Determine the respective efficiencies of

1. Introduction

β2-AR activation induces antiapoptotic effects in cardiomyocytes mediated by stimulation of the PI-3K pathway [1]. The proposed mechanism by which this activation occurs is dependent on G protein-coupled receptor kinases (GRKs). Under basal conditions, GRK2 forms a complex with PI-3K in the cytosol [2]. When β2-AR is occupied by an agonist, GRK2 is translocated to the membrane by a Gβγ subunit-dependent mechanism and subsequently recruits PI-3K from the cytosol to the membrane [2]. The proximity of the PI-3K and the stimulated receptor induces the activation of the enzyme. In contrast, β1-AR has proapoptotic activity in cardiomyocytes due to stimulation of PKA or CAM KII [3]. Indeed, it has been observed that stimulation of β1-AR with an agonist induces apoptosis and this can be prevented in presence of inhibitors of either PKA or CAM-KII [4].

Interestingly it has been shown that stimulated β2-AR can also recruit GRK to the membrane. The interaction of either β-AR subtype with GRKs is mainly via the intracellular loops and carboxy tail of the receptor [5]. When activated, GRK induces the phosphorylation of certain serine/threonine residues in the carboxy tail of both β-AR subtypes resulting in functional uncoupling of the receptor from their primary signalling pathways. GRK2 phosphorylation also favours subsequent interaction with β-arrestin. This interaction with β-arrestin further desensitizes the receptor and is subsequently involved in receptor endocytosis [6, 7].

As GRK interacts with molecular determinants in carboxy tail of the receptor, we hypothesized that differences in the coupling of the β1- and β2-AR with PI-3K may be due to determinants located in this portion of the receptor. This study was designed to determine the respective efficiencies of
β₁-AR and β₂-AR to stimulate the PI-3K pathway and to test the above hypothesis.

2. Material and Methods

2.1. DNA Constructions, Cell Transfection, and Culture. Murine β₁-AR and human β₂-AR subcloned into pcDNA3 were used in this study. Two chimeric receptors consisting of the β₁-AR with the seventh transmembrane domain and the carboxyl-terminal tail of the β₂-AR and the reciprocal β₂-AR with the seventh transmembrane domain and the carboxyl-terminal tail of the β₁-AR were constructed as follows. A restriction site for Hpa I was created by polymerase chain reaction (PCR) at position 2070 in the β₁-AR. The new restriction site in the β₁-AR was created with three primers. Two primers were used for the hybridization with the receptor sequence. These primers contained 21 base pairs and had, respectively, CGGCTAGAAGGCCATAGGCC and TCG-TGGCAACAGTGTCGGCCA sequences. The third primer was utilized to introduce the restriction site. This primer which contained 24 base pairs had the following sequence: GGTGGAAGGGTTAACACGTTG. The mutated β₁-AR and the β₂-AR wt were double digested with Hpa I and Xho I. The result of this digestion is two fragments of 6540 bp and 486 bp for the mutated β₁-AR and two fragments 5998 bp and 1362 bp for the β₂-AR wt. The appropriate restriction fragments (containing the seventh transmembrane domain and the carboxy-terminal portion of the receptor) were isolated, exchanged for their counterparts, and religated. Positive clones were selected by enzymatic digestion and confirmed by sequencing.

The β-AR wild type (wt), chimeric receptors, dominant-negative PI-3 kinase (p85ΔPI-3K), and/or carboxy-terminal domain of GRK2 (ct-GRK2) were transiently transfected in human embryonic kidney (HEK 293) cells using the calcium phosphate precipitation method. We performed all experiments at 48 hours posttransfection, that is, at maximal receptor expression determined by ligand binding. Cells were starved overnight 24 hours before the experiments in a medium without fetal bovine serum. HEK 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, 1 mM glutamine, 0.25 mg/mL fungizone in an atmosphere of 95% air/5% CO₂ at 37°C. On the day of the experiment, cells were treated with 1 μM isoproterenol for the indicated times and fractionated for the cytosolic or membrane compartments. In some experiments, cells were pretreated with pertussis toxin (0.1 μg/mL; Sigma), 18 hours before stimulation with isoproterenol.

