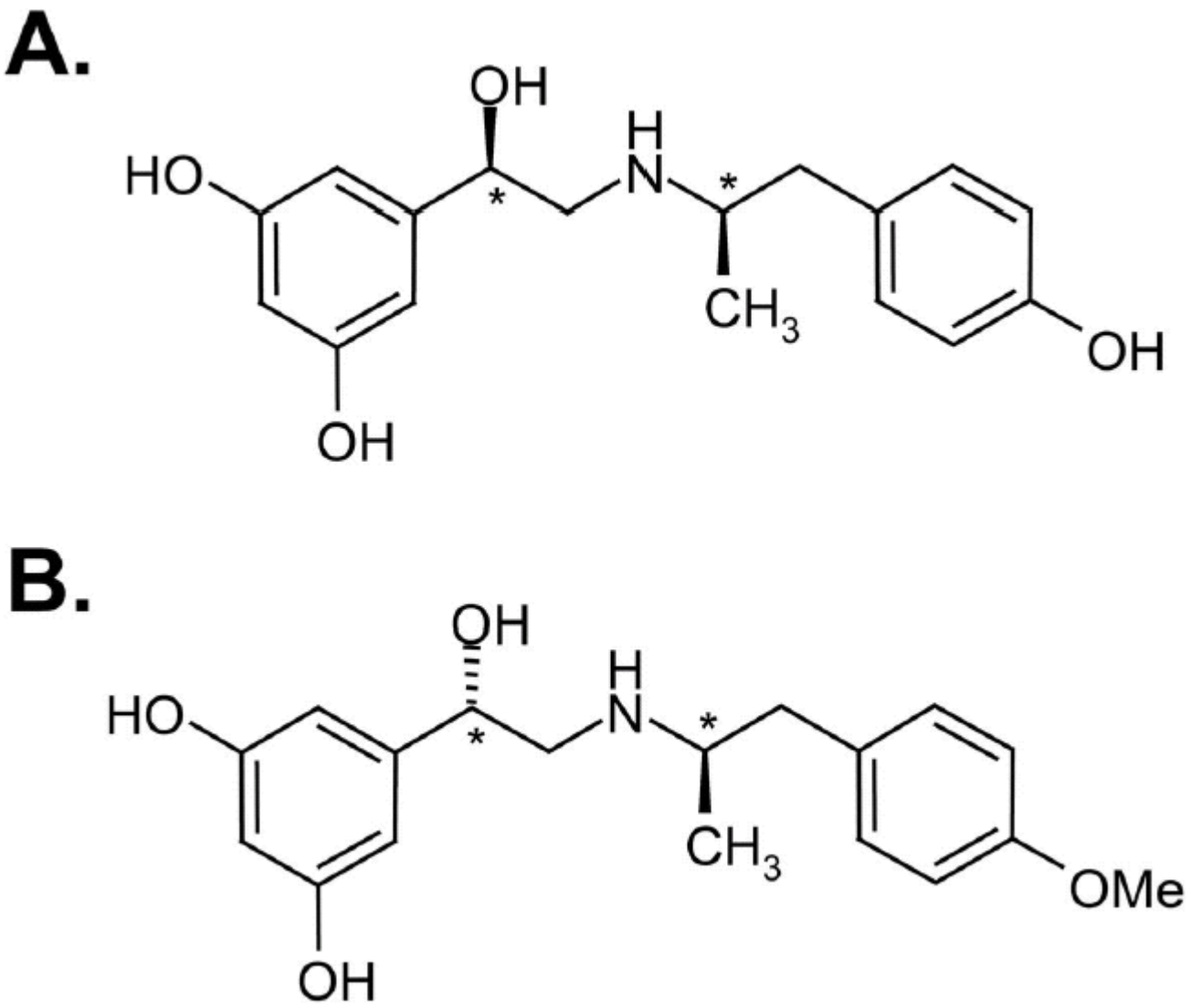

Figure 1

