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Troglitazone stimulates beta-arrestin-dependent cardiomyocyte contractility via the angiotensin II type 1A receptor.

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**“Troglitazone stimulates β -arrestin-dependent cardiomyocyte contractility via
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Abstract

Peroxisome proliferator-activated receptor γ (PPAR γ) agonists are commonly used to treat cardiovascular diseases, and are reported to have several effects on cardiovascular function that may be due to PPAR γ -independent signaling events. Select angiotensin receptor blockers (ARBs) interact with and modulate PPAR γ activity, thus we hypothesized that a PPAR γ agonist may exert physiologic effects via the angiotensin II type 1A receptor (AT1_AR). In AT1_AR-overexpressing HEK 293 cells, both angiotensin II (Ang II) and the PPAR γ agonist troglitazone (Trog) enhanced AT1_AR internalization and recruitment of endogenous β -arrestin1/2 (β arr1/2) to the AT1_AR. A fluorescence assay to measure diacylglycerol (DAG) accumulation showed that although Ang II induced AT1_AR-G_q protein-mediated DAG accumulation, Trog had no impact on DAG generation. Trog-mediated recruitment of β arr1/2 was selective to AT1_AR as the response was prevented by an ARB and Trog-mediated β arr1/2 recruitment to β 1-adrenergic receptor (β 1AR) was not observed. In isolated mouse cardiomyocytes, Trog increased both % and rate of cell shortening to a similar extent as Ang II, effects which were blocked with an ARB. Additionally, these effects were found to be β arr2-dependent, as cardiomyocytes isolated from β arr2-KO mice showed blunted contractile responses to Trog. These findings show for the first time that the PPAR γ agonist Trog acts at the AT1_AR to simultaneously block G_q protein

activation and induce the recruitment of β arr1/2, which leads to an increase in cardiomyocyte contractility.

Keywords: PPAR γ ; troglitazone; AT1 $_A$ R; β -arrestin; cardiomyocyte; contractility.

Introduction

Angiotensin II (Ang II)-mediated activation of the angiotensin type 1 $_A$ receptor (AT1 $_A$ R), a member of the seven-transmembrane receptor (7TMR) family, leads to the initiation of both G $_q$ protein-dependent and -independent signaling cascades. G $_q$ protein-dependent signaling involves 2nd messenger generation (e.g. diacylglycerol (DAG) accumulation), ultimately leading to a hypertrophic response that can be maladaptive over time, but that can be attenuated via the use of angiotensin receptor blockers (ARBs) [1; 2]. G protein-independent signaling involves recruitment of β -arrestins 1 and/or 2 (β arr1/2) [3], multifunctional scaffold proteins that are involved in numerous cell signaling events including initiation of receptor internalization, activation of protein kinase and anti-apoptotic cascades and transactivation of epidermal growth factor receptor [3; 4; 5]. β -arrestin-dependent signaling has been shown to be both physiologically relevant and beneficial in the cardiovascular system. In cardiomyocytes, β -arrestin signaling mediates protection against chronic sympathetic stimulation [6] and increases contractility in response to AT1 $_A$ R stimulation [7]. Additionally, β -arrestin signaling has been shown to activate anti-apoptotic pathways downstream of AT1 $_A$ R in vascular smooth muscle cells [5]. Thus, an agent that acts selectively at the AT1 $_A$ R to induce beneficial β -arrestin

signaling without increasing detrimental G_q protein activity could be effective in the treatment of cardiovascular disorders.

Recent studies have shown that synthetic agonists for the transcription factor peroxisome proliferator-activated receptor γ (PPAR γ) oppose Ang II-mediated effects, reducing the hypertrophic response of cardiomyocytes to administration of Ang II as well as decreasing Ang II-mediated signaling in blood vessels and subsequent development of hypertension [8; 9; 10; 11]. Synthetic agonists for PPAR γ are typically used in the treatment of Type II diabetes to increase insulin sensitivity [12]. These agents have been shown experimentally and clinically to improve cardiovascular function via increased vascular reactivity and cardiac function, decreased inflammation and reduced visceral fat storage (as reviewed in [13]). The mechanisms by which PPAR γ agonists oppose Ang II-mediated signaling are not fully understood, but may be independent of PPAR γ itself. In fact, PPAR γ agonists such as troglitazone (Trog) have been shown to induce acute intracellular signaling responses independent of the effects of PPAR γ on transcriptional processes, though a role for 7TMRs in mediating these responses has not been explored [14; 15].

While PPAR γ agonists have been shown to oppose Ang II signaling, a subset of angiotensin receptor blockers (ARBs) have been shown to be selective PPAR γ modulators (SPARRMs), increasing gene transcription and modulating metabolic pathways to reduce glucose and triglyceride levels and increase insulin sensitivity [16; 17; 18]. Since PPAR γ agonists can induce PPAR γ -independent signaling, and select ARBs can activate PPAR γ , we tested the hypothesis that PPAR γ agonists activate AT1_AR signaling. Here, we show that Trog stimulates the AT1_AR to induce endogenous β arr1/2 recruitment and AT1_AR internalization without G_q protein activation. Moreover, cardiomyocyte contractility is increased in response to

Trog, an effect that is sensitive to both an ARB and β arr2 expression, indicating that activation of AT1_AR- β -arrestin-mediated signaling is a mechanism by which PPAR γ agonists can induce PPAR γ -independent physiologic effects.