2.2. Preparation of Cytosolic and Membrane Fractions. Cells were washed three times with 10 mL of phosphate-buffered saline at 4°C and mechanically detached in 1 mL of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 10 μg/mL benzamidine. Cells were then lysed with a sonicator (3 bursts of 10 seconds at max speed), and the lysates were centrifuged at 1000 × g for 5 minutes at 4°C. The supernatant was centrifuged at 45 000 × g for 20 minutes and was considered as the cytosolic preparation. Protein content was assessed using the Bradford method (Bio-Rad). The pelleted membranes were resuspended in 250 μL of a solubilization buffer (buffer A) containing 50 mM Tris pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1 mM Na₂VO₃, 10 mM benzamidine, 0.5 mM PMSF, 10 μg/mL leupeptin, 5 mM DTT, 1 μM microcystin LR, and 1% Triton X-100; and solubilized for 2 hours at 4°C. Then the membranes were centrifuged at 10 000 × g for 15 minutes. The protein content was assessed using the Lowry method (Bio-Rad).

2.3. Radioligand Binding Assay. Radioligand binding assays were conducted essentially as described previously [8] with ∼5 μg of membrane proteins in a total volume of 0.5 mL containing 250 pM [125I]CYP in the presence or absence of 10 μM alprenolol to define nonspecific binding. The binding reactions were incubated at room temperature for 90 minutes and terminated by rapid filtration with ice-cold 25 mM Tris-HCl, pH 7.4, over Whatman GF/C glass fiber filters preincubated for ≥30 minutes in a buffer containing 25 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, and 0.3% polyethyleneimine.

2.4. Western Blotting. Western blotting was conducted as described previously [9]. Briefly, aliquots of 50–75 μg of cytosolic or membrane protein preparations were subjected to 10% denaturing polyacrylamide gel electrophoresis as previously described. Transfer was performed with a Trans-Blot SD Semi-dry transfer cell (Bio-Rad) on Protran nitrocellulose membrane (Mandel, Montréal, QC, Canada). Protein transfer efficiency was assessed using Ponceau S staining. Membranes were blocked using 5% nonfat dry milk in TBS-T (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.05% Tween 20) and membranes were incubated at 4°C overnight with primary antibody (anti-Phospho-Akt (Ser 473) and anti-Akt from Cell Signaling Technology, (Mississauga, Canada) or anti-PI-3K, anti-β₁- or anti-β₂-AR from Santa-Cruz (Calif, USA) diluted 1:1000 in 5% nonfat dry milk into TBS-T. Subsequently, membranes were washed and incubated for 45 minutes at room temperature with the secondary antibody (diluted 1:5000 in 5% nonfat dry milk into TBS-T) conjugated to horseradish peroxidase. Membranes were washed and exposed to scientific imaging film (Perkin Elmer Life Sciences, ON) or quantified using a Kodak ImageStation 440CF using enhanced chemiluminescence reagent (Perkin Elmer Life Sciences, Band). Intensities were analyzed using Kodak 1D v.3.5.5 Scientific Imaging Software.

2.5. Immunohistochemistry and Receptor Internalization. Sequestration of β-AR was observed by immunolocalisation. After agonist treatment, cells were washed with PBS and fixed with 3% paraformaldehyde for 15 minutes. After these washes, nonspecific sites were blocked with 0.2% BSA and 0.15% Triton x-100 (blocking solution) for 10 minutes. Primary antibody (β₁- and β₂-AR from Santa-Cruz), prepared in the blocking solution (1:200), was added for 30 minutes at room temperature. After another series
of washes, secondary antibody (antirabbit, Santa-Cruz,) also
prepared in the blocking solution (1:500) was added for 30
minutes. After a final series of washes, slides were mounted
and viewed using a Leica epi-illumination microscope.

2.6. PI-3K Activity. PI-3K activity was measured as pre-
viously described [10]. Briefly, 250–375 μg of cytosolic and membrane proteins were precipitated with anti-
phosphotyrosine antibody conjuged to biotin (1:50, Santa-
Cruz, Calif, USA) overnight at 4°C. The immune complex
was pelleted (with streptavidin beads) and washed three
times with lysis buffer and twice with phosphate-buffered
saline buffer containing 0.1 mM Na3VO4. The immune
pellet was then suspended in activation buffer (35 mM
ATP, 0.2 mM adenosine, 30 mM MgCl2, 10 mg/mL L-α-
phosphatidylinositol, and 20 μCi [γ-32P]-ATP; (Amersham
Pharmacia Biotech, Baie-d’Urfé, Canada) and incubated
at room temperature for 20 minutes. The reaction was
stopped with the addition of 100 μL HCl 1 M and 200 μL
of chloroform:methanol (1:1). The aqueous phase was then
resolved by thin layer chromatography on K6 Silica Gel plates
(Whatman, Clifton, NJ, USA) in a solvent system contain-
ing chloroform:methanol:ammonium hydroxide (45:35:10).
Plates were exposed to film for three to five days (−80°C).