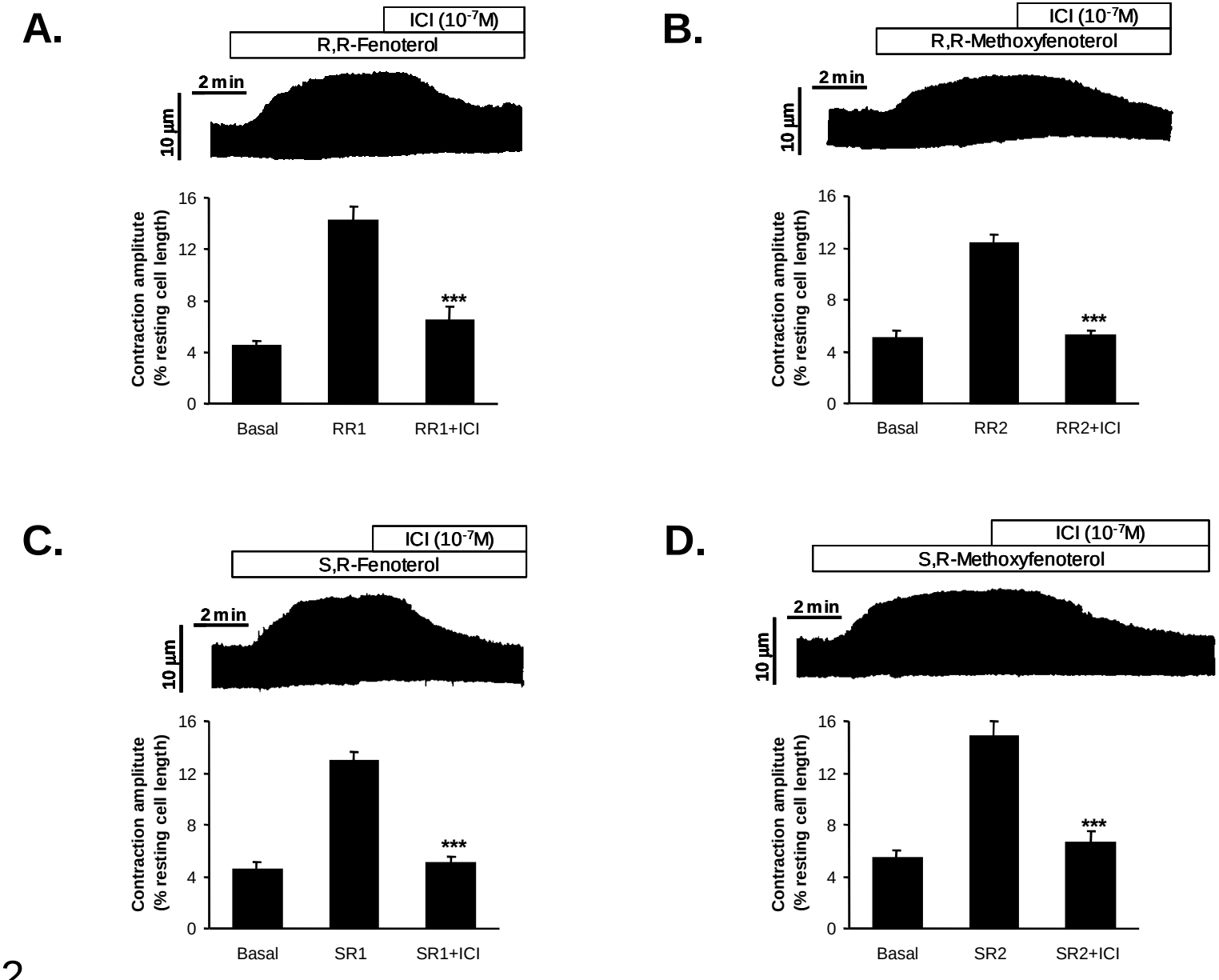

Figure 2