Materials and Methods

Materials. HEK 293 cells stably expressing hemagglutinin-tagged AT1_AR (HA-AT1_AR cells), the plasmid construct for monomeric cyan fluorescent protein (mCFP)/mYFP-tagged diacylglycerol reporter (DAGR) and β arr2 knockout mice were kindly provided by Dr. RJ Lefkowitz (Duke University Medical Center). Troglitazone (Trog) was obtained from Calbiochem (San Diego, CA). Ang II, isoproterenol and losartan were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and pharmacological treatment. HEK 293 cells stably expressing FLAG-tagged β 1-adrenergic receptor (FLAG- β 1AR cells) and HA-AT1_AR cells were maintained in MEM supplemented with 10% FBS and 1% pen/strep at 37°C. Cells were serum-starved for 1hr prior to drug treatments outlined in figure legends.

Fluorescent microscopy. HA-AT1_AR cells were seeded into 35mm glass-bottom confocal dishes (MatTek Corporation, MA) coated with 10 μ g/mL collagen (Sigma-Aldrich). After 5 min stimulation, cells were fixed with 4% paraformaldehyde/PBS for 20 min and permeabilized for 5 min with ice-cold 0.2% Triton-X 100 in PBS. Cells were blocked for 1hr and all antibodies were diluted in 0.1% BSA in PBS. HA-AT1_AR was visualized using monoclonal anti-HA clone HA-7 (Sigma-Aldrich) at 1:1,000 overnight, 4°C, followed by goat anti-mouse IgG, Dylight594

(Thermo Scientific, Rockford IL) at 1:1000 for 1hr. Samples were imaged using a Leica DMI6000B inverted microscope with the Leica DFC360 FX 1.4-megapixel monochrome digital camera. This system was controlled by and deconvolution carried out using Leica AF6000 software. Each condition was performed independently 3 times.

Chemical crosslinking and immunoprecipitation. Crosslinking was performed as previously described [19]. Immunoprecipitation (IP) of samples was performed using 200 to 500 μ g protein and overnight incubation at 4°C with 25 μ L of monoclonal anti-HA agarose or anti-FLAG M2 agarose (Sigma-Aldrich). β arr1/2 recruitment was normalized to total β arr1/2 levels and amount of immunoprecipitated HA-AT1_AR or FLAG- β 1AR.

Immunoblotting. Following drug treatment, cells were rinsed in ice-cold PBS then collected and lysed in buffer containing 1% NP40, 20mM Tris, pH 7.4, 137mM NaCl, 100 μ g/mL PMSF, 10% Glycerol, 10mM NaF, 0.36mg/mL Na₃VO₄ and EDTA-free HALT protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein estimation was carried using Pierce 660nm Protein Assay Reagent (Thermo Scientific), and immunoblotting of samples performed as previously described [19]. The LI-COR biosciences Odyssey system was used for detection of immunoblots using anti- β -arrestin1/2 (D24H9) at 1:5,000 (Cell Signaling Technology, Danvers, MA) and anti-FLAG M2 at 1:10,000 (Sigma-Aldrich). IRDye800 conjugated anti-HA epitope tag was used at 1:20,000 (Rockland, Gilbertsville, PA). Non-conjugated primary antibodies were detected with IRDye680 Donkey anti-rabbit IgG (H+L) at 1:5,000 in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE).

DAGR assay. HA-AT1_AR cells transfected with DAGR were seeded into 35mm glass-bottom confocal dishes (MatTek Corporation, MA) coated with 10 µg/mL fibronectin. Prior to assay, cells were rinsed and incubated in buffer containing 125mM NaCl, 5mM KCl, 1.5mM MgCl₂, 1.5mM CaCl₂, 10mM glucose, 0.2% BSA, 10mM HEPES, pH 7.4. Cells were stimulated with drugs and underwent fluorescence resonance energy transfer (FRET) analysis as described previously [20], with quantification of the DAGR ratio over time calculated as FRET intensity/mCFP intensity, normalized to baseline.

Cardiomyocyte Contractility

Mice used in these studies were C57BL/6 mice and β arr2 knockout mice (described previously, [7; 21]), 3-6 months old and 25-40g. 10-15 cardiomyocytes were used per treatment per heart. Animals were handled according to approved protocols and animal welfare regulations of the Institutional Review Board at Duke University Medical Center. Myocyte isolation, visualization and analysis were carried out as previously described [22; 23].

Statistical Analysis

Data are presented as mean \pm s.e.m. All statistical analyses were performed using One-way ANOVA with Tukey's multiple comparisons test via Prism 5.0 software.

Results

Troglitazone induces AT1_AR internalization and β -arrestin recruitment without activation of G_q protein.

To determine if a PPAR γ agonist can stimulate the AT1_AR, we performed several assays to explore different facets of receptor activation. A common response to 7TMR stimulation is internalization, thus we initially aimed to determine if the PPAR γ agonist Trog induces AT1_AR internalization. HA-AT1_AR cells were treated with Trog or Ang II, as a positive control for internalization, and HA-AT1_AR puncta formation was assessed via immunofluorescence analysis. HA-AT1_AR puncta formation was increased in response to both Ang II and Trog (Fig. 1, panels 2 and 3 arrowheads), indicating that the PPAR γ agonist stimulates AT1_AR internalization.