2.7. Statistical Analysis. Results are expressed as mean ±
SEM and were evaluated using analysis of variance adapted
for factorial experimental design. Orthogonalization was
performed when necessary [11]. P < .05 was considered
significant.

3. Results

3.1. Expression of β-AR Subtypes and p85ΔPI-3K in HEK
293 Cells. HEK 293 cells were transiently transfected with
cDNAs encoding for β1-AR Wt, β2-AR Wt, β1 × β2-AR, or
β2 × β1-AR and in some case, p85ΔPI-3K. Forty-eight hours
after transfection β-AR expression levels were approximately
500 fmol/mg of proteins (compared to 10–20 fmol/mg in
untransfected cells). Expression of p85ΔPI-3K determined by
western blot was increased 3.24 times as compared to wild-
type cells (data not shown, n = 3).

3.2. Stimulation of β2-AR but not β1-AR Induces Activation
of PI-3K/Akt Pathway. PI-3K activation by the β1- or β2-
AR was measured by in vitro phosphorylation of L-α-
phosphatidylinositol. Transfected cells were stimulated with
isoproterenol 1 μM for 0, 5, or 15 minutes at 37°C. Stimulation
of β2-AR- for 5 or 15 minutes induced a significant
augmentation in PI-3K activity compared to basal conditions
(nonstimulated; Figure 1). In contrast, β1-AR stimulation
had no effect on PI-3K activity. Thus, the stimulation of
β2-AR but not β1-AR by isoproterenol induces activation
of PI-3K in our model. We suspect that the apparent
high basal level of PI-3K activation observed in our cell
line may be due to the phosphotyrosine antibody used to
immunoprecipitate the activated PI-3K. Using this antibody,
we immunoprecipitate other PI-3K subtypes as well as
phosphotyrosine proteins that although not activated by β-
AR may still contribute to the basal level of activation.

To confirm differences between stimulation of the two β-
AR subtypes on PI-3K activity, we used a measure of Akt
(a downstream PI-3K effector) activation. Akt activation,
as determined by the phosphorylation status of Serine 473,
was significantly increased after β2-AR-stimulation for 5
and 15 minutes as compared to the basal state (Figure 2).
Stimulation of β1-AR did not modify the phosphorylation
status of Akt as compared to control, confirming that
β1-AR activation cannot stimulate PI3-kinase/Akt pathway
activation. Stimulation of untransfected HEK293 with
isoproterenol did not result in any significant activation of Akt
(data not shown).

3.3. Stimulation of Either β1- or β2-AR Induces PI-3K Recruit-
ment to the Plasma Membrane. To determine whether β1-
AR can recruit PI-3K to the plasma membrane, immunolo-
calisation of PI-3K was performed using Western blotting
with anti-P110γ antibody. Transiently transfected cells with
β1- or β2-AR were stimulated by isoproterenol 1 μM for 0,
5, or 15 minutes at 37°C. Both β1- and β2-AR stimulation
results in recruitment of PI-3K to the particulate fraction
(Figure 3). Compared with control (i.e., nonstimulated
cells), the presence of PI-3K was significantly increased (P <
.05) in membrane fractions by agonist stimulation of either
β1-AR or β2-AR for 5 or 15 minutes. No significant difference
was detected between 5 and 15 minutes of stimulation. Thus,
either subtype of β-AR can recruit PI-3K to the membrane
after agonist stimulation but only the β2-AR results in PI-3K activation.

