Published in final edited form as:

J Recept Signal Transduct Res. 2012 April ; 32(2): 76–86. doi:10.3109/10799893.2011.647353.

α_{1A} -Adrenergic receptor differentially regulates STAT3 phosphorylation through PKC ϵ and PKC δ in myocytes

Ting Shi, Robert S. Papay, and Dianne M. Perez

Department of Molecular Cardiology, NB50, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

Abstract

Previous studies demonstrated α_1 -adrenergic receptors (ARs) increase STAT3 activation in transfected and non-cardiac primary cell lines. However, the mechanism used by α_1 -ARs resulting in STAT3 activation is unknown. While other G-protein-coupled receptors (GPCRs) can couple to STAT3, these mechanisms demonstrate coupling through SRC, TYK, Rac, or complex formation with Gq and used only transfected cell lines. Using normal and transgenic mice containing constitutively active mutations (CAM) of the α_{1A} -AR subtype, neonatal mouse myocytes and whole hearts were analyzed for the mechanism to couple to STAT3 activation. α_1 -ARs stimulated time-dependent increases in p-SRC, p-JAK2, and p-STAT3 in normal neonatal myocytes. Using various kinase inhibitors and siRNA, we determined that the α_{1A} -AR coupled to STAT3 through distinct and unique pathways in neonatal myocytes. We found that PKC ϵ inhibition decreased p-ERK and p-Ser STAT3 levels without affecting p-Tyr STAT3. In contrast, we found that PKC δ inhibition affected p-SRC and p-JAK2 resulting in decreased p-Tyr and p-Ser STAT3 levels. We suggest a novel α_{1A} -AR mediated PKC ϵ /ERK pathway that regulates the phosphorylation status of STAT3 at Ser-727 while PKC δ couples to SRC/JAK2 to affect Tyr-705 phosphorylation. Furthermore, this pathway has not been previously described in a GPCR system that couples to STAT3. Given cell survival and protective cardiac effects induced by PKC, STAT3 and ERK signaling, our results could explain the neuroprotective and cardiac protective pathways that are enhanced with α_{1A} -AR agonism.

Keywords

Signal transduction; epinephrine; cross talk; cytokine signaling; cardiac protection

Introduction

Adrenergic receptors (ARs) belong to the superfamily of G-protein coupled receptors (GPCR). ARs have been classified into α_1 -ARs, α_2 -ARs and β -ARs. Three subtypes of α_1 -ARs (α_{1A} -AR, α_{1B} -AR, and α_{1D} -AR) have been cloned and characterized (2–4). They can all couple to Gq to regulate phospholipase C and release intracellular calcium. α_1 -ARs are expressed in various tissues and cell types including cardiomyocytes (CMs) (5). α_1 -ARs mediate the action of the endogenous catecholamines and induce a variety of organ-specific physiological effects such as regulating blood pressure, cardiac functions, and sympathetic neurotransmission.

© 2012 Informa Healthcare USA, Inc.

Address for Correspondence: Dianne M. Perez, PhD, Department of Molecular Cardiology, NB50, 9500 Euclid Ave., Cleveland Clinic Foundation, Cleveland, OH, 44195, USA. Phone: 216–444–2058. Fax: 216–444–9263. perezd@ccf.org.

Declaration of interest

The authors report no declarations of interest.

STAT3 is activated by many growth factors and cytokines, most notably by the IL-6 cytokine family. Investigators demonstrated that JAK/STAT3 activity participates in cardiac regulation and promotes compensatory CM hypertrophy, survival, and thus protects against cardiovascular stress (6,7). Knockout of STAT3 enhanced myocardial injury, induced heart failure, and abolished preconditioning, supporting a crucial role of STAT3 in heart-protective pathways (7–9). Our previous data showed that α_1 -ARs modulate STAT3 activity in transfected and transformed Rat-1 fibroblasts (10) but the signaling pathways involved are not known. One recent manuscript suggests that α_1 -ARs transactivates the epidermal growth factor receptor (EGFR) leading to STAT3 activation and cardiac hypertrophy in rat neonatal myocytes but do not show the mechanism of the coupling (11). Demonstration of the mechanism of α_{1A} -AR coupling to STAT3 pathways in physiological settings would support the current theory of the α_{1A} -AR mediating adaptive cardiac hypertrophy and protection against ischemia and heart failure and provide rationale for novel therapeutic intervention (1).

In addition to STAT3, cardioprotective effects are also reported for PKC ϵ and ERK (12–14). While PKC δ has often been associated with reperfusion cell death (13), one recent study indicated that α_1 -ARs use PKC δ to modulate the increase in sarcolemmal ATP-sensitive potassium channel current needed in ischemic preconditioning (15). ERK has been shown to mediate α_{1A} -AR survival effects in myocytes (14), but there are no studies to suggest that α_{1A} -AR regulates four signaling distinct molecules (PKC ϵ , PKC δ , SRC, ERK) that are responsible for its coupling to STAT3 and its potential mechanism of cardiac protection.