Downstream effects of AT1_AR activation are mediated by both G_q protein-dependent and β arr1/2-dependent signaling [24]. G_q protein activity can be assessed using DAGR, a fluorescent biosensor containing a diacylglycerol (DAG) binding domain flanked by mCFP and mYFP that produces an increase in intermolecular FRET in response to DAG generation at the membrane [20]. HA-AT1_AR cells expressing DAGR underwent stimulation with Trog, or Ang II as a positive control for G_q protein activation. In response to Ang II, the FRET ratio increased sharply, indicating a rapid generation of DAG at the membrane (Fig. 2A) however stimulation with the same concentration of Trog (100 μ M) that induced AT1_AR internalization did not increase DAG production, as shown by an unaltered FRET ratio. These results indicate that, while Trog induces AT1_AR internalization, it does not stimulate AT1_AR-mediated G_q protein activity.

Since β arr1/2 recruitment to activated AT1_ARs is required for receptor internalization [24], we tested whether Trog acts to recruit endogenous β arr1/2 to the AT1_AR. HA-AT1_AR cells were stimulated with increasing concentrations of Trog, or Ang II as a positive control for β arr1/2 recruitment, and underwent chemical crosslinking, immunoprecipitation of HA-AT1_AR

and immunoblotting analysis. Trog and Ang II each significantly increased β arr1/2 recruitment to HA-AT1_AR in a concentration-dependent manner (Fig. 2B). Concentrations of Ang II in the low nanomolar range (5-10nM) produced equivalent β arr1/2 recruitment elicited by concentrations of Trog in the high micromolar range (50-100 μ M), concentrations shown to induce AT1_AR internalization (Fig. 1) and previously shown by others to mediate PPAR γ -independent effects [14; 25]. These data show that while Trog has no effect on AT1_AR-coupled G_q protein activity, it does induce β arr1/2 recruitment to the AT1_AR and promote receptor internalization.

Troglitazone-mediated β -arrestin recruitment is selective for the AT1_AR

Trog-mediated β arr1/2 recruitment to the AT1_AR suggests that Trog acts at the AT1_AR itself to stimulate the response, thus we tested whether β -arrestin recruitment could be blocked with an ARB. Using matched submaximal concentrations of Trog (50 μ M) and Ang II (5nM) for β arr1/2 recruitment, the ability of the ARB, losartan (Los), to block Trog-mediated β arr1/2 recruitment to AT1_AR was assessed via immunoprecipitation. As a positive control for Los-mediated inhibition of the AT1_AR, Ang II-mediated β arr1/2 recruitment to HA-AT1_AR was prevented by Los pretreatment (Fig. 3A). Similarly, Trog-mediated β arr1/2 recruitment to HA-AT1_AR was ablated by pretreatment with Los. These results indicate that Trog acts directly at the AT1_AR to induce β arr1/2 recruitment, an effect that can be surmounted with an ARB.

To investigate whether β arr1/2 recruitment in response to Trog is selective to the AT1_AR or is a nonselective response to high concentrations of Trog at 7TMRs in general, we tested the ability of Trog to induce recruitment of β arr1/2 to the β 1-adrenergic receptor (β 1AR). FLAG- β 1AR cells were stimulated with the highest concentration of Trog (100 μ M) shown to induce β arr1/2 recruitment to the AT1_AR, or isoproterenol (ISO, 1 μ M) as a positive control for β arr1/2

recruitment to the β 1AR. While ISO stimulation induced a significant increase in β arr1/2 recruitment to the β 1AR, Trog was unable to promote β -arrestin recruitment to the β 1AR (Fig. 3B), indicating that Trog-induced β arr1/2 recruitment is selective for the AT1_AR.

Troglitazone increases cardiomyocyte contractility via the AT1_AR in a β -arrestin-dependent manner

To determine if a PPAR γ agonist induces a physiologic effect mediated by AT1_AR signaling, we measured the effect of Trog on the contractility of freshly isolated murine cardiomyocytes. Treatment of wild-type cardiomyocytes with concentrations of Trog that were shown to induce β arr1/2 recruitment to AT1_AR (50-100 μ M, Fig. 2B) resulted in a significant increase in both the percentage and rate of cell shortening (Fig. 4A, black bars). These Trog-mediated effects on contractility were statistically indistinguishable from those of Ang II. Importantly, the Trog-mediated increase in cardiomyocyte contractility was blocked by pretreatment with an ARB (valsartan, Val). These results confirm our observation that Trog acts via the AT1_AR and indicate that this interaction is capable of mediating a physiologic increase in cardiomyocyte contractility.