3.4. Recruitment of PI-3K to the Plasma Membrane is Gβγ Subunit-Dependent. It has been shown that PI-3K and GRK2 form a cytosolic complex [2]. To determine whether the mechanism of PI-3K recruitment to the plasma membrane was Gβγ subunit-dependent, β1- or β2-AR was transiently cotransfected with the carboxyl-terminal portion (ct) of GRK2 (a sequestering agent for Gβγ) in HEK 293 cells. Cells cotransfected with β1- or β2-AR and ct-GRK2 were stimulated with 1 μM isoproterenol for 5 minutes. ct-GRK2 when transfected alone had no effect on PI-3K activity. Stimulation of the β2-AR for 5 minutes with isoproterenol in cells cotransfected with ct-GRK2 resulted in a significantly decreased (P < .001) PI-3K recruitment to the particulate fraction (Figure 4) as compared to stimulation of the β2-AR expressed alone. Similar results were obtained when cells expressing the β1-AR were stimulated for 5 minutes with the agonist, that is, the presence of ct-GRK2 significantly decreased (P < .001) PI-3K recruitment to membranes (Figure 4). Thus, PI-3K recruitment to the plasma membrane by either β1- or β2-AR stimulation is Gβγ subunit-dependent although only β2-AR-dependent recruitment results in subsequent PI-3K activation.

3.5. PI-3K/Akt Pathway Activation Following Stimulation of Chimeric β1 × β2-AR and β1 × β1-AR. To determine the importance of receptor-specific determinants in the β-AR carboxy tail for PI-3K activation, we constructed two chimeric receptors which consisted of the β1-AR with the proximal seventh transmembrane domain and carboxy-terminal tail of the β2-AR (β1 × β2-AR) as well as the reciprocal receptor which consisted of β2-AR with the carboxy-terminal tail of the β1-AR (β2 × β1-AR). When the β1 × β2-AR was stimulated by 1 μM isoproterenol for 5 or 15 minutes, PI3-kinase activity was significantly increased (Figure 5; P < .05). On the other hand, agonist stimulation of the β2 × β1-AR for 5 or 15 minutes did not result in increased PI3-kinase activity. These results suggest that the carboxy-terminal tail of the β2-AR contains molecular determinants which are required for PI3-kinase activation.

**Figure 2:** Akt activation following β-AR stimulation. HEK 293 cells were transfected with β1-AR or β2-AR. Forty-eight hours after the transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. Akt activation status was determined by immunoblot for the phosphorylated form of Akt compared to the total amount of Akt after stimulation of β1-AR (□) or β2-AR (■) expressing cells. Top panel is a representative immunoblot of the experiments (n = 4–5). Data from these experiments is quantitated in the bottom panel as described in Section 2. *P < .05 versus 0 minute.

**Figure 3:** PI-3K Partitioning of PI-3K to the membrane. Forty-eight hours after transfection, cells were stimulated with 1 μM isoproterenol for the indicated times. Membranes were isolated, solubilized, and approximately 100 μg of membrane proteins were separated on a 10% SDS-PAGE gel. Membrane-associated PI-3K levels increased with cells expressing β1-AR (□) or β2-AR (■). Top panel is a representative immunoblot of the experiments (n = 3–4). Data from these experiments is quantitated in the bottom panel as described in Section 2. *P < .05 versus 0 minute.

**Figure 4:** GRK2 carboxy-terminal domain reduced PI-3K recruitment to membrane fractions in response to β-AR stimulation. HEK 293 cells were cotransfected with ct-GRK-2 and β-AR subtypes. Forty-eight hours after the transfection, cells were stimulated with 1 μM isoproterenol for 5 minutes. PI3-kinase recruitment to particulate fractions decreased with cells expressing either β1-AR (□) or β2-AR (■) in presence of cotransfected ct-GRK2. Top panel is a representative immunoblot of the experiments (n = 4–6). Data from these experiments is quantitated in the bottom panel as described in Section 2. *P < .05 versus 0 minute.
To confirm results obtained for the PI-3K activation mediated by the two chimeric β-ARs, we again determined the activation status of Akt. Chimeric receptors were stimulated with 1 μM isoproterenol for 0, 5, or 15 minutes at 37°C. Akt phosphorylation was significantly increased (P < .05) in HEK 293 cells expressing β1 × β2-AR (Figure 6). Stimulation of β2 × β1-AR by isoproterenol had no effect on Akt phosphorylation as compared to basal conditions. Thus chimeric β1 × β2-AR increased Akt phosphorylation and confirmed that the carboxyl-terminal tail of the β2-AR contained molecular determinants necessary and sufficient for PI-3K activation.