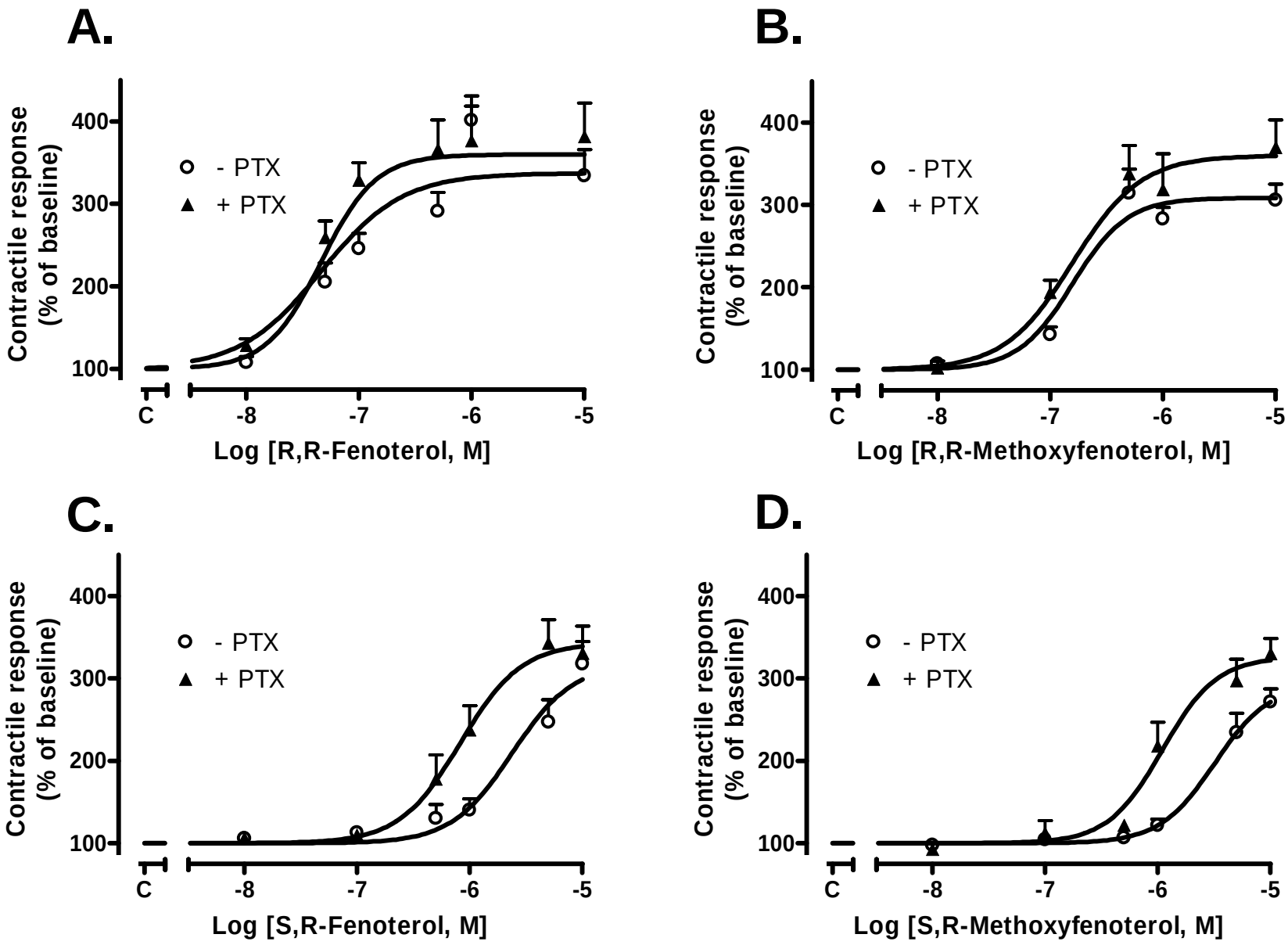

Figure 3