Since β arr2 plays a role in AT1_AR-mediated myocyte contractility [7], we tested whether β arr2 signaling is required for Trog-mediated effects on myocyte contractility. Cardiomyocytes isolated from β arr2 knockout mice (β arr2-KO) exhibited blunted cell shortening responses to both Ang II and Trog stimulation that were not statistically different from untreated cells (Fig. 4A, grey bars). To ensure that β arr2-KO cardiomyocytes were responsive to a contractile stimulus, they were treated with ISO, which produced a significant increase in contractile response. Comparison of the contractility increases achieved in wild-type versus β arr2-KO

cardiomyocytes revealed a significantly diminished response to both Ang II and Trog in the absence of β -arr2 (Fig. 4B). Thus, β arr2 is a key mediator of Trog-induced cardiomyocyte contractility.

Discussion

In this study, we show that the PPAR γ agonist Trog acts at the AT1_AR to induce AT1_AR internalization and ARB-sensitive recruitment of β arr1/2, in the absence of G_q protein activation. Moreover, we show that Trog increases cardiomyocyte contractility in an ARB-sensitive manner and is, at least partially, β -arrestin2-dependent. Although PPAR γ agonists classically activate PPAR γ to mediate gene transcription, it has also been shown that these agents can exert PPAR γ -independent cell signaling processes [14; 15; 26]. Our observation that a PPAR γ agonist acts at the AT1_AR to rapidly recruit β arr1/2 suggests a lack of role for PPAR γ -mediated transcriptional events in this process. With regard to cardiomyocyte function, our data highlights the ability of Trog to induce an acute physiologic response via AT1_AR signaling, increasing cardiomyocyte contractility. These data, coupled with the ARB-sensitive nature of Trog-mediated AT1_AR- β -arrestin signaling, suggest that Trog-mediated effects at the level of AT1_AR are independent of PPAR γ activation.

PPAR γ agonists, including Trog and ciglitazone, have been previously reported to exert off-target effects, influencing intracellular processes independent of PPAR γ activation in different cell lines and tissue [14; 15; 26]. One of these processes involved alterations in intracellular and/or extracellular Ca²⁺-handling, dependent upon the PPAR γ agonist, though a role for AT1_AR in this process was not explored [14]. Such effects on Ca²⁺-handling may explain our observation that a portion of the Trog-induced cardiomyocyte contractility was

β arr2-independent, as approximately half the contractile response remained in the absence of β arr2 (Fig. 4C). In our study, we show Trog-mediated AT1_AR effects are independent of G_q protein activation but dependent on β -arrestin recruitment. The ability of a 7TMR to utilize β -arrestin to exert downstream signaling is now being recognized as a mechanism to stimulate physiologic effects and accumulating data suggests that β -arrestin-mediated signaling opposes detrimental G protein-mediated activity to confer beneficial cellular effects [3; 6; 27].

The ability of ligands to stabilize receptors in certain conformations that activate particular signaling pathways but not others has been increasingly explored [28; 29]. [Sar¹, Ile⁴, Ile⁸]-Ang (SII) was shown to act at the AT1_AR to preferentially enhance β -arrestin-mediated signaling with no effect on G_q protein activity and has been suggested to be more cardioprotective than conventional ARBs that prevent both G protein- and β -arrestin-dependent signaling [7; 19; 30]. Our study shows that the PPAR γ agonist Trog acts similarly to SII by preferentially enhancing β -arrestin recruitment to AT1_AR and subsequent cardiomyocyte contractility. We postulate that PPAR γ -mediated activation of AT1_AR- β -arrestin signaling may in part provide an explanation for the cardioprotective effects of PPAR γ agonists reported in the literature [8; 9; 10]. An additional benefit of PPAR γ agonist action at the AT1_AR may be the lack of potentially detrimental G_q protein-mediated signaling, especially under conditions of heightened Ang II stimulation, such as the development of hypertension. Others have suggested that the use of SPARRMs to simultaneously block AT1_AR activation and induce PPAR γ activity would be clinically more beneficial than conventional PPAR γ agonists by exerting both antihypertensive and antidiabetic effects [17; 18]. Alternatively, using Trog as an example, we suggest PPAR γ agonists may show enhanced clinical usefulness by simultaneously inducing

beneficial PPAR γ - and AT1_AR- β arrestin-mediated effects without a concomitant increase in potentially detrimental AT1_AR-G_q protein-mediated signaling.

Conclusions

Our study shows that the PPAR γ agonist Trog stimulates β -arrestin recruitment to the AT1_AR and induces receptor internalization independent of G_q protein activity. Effects of PPAR γ agonists on acute cardiomyocyte function have not previously been reported, and here we show Trog increases cardiomyocyte contractility in a β arr2-dependent manner. We propose that a PPAR γ agonist with enhanced efficiency at inducing β -arrestin recruitment to the AT1_AR, in the absence of G_q protein-mediated effects, could provide a means of potentiating the beneficial effects of both PPAR γ agonists and ARBs in the treatment of cardiovascular ailments.

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References

- [1] G.W. Dorn, 2nd, and T. Force, Protein kinase cascades in the regulation of cardiac hypertrophy. *J Clin Invest* 115 (2005) 527-37.
- [2] B.J. Hoogwerf, Renin-angiotensin system blockade and cardiovascular and renal protection. *Am J Cardiol* 105 30A-5A.
- [3] S.M. DeWire, S. Ahn, R.J. Lefkowitz, and S.K. Shenoy, Beta-arrestins and cell signaling. *Annu Rev Physiol* 69 (2007) 483-510.
- [4] D.G. Tilley, I.M. Kim, P.A. Patel, J.D. Violin, and H.A. Rockman, beta-Arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. *J Biol Chem* 284 (2009) 20375-86.