3.6. Effect of Pertussis Toxin Treatment on β-AR Stimulation of PI3-K Activity. It has been proposed that stimulation of PI-3K by β2-AR is pertussis toxin sensitive [1, 12]. To determine the effect of pertussis toxin on β-AR induced stimulation of PI-3K, we incubated the cells with PTX for 18 hours followed by a stimulation with 1 μM isoproterenol for 15 minutes. Our results indicate that, in presence of PTX, no activation of PI-3K was observed with β2-AR stimulation (Figure 7(b)). In presence of the chimeric receptors, PTX treatment abolished the activation of PI-3K observed with the chimeric β1 × β2-AR (Figure 7(d)). Consistent with our earlier data, neither the β1-AR (Figure 7(a)) nor the β2 × β1-AR (Figure 7(c)) was sensitive to PTX pretreatment. These results indicate that stimulation of PI-3K by β2-AR is pertussis toxin sensitive.

3.7. Involvement of PI3-K Activity on β-AR Sequestration. Cells expressing β1-AR were treated with isoproterenol for 15 minutes. After stimulation, very little β1-AR was observed in the interior compartments of treated cells and no significant difference was observed in presence of either cotransfected p85ΔPI-3K or PTX pretreatment (Figures 8(a) to 8(d)). In contrast, we observed a significant internalization of β2-AR after a similar period of agonist stimulation as shown by clustered distribution of the receptor (Figure 8(f); outline of the cell is poorly defined in this particular condition). β2-AR internalization was inhibited in the presence of either cotransfected p85ΔPI-3K or PTX pretreatment (Figures 8(g) and 8(h)). These results confirmed that functional PI-3K is important for the sequestration of β2-AR and attests to the effectiveness of our p85ΔPI-3K cotransfection or PTX pretreatment.

4. Discussion

The results of the current study demonstrate that stimulation of β2-AR, but not β1-AR, induces PI-3K activation by a pertussis toxin-sensitive mechanism. This subtype-dependent activation has been reinforced by the fact that Akt, a downstream PI-3K effector, was also selectively activated by β2-AR stimulation. However, both receptor subtypes can recruit the PI-3K to the plasma membrane via a Gβγ subunit-dependent mechanism. Naga Prasad et al. [2] had previously demonstrated that PI-3K and GRK2 formed a cytosolic complex and the recruitment of the enzyme to the plasma membrane was facilitated by Gβγ subunits in an agonist-dependent manner. Our results confirmed that PI-3K is recruited to the plasma membrane via a Gβγ subunit-dependent mechanism. GRK-2 is important for PI-3K recruitment to the plasma membrane, thus we thought this might suggest that the carboxyl-terminal domain of the receptor is important for the PI-3K activation because the carboxyl-terminal domain of the β-AR contains sites phosphorylated by GRK2. Furthermore, Shiina et al. [13] had demonstrated that the carboxyl-terminal domain and
Figure 7: Pertussis toxin effects on β-AR-mediated PI-3K activation. Twenty-six hours after transfection, cells were incubated with 0.1 μg/mL pertussis toxin for 18 hours. Cells were stimulated for 5 minutes with 1 μM isoproterenol prior to processing for TLC as described in Figure 1 and Section 2. PI-3K activity was determined by the level of [32P]-PI produced by the stimulation of β1-AR (a), β2-AR (b), β2 × β1-AR (c), and β1 × β2-AR expressing cells. (n = 3-4; *P < .05 versus basal and PTX-Iso conditions).

the third cytosolic loop are the regions mostly responsible for the difference in internalization behavior between both β-AR subtypes.

To determine the potential contribution of the carboxyl-terminal domains of the β1- and β2-AR in the PI-3K activation, chimeric β1- and β2-AR in which the seventh transmembrane domain and the carboxyl-terminal domain have been exchanged were constructed. We observed that the chimeric β1 × β2-AR could activate PI-3K in contrast to the wild type β1-AR. Reciprocally, β2-AR lost its ability to activate PI-3K when its carboxy-terminal domain was exchanged for that of the β1-AR. This result demonstrated that the β2-AR carboxy-terminal domain contains important molecular determinants for PI-3K activation.