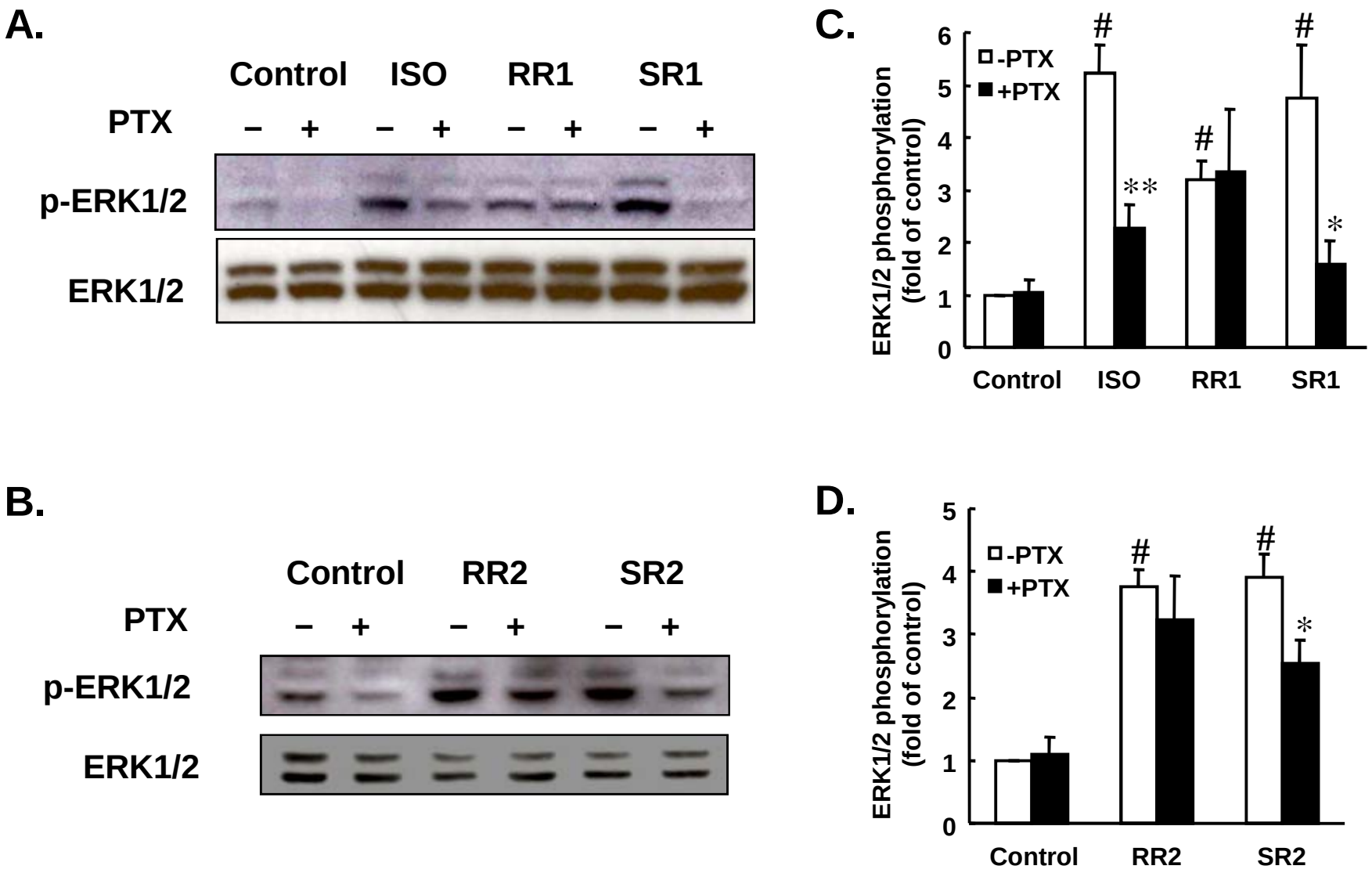

Figure 4