- [5] S. Ahn, J. Kim, M.R. Hara, X.R. Ren, and R.J. Lefkowitz, β -Arrestin-2 Mediates Anti-apoptotic Signaling through Regulation of BAD Phosphorylation. *J Biol Chem* 284 (2009) 8855-65.
- [6] T. Noma, A. Lemaire, S.V. Naga Prasad, L. Barki-Harrington, D.G. Tilley, J. Chen, P. Le Corvoisier, J.D. Violin, H. Wei, R.J. Lefkowitz, and H.A. Rockman, Beta-arrestin-mediated β_1 -adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest* 117 (2007) 2445-58.
- [7] K. Rajagopal, E.J. Whalen, J.D. Violin, J.A. Stiber, P.B. Rosenberg, R.T. Premont, T.M. Coffman, H.A. Rockman, and R.J. Lefkowitz, Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci U S A* 103 (2006) 16284-9.
- [8] C. De Ciuceis, F. Amiri, M. Iglarz, J.S. Cohn, R.M. Touyz, and E.L. Schiffrin, Synergistic vascular protective effects of combined low doses of PPAR α and PPAR γ activators in angiotensin II-induced hypertension in rats. *Br J Pharmacol* 151 (2007) 45-53.
- [9] M. Asakawa, H. Takano, T. Nagai, H. Uozumi, H. Hasegawa, N. Kubota, T. Saito, Y. Masuda, T. Kadowaki, and I. Komuro, Peroxisome proliferator-activated receptor gamma plays a critical role in inhibition of cardiac hypertrophy in vitro and in vivo. *Circulation* 105 (2002) 1240-6.
- [10] K. Benkirane, E.C. Viel, F. Amiri, and E.L. Schiffrin, Peroxisome proliferator-activated receptor gamma regulates angiotensin II-stimulated phosphatidylinositol 3-kinase and mitogen-activated protein kinase in blood vessels in vivo. *Hypertension* 47 (2006) 102-8.
- [11] Z. Wei-Guo, Y. Hui, L. Shan, Z. Yun, N. Wen-Cheng, Y. Fu-Lin, F. Fang-Yan, G. Jun-Hua, and Z. Jian-Hua, PPAR-gamma agonist inhibits Ang II-induced activation of dendritic cells via the MAPK and NF-kappaB pathways. *Immunol Cell Biol* (2009).
- [12] M. Abdelrahman, A. Sivarajah, and C. Thiernemann, Beneficial effects of PPAR-gamma ligands in ischemia-reperfusion injury, inflammation and shock. *Cardiovasc Res* 65 (2005) 772-81.
- [13] C.B. Patel, J.A. De Lemos, K.L. Wyne, and D.K. McGuire, Thiazolidinediones and risk for atherosclerosis: pleiotropic effects of PPAR gamma agonism. *Diab Vasc Dis Res* 3 (2006) 65-71.
- [14] B.J. Dewar, O.S. Gardner, C.S. Chen, H.S. Earp, J.M. Samet, and L.M. Graves, Capacitative calcium entry contributes to the differential transactivation of the epidermal growth factor receptor in response to thiazolidinediones. *Mol Pharmacol* 72 (2007) 1146-56.
- [15] O.S. Gardner, C.W. Shiao, C.S. Chen, and L.M. Graves, Peroxisome proliferator-activated receptor gamma-independent activation of p38 MAPK by thiazolidinediones involves calcium/calmodulin-dependent protein kinase II and protein kinase R: correlation with endoplasmic reticulum stress. *J Biol Chem* 280 (2005) 10109-18.
- [16] S.C. Benson, H.A. Pershadsingh, C.I. Ho, A. Chittiboyina, P. Desai, M. Pravenec, N. Qi, J. Wang, M.A. Avery, and T.W. Kurtz, Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. *Hypertension* 43 (2004) 993-1002.
- [17] M. Schupp, M. Clemenz, R. Gineste, H. Witt, J. Janke, S. Helleboid, N. Hennuyer, P. Ruiz, T. Unger, B. Staels, and U. Kintscher, Molecular characterization of new selective peroxisome proliferator-activated receptor gamma modulators with angiotensin receptor blocking activity. *Diabetes* 54 (2005) 3442-52.