The present study confirms PI-3K activation by β2-AR stimulation as observed in other studies [2, 12]. However, the kinetics of PI-3K activation previously reported for β2-AR stimulation was different to that observed in the present study. PI-3K was rapidly activated following β2-AR stimulation and returned to basal levels after 10 minutes. In our study, we observed a significant PI-3K activation with a 5-minute β2-AR stimulation that was maintained after 15 minutes. The difference in activation patterns may be due to the agonist concentration used. In the previous study [2], 10 μM isoproterenol was used which is 10 times higher than the concentration used here. Higher concentration of agonist may induce more rapid PI-3K recruitment to the membrane and thus activation of the enzyme but may also more rapidly stimulate mechanisms which terminate these signals as well.

Pretreatment with pertussis toxin results in a loss of PI-3K activation by β2-AR stimulation confirming results obtained in other studies [1, 12]. The new information afforded by the present study is that the carboxy-terminal portion of the receptor is important for the interaction between the receptor, Gi heterotrimers (as stimulation depends on both the Gα and Gβγ subunits), and PI-3K. Indeed, the chimeric receptor β1 × β2-AR can activate the PI-3K by a pertussis toxin sensitive mechanism whereas the chimeric receptor β2 × β1-AR is unable to activate PI-3K. However, other regions of the receptor may also be important for full activation or regulation of the response by the desensitization machinery.

Our results demonstrate that isoproterenol-stimulated β1-AR (for up to 15 minutes) cannot activate PI-3K. These
Figure 8: p85ΔPI-3K reduced agonist-induced β2-AR sequestration. HEK 293 cells were transfected with p85ΔPI-3K and the two β-AR subtypes. Cells were stimulated with 1 μM isoproterenol for 15 minutes (b) and (f). Stimulation of β2-AR-transfected cells induced a cluster distribution which is characteristic of receptor internalization (f). This sequestration was abolished in presence of either cotransfected p85ΔPI-3K (g) or PTX pretreatment (h). No significant β1-AR sequestration was observed under the different conditions used in these experiments (a)–(d). Representative images of several experiments (n = 8–15) are shown.

Results contrast with those reported in a previous study in which activation of PI-3K was observed after stimulation of β1-AR transfected into HEK 293 cells [2]. Several possibilities can explain the discrepancy between both studies. First, 1 μM isoproterenol although sufficient to induce submaximal adenylyl cyclase activity [8] cannot induce PI-3K activation. Secondly, in our study we use transiently transfected cells that express approximately 500 fmol receptor/mg of membrane proteins, which is closer to physiological levels than seen with the study of Naga Prasad et al. [2]. Also, it is clear that the interplay between the signalling and internalization machinery is different for the two receptors even though they can interact with many of the same proteins. This study provides another such example that both β1-AR and β2-AR lead to PI-3K and GRK2 recruitment; both the signalling and desensitization outcomes for these events are markedly different. Some studies have also shown that β1-AR, in contrast to the β2-AR, cannot activate Gi [14] while others suggest that it can [15]. It is possible that other cell- and tissue-specific factors might regulate these events as well.

In conclusion, we observed that PI-3K activation is β-AR subtype specific and the difference is due to molecular determinants present in the carboxy-terminal tail of the receptors. PI-3K activation is pertussis toxin sensitive and is necessary for the sequestration of the β2-AR.

Acknowledgments

The second author is a scholar of the Fonds de la Recherche en Santé du Québec, Heart and Stroke Foundation, and Réseau en Santé Cardiovasculaire. The fourth author is a Chercheur National of the Fonds de la Recherche en Santé du Québec. The fifth author is a scholar of the Fonds de la Recherche en Santé du Québec. This work was supported by National Sciences and Engineering Research Council of Canada (250234-07).
References

[1] A. Chesley, M. S. Lundberg, T. Asai, et al., “The β2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through Gi-dependent coupling to phosphatidylinositol 3′-kinase,” *Circulation Research*, vol. 87, no. 12, pp. 1172–1179, 2000.

[2] S. V. Naga Prasad, L. S. Barak, A. Rapacciuolo, M. G. Caron, and H. A. Rockman, “Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by β-adrenergic receptor kinase 1: a role in receptor sequestration,” *The Journal of Biological Chemistry*, vol. 276, no. 22, pp. 18953–18959, 2001.

[3] R.-P. Xiao, W. Zhu, M. Zheng, et al., “Subtype-specific β-adrenoceptor signaling pathways in the heart and their potential clinical implications,” *Trends in Pharmacological Sciences*, vol. 25, no. 7, pp. 358–365, 2004.