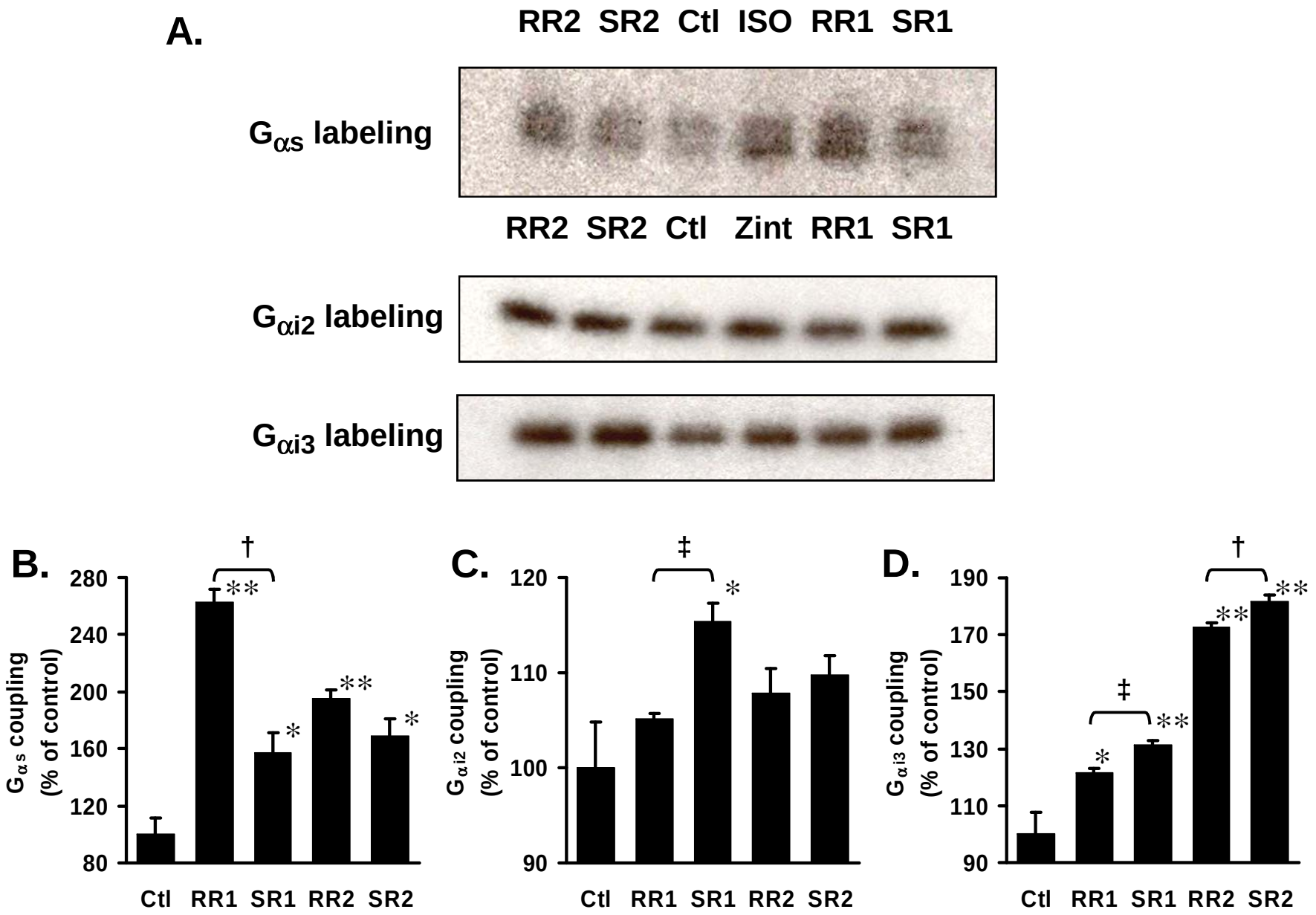

Figure 5