- [18] T.G. Marshall, R.E. Lee, and F.E. Marshall, Common angiotensin receptor blockers may directly modulate the immune system via VDR, PPAR and CCR2b. *Theor Biol Med Model* 3 (2006) 1.
- [19] I.M. Kim, D.G. Tilley, J. Chen, N.C. Salazar, E.J. Whalen, J.D. Violin, and H.A. Rockman, Beta-blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. *Proc Natl Acad Sci U S A* 105 (2008) 14555-60.
- [20] J.D. Violin, S.M. Dewire, W.G. Barnes, and R.J. Lefkowitz, G protein-coupled receptor kinase and beta-arrestin-mediated desensitization of the angiotensin II type 1A receptor elucidated by diacylglycerol dynamics. *J Biol Chem* 281 (2006) 36411-9.
- [21] L.M. Bohn, R.J. Lefkowitz, R.R. Gainetdinov, K. Peppel, M.G. Caron, and F.T. Lin, Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286 (1999) 2495-8.
- [22] H.A. Rockman, D.J. Choi, S.A. Akhter, M. Jaber, B. Giros, R.J. Lefkowitz, M.G. Caron, and W.J. Koch, Control of myocardial contractile function by the level of beta-adrenergic receptor kinase 1 in gene-targeted mice. *J Biol Chem* 273 (1998) 18180-4.
- [23] L. Barki-Harrington, L.M. Luttrell, and H.A. Rockman, Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction in vivo. *Circulation* 108 (2003) 1611-8.
- [24] S. Ahn, S.K. Shenoy, H. Wei, and R.J. Lefkowitz, Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279 (2004) 35518-25.
- [25] O.S. Gardner, B.J. Dewar, H.S. Earp, J.M. Samet, and L.M. Graves, Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation. *J Biol Chem* 278 (2003) 46261-9.
- [26] A.O. Rosa, J. Egea, A. Martinez, A.G. Garcia, and M.G. Lopez, Neuroprotective effect of the new thiadiazolidinone NP00111 against oxygen-glucose deprivation in rat hippocampal slices: implication of ERK1/2 and PPARgamma receptors. *Exp Neurol* 212 (2008) 93-9.
- [27] M.H. Lee, H.M. El-Shewy, D.K. Luttrell, and L.M. Luttrell, Role of beta-arrestin-mediated desensitization and signaling in the control of angiotensin AT1a receptor-stimulated transcription. *J Biol Chem* 283 (2008) 2088-97.
- [28] S. Galandrin, G. Oligny-Longpre, and M. Bouvier, The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* 28 (2007) 423-30.
- [29] S. Galandrin, and M. Bouvier, Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* 70 (2006) 1575-84.
- [30] J.W. Wisler, S.M. DeWire, E.J. Whalen, J.D. Violin, M.T. Drake, S. Ahn, S.K. Shenoy, and R.J. Lefkowitz, A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci U S A* 104 (2007) 16657-62.

Figure 1

HA-AT1_AR stable cells

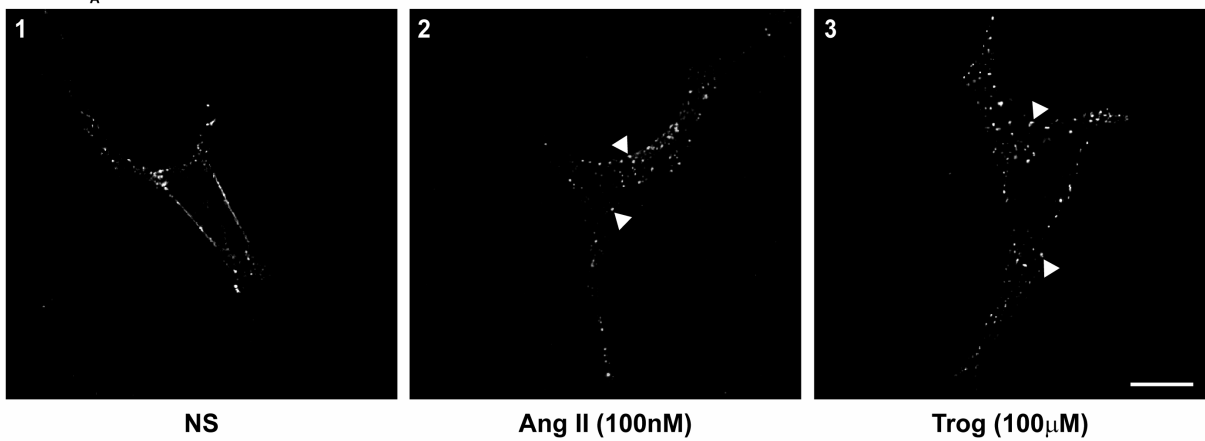


Figure 1: The PPAR γ agonist troglitazone increases AT1_AR internalization. HA-AT1_AR cells stimulated with Ang II (100nM, panel 2) or Trog (100μM, panel 3) for 5 min underwent immunofluorescent analysis for HA-AT1_AR localization. Ang II and Trog stimulation each enhanced AT1_AR internalization as indicated by the increased formation of HA-AT1_AR-

containing puncta (arrowheads), as compared to non-stimulated cells (NS, panel 1).
 Representative images shown, n = 3 of each condition, scale bar = 10μm.