[4] W.-Z. Zhu, S.-Q. Wang, K. Chakir, et al., “Linkage of β1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II,” *The Journal of Clinical Investigation*, vol. 111, no. 5, pp. 617–625, 2003.

[5] L. S. Barak, A. M. Wilbanks, and M. G. Caron, “Constitutive desensitization: a new paradigm for g protein-coupled receptor regulation,” *ASSAY and Drug Development Technologies*, vol. 1, no. 2, pp. 339–346, 2003.

[6] T. A. Kohout, F.-T. Lin, S. J. Perry, D. A. Conner, and R. J. Lefkowitz, “β-arrestin 1 and 2 differentially regulate heptahelical receptor signaling trafficking,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 4, pp. 1601–1606, 2001.

[7] E. R. Prossnitz, “Novel roles for arrestins in the post-endocytic trafficking of G protein-coupled receptors,” *Life Sciences*, vol. 75, no. 8, pp. 893–899, 2004.

[8] G. Rousseau, F. Nantel, and M. Bouvier, “Distinct receptor domains determine subtype-specific coupling and desensitization phenotypes for human β1- and β2-adrenergic receptors,” *Molecular Pharmacology*, vol. 49, no. 4, pp. 752–760, 1996.

[9] M. Boucher, S. Nim, C. de Montigny, and G. Rousseau, “Alterations of β-adrenoceptor responsiveness in postischemic myocardium after 72 h of reperfusion,” *European Journal of Pharmacology*, vol. 495, no. 2-3, pp. 185–191, 2004.

[10] M. Boucher, S. Pesant, S. Falcao, et al., “Post-Ischemic cardioprotection by A2A adenosine receptors: dependent of phosphatidylinositol 3-kinase pathway,” *Journal of Cardiovascular Pharmacology*, vol. 43, no. 3, pp. 416–422, 2004.

[11] B. J. Winer, *Statistical Principles in Experimental Design*, McGraw-Hill, New York, NY, USA, 1971.

[12] S.-H. Jo, V. Leblais, P. H. Wang, M. T. Crow, and R.-P. Xiao, “Phosphatidylinositol 3-kinase functionally compartmentalizes the concurrent Gs signaling during β2-adrenergic stimulation,” *Circulation Research*, vol. 91, no. 1, pp. 46–53, 2002.

[13] T. Shiina, A. Kawasaki, T. Nagao, and H. Kurose, “Interaction with beta-arrestin determines the difference in internalization behavior between beta1- and beta2-adrenergic receptors,” *The Journal of Biological Chemistry*, vol. 275, no. 37, pp. 29082–29090, 2000.

[14] M. Kuschel, Y.-Y. Zhou, H. Cheng, et al., “Gs protein-mediated functional compartmentalization of cardiac β1-adrenergic signaling,” *The Journal of Biological Chemistry*, vol. 274, no. 31, pp. 22048–22052, 1999.

[15] N. P. Martin, E. J. Whalen, M. A. Zamah, K. L. Pierce, and R. J. Lefkowitz, “PKA-mediated phosphorylation of the β1-adrenergic receptor promotes Gi/Gs switching,” *Cellular Signalling*, vol. 16, no. 12, pp. 1397–1403, 2004.

[16] I. Gaidarov, J. G. Krupnick, J. R. Fack, J. L. Benovic, and J. H. Keen, “Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding,” *The EMBO Journal*, vol. 18, no. 4, pp. 871–881, 1999.

[17] T. Suzuki, C. T. Nguyen, F. Nantel, et al., “Distinct regulation of beta 1- and beta 2-adrenergic receptors in Chinese hamster fibroblasts,” *Molecular Pharmacology*, vol. 41, no. 3, pp. 542–548, 1992.

[18] A. Marchese, C. Chen, Y.-M. Kim, and J. L. Benovic, “The ins and outs of G protein-coupled receptor trafficking,” *Trends in Biochemical Sciences*, vol. 28, no. 7, pp. 369–376, 2003.

[19] S. V. Naga Prasad, S. A. Laporte, D. Chamberlain, M. G. Caron, L. Barak, and H. A. Rockman, “Phosphoinositol 3-kinase regulates β1-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex,” *The Journal of Cell Biology*, vol. 158, no. 3, pp. 563–575, 2002.