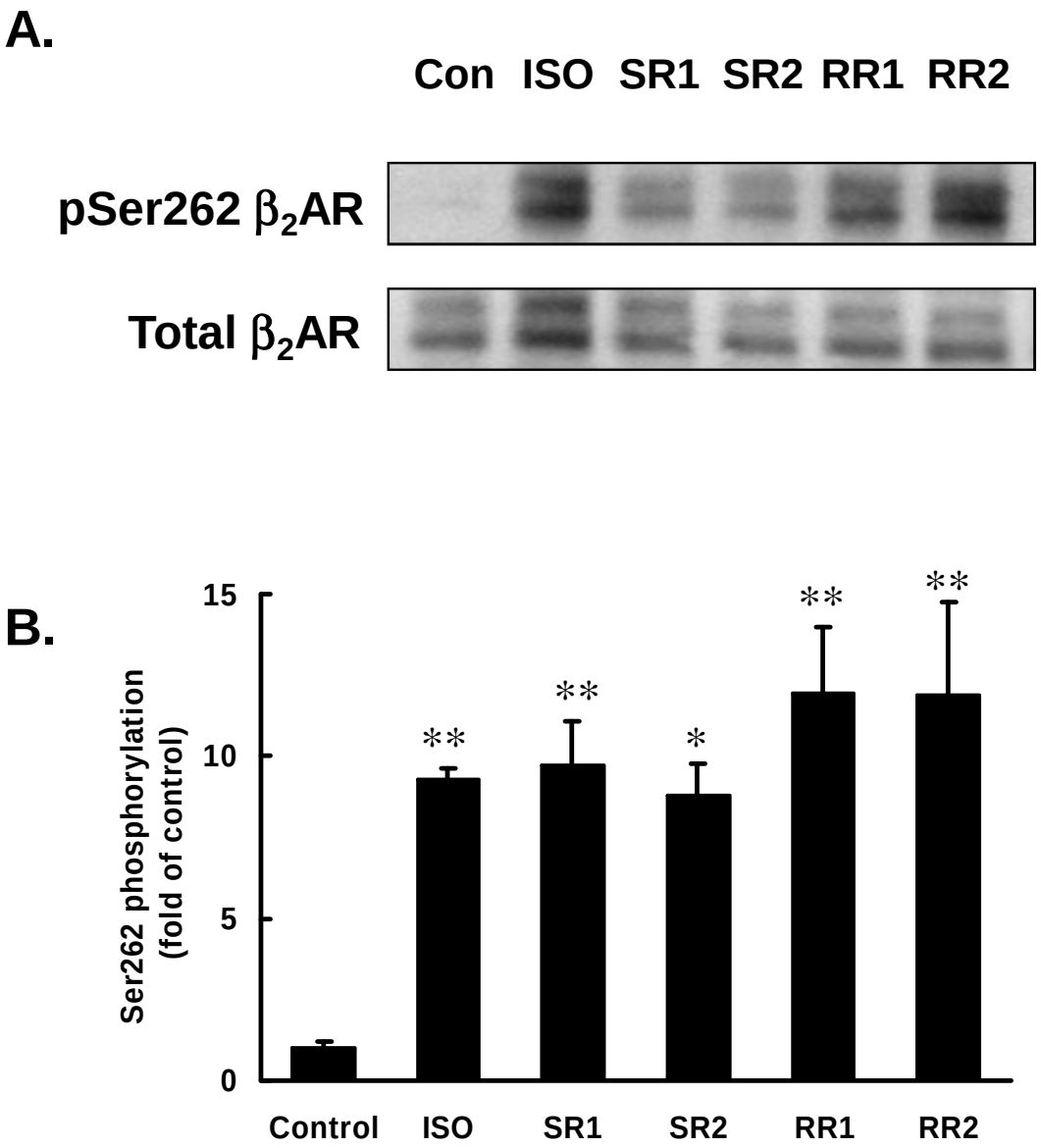

Figure 6