Figure 2

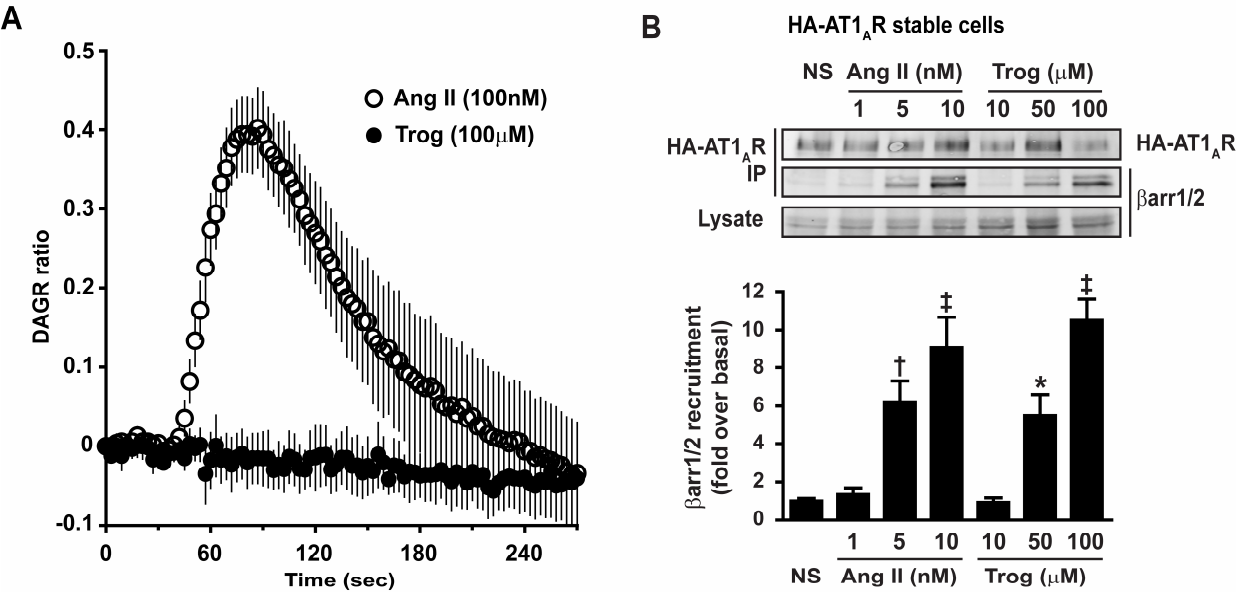


Figure 2: The PPAR γ agonist troglitazone induces β arr1/2 recruitment to the AT1_AR in a G_q protein-independent manner. (A) FRET analysis was used to assess the G_q protein-

mediated response to agonist stimulation by detecting changes in the FRET ratio of transiently-transfected DAGR in HA-AT1_AR cells. Ang II (100nM) stimulation induced a rapid increase in DAGR ratio which peaked at 90 sec, while Trog (100μM) stimulation produced no response in DAGR ratio, n = 3 independent experiments. (B) HA-AT1_AR cells were stimulated for 5 min with Ang II (1-10nM) or Trog (10-100μM) and underwent crosslink/IP with HA-agarose gel. βarr1/2 was recruited to HA-AT1_AR in a concentration-dependent manner in response to both Ang II and Trog, as summarized in histogram. *P<0.05, †P<0.01 and ‡ P<0.001 versus non-stimulated cells (NS), n = 6 each.

Figure 3

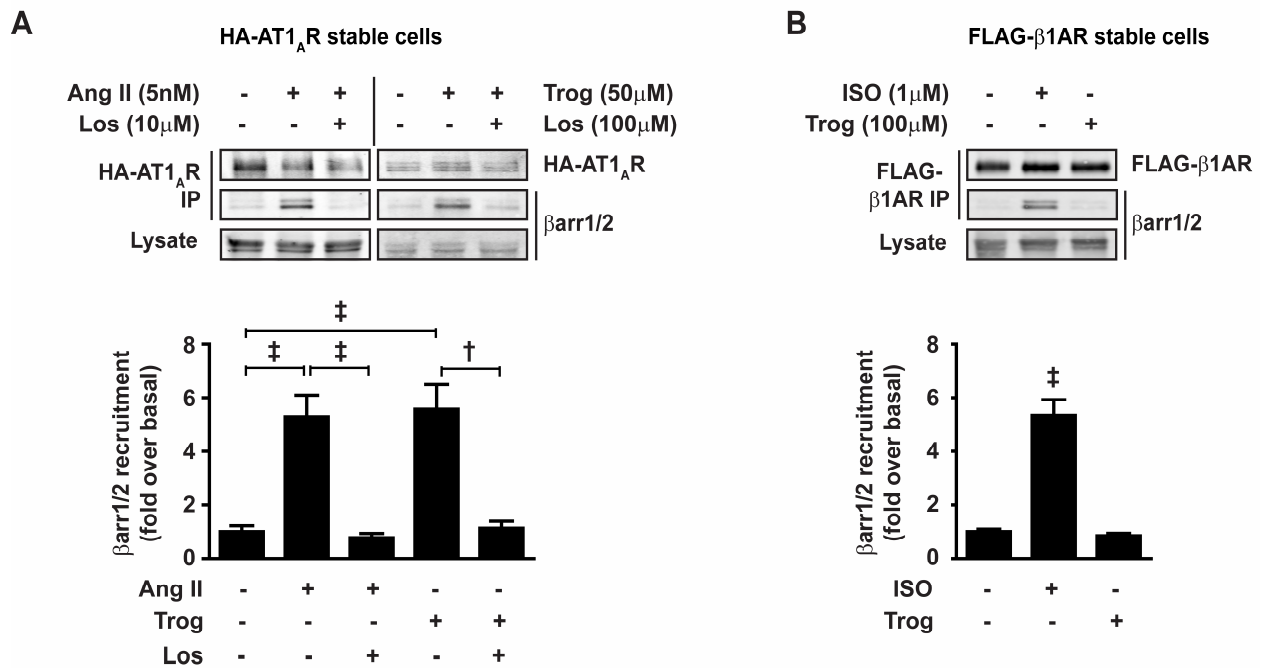


Figure 3: The PPAR_γ agonist troglitazone recruits β-arrestin to the AT1_AR selectively. (A)

HA-AT1_AR cells were stimulated with Ang II (5nM) ± Losartan (Los, 10μM) or Trog (50μM) ±

Los (100 μ M) for 5 min and underwent crosslink/IP as described in Figure 2B. β arr1/2 recruitment to AT1_AR was significantly increased following stimulation with either Ang II or Trog, an effect blocked by 5 min Los pretreatment, as summarized in histogram. †P<0.01 and ‡P<0.001, n \geq 3 each. (B) FLAG- β 1AR cells were stimulated with ISO (1 μ M) or Trog (100 μ M) for 5 min and underwent crosslink/IP with FLAG-M2 agarose. β arr1/2 recruitment to β 1AR was significantly increased following stimulation with ISO, but not Trog, as summarized in histogram. ‡ P<0.001 versus cells with no pharmacological treatment, n = 3 each.

Figure 4

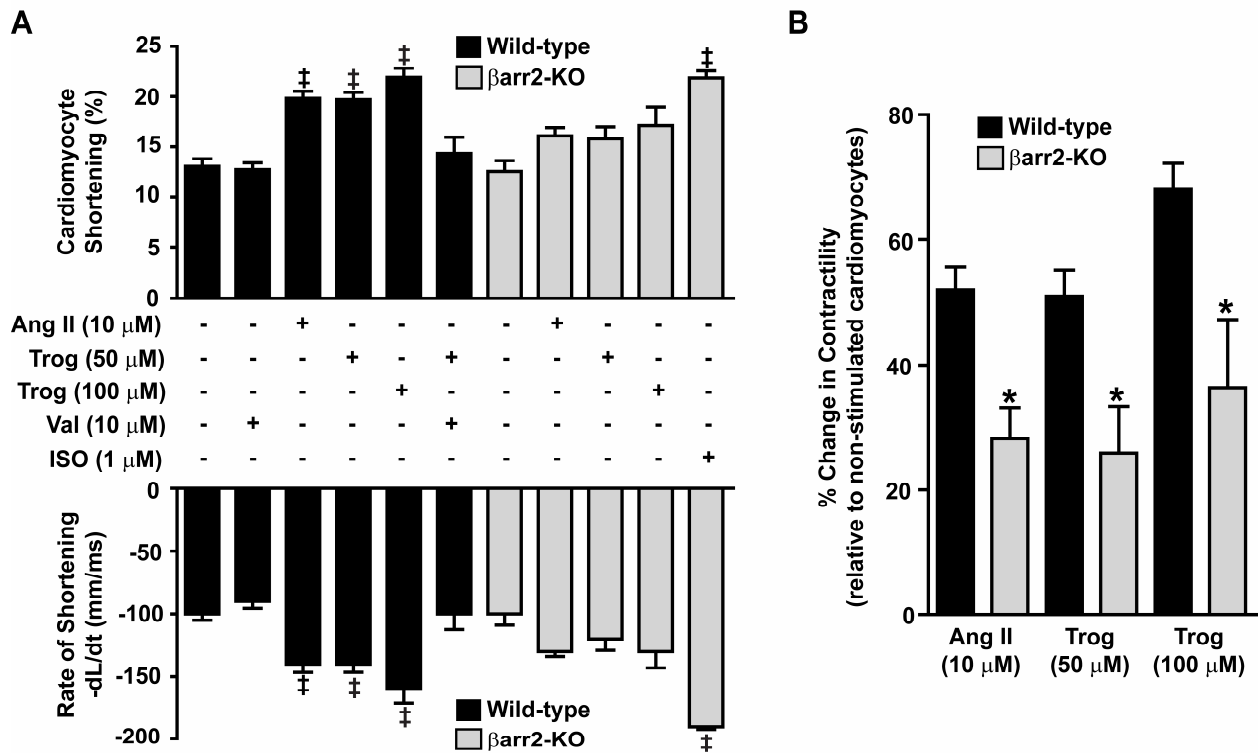


Figure 4: The PPAR γ agonist troglitazone induces AT1 $_A$ R- β -arrestin-dependent cardiomyocyte contractility. (A) Isolated wild-type or β arr2-KO cardiomyocytes were field-stimulated at 0.5 Hz basally or following treatment with Ang II (10 μ M), Trog (50 or 100 μ M), Val (10 μ M) or ISO (1 μ M). Histograms summarize % (upper) and rate (lower) of cardiomyocyte shortening. $\dagger P < 0.001$ versus cells with no pharmacological treatment, $n \geq 3$ individual hearts. (B) Comparison of the % change in contractility, relative to cells with no pharmacological treatment, in response to Ang II (10 μ M) or Trog (50 or 100 μ M) in wild-type versus β arr2-KO cardiomyocytes. $*P < 0.05$ versus wild-type within corresponding treatment group.