While previous studies have demonstrated other GPCRs coupling to STAT3, these mechanisms involved either a direct coupling of Gq or a receptor complex to JAK involving reactive oxygen species, Rac, SRC or Tyk (16,17) and all of the cell types used were transformed cell lines. PKC, let alone any specific isoform has not been previously associated with GPCR coupling to STAT3. In the current study, we show a novel α_1 -AR coupling to STAT3 through a differential signaling mechanism involving PKC ϵ , PKC δ , SRC and ERK in primary cardiomyocytes. Given the cardioprotective effects of the α_{1A} -AR, our results suggests that α_{1A} -AR coupling to STAT3 may be responsible for at least part of its protective effects in the heart.

Methods

Mouse models

Transgenic mouse models that systemically over express the α_{1A} -AR or α_{1B} -AR subtypes have been characterized and previously described (18–20). These mice express constitutively active mutants (CAM) of the receptors under the control of their native mouse promoter to increase basal subtype-specific signaling in tissues that naturally express that subtype. All animal procedures were conducted as humanely as possible, conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and approved by the Animal Research Committee of The Cleveland Clinic Foundation.

Isolation of primary cultures of neonatal cardiomyocytes (CMs)

Neonatal CMs were isolated using kits from Cellutron, Inc. (Princeton Junction, NJ) following the procedures provided by the manufacturer. Briefly, hearts from 1 to 3 day neonatal mouse pups were aseptically excised, the atria removed, and the ventricles digested at 37°C for 15 min in buffered solution containing collagenase type II. The liquid portion of the digestion mixture was centrifuged and the resulting pellet containing CMs was resuspended in Dulbecco's modified Eagle's medium (DMEM-F12) based solution from the

company. The remaining ventricle tissue was subjected up to a total of six more rounds of digestion and the resulting pellet from each digestion was pooled, centrifuged, and resuspended in DMEM-F12 medium containing 10% fetal bovine serum. The suspension containing CMs was pre-plated in a sterile tissue-culture flask at 37°C in the presence of 5% CO₂ for 1 h to reduce fibroblast contamination. The CM-enriched cell suspension after pre-plating was transferred to six-well plates pre-coated for 2 h with the SureCoat coating solution provided by the company and incubated for 48 h in serum-free plating media before experimental treatments.

Cell culture and treatments

The β -AR blocker propranolol (Sigma, St. Louis, MO) and the α_2 -AR blocker rauwolscine (Sigma) were added into the cell culture medium at final concentrations of 1 and 0.1 μ M, respectively, and incubated for 30 min before adding other agents. For α_1 -AR agonist-treated cells, phenylephrine (PE, Sigma) was added into the culture medium at a final concentration of 100 μ M and incubation continued for designated time periods as described in the figure legends. For treatment with kinase inhibitors or the α_1 -AR antagonist prazosin (1 μ M), cells were pre-incubated with the blockers for 30 min. Different concentrations of the agents were pre-tested based on the literature or the IC₅₀ of each chemical inhibitor. Cytotoxicity assays were performed using the XTT cell viability assay kit (Biotium (Hayward, CA, USA)) following the manufacturer's procedures to decide the optimum final concentration(s) of the inhibitors to be used as follows: Adenylate cyclase (2'-5'-Dideoxyadenosine, 100 μ M); ERK (PD98059 or 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, Calbiochem, 25 μ M); JAK2 (Tyrphostin AG490 or 2-Cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide, 2-Cyano-3-(3,4-dihydroxyphenyl)-N-(phenylmethyl)-2-propenamide, Calbiochem, 25 μ M); p38 (SB203580 or 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole, Calbiochem, 10 μ M); PKC δ (rottlerin or 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one, Tocris, 10 μ M); PKC ϵ (Ro-31-8220 or 2-{1-[3-(Amidiniothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)maleimide methanesulfonate salt, Bisindoylmaleimidine IX, Tocris, 12 μ M); Propranolol (Sigma, 1 μ M); Rauwolscine (Sigma, 0.1 μ M); c-SRC (PP2 or 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, Calbiochem, 10 μ M).

Immunoblotting

Heart tissue or CM extracts were washed in phosphate-buffered saline and homogenized in a SDS-based lysis buffer (50 mM Tris, 100 mM DTT, 2% SDS, 10% glycerol). Fresh solutions of proteinase inhibitors (final concentrations in lysis buffer: 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 0.15 μ M aprotinin, 0.5 mM EDTA, 1 μ M leupeptin) and phosphatase inhibitors (final concentrations in lysis buffer: 10 mM sodium fluoride, 2 mM β -glycerophosphate, 2 mM sodium pyrophosphate decahydrate, 1 mM sodium orthovanadate) were added to the lysis buffer immediately before use. Equal amounts of protein were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted with primary antibodies overnight at 4°C. After removal of blotting solution containing primary antibody, the blot was incubated with an HRP-conjugated secondary antibodies at room temperature for 1 h, and the signal was detected by chemiluminescence (Pierce). In all cases, total amounts of the non-phosphorylated signaling protein analyzed was performed and phosphorylated protein amounts normalized first to the total protein, then to GAPDH. Images were scanned and analyzed using Image J software. Relative expression levels were measured and compared to unstimulated controls, which was assigned an unit of 1.

siRNA experiments

Neonatal mouse myocytes (1–3 day old) were seeded into eight 35 mm dishes in NS media (Cellutrone). After a day or two, the media was replaced with fresh NS media (2.3 ml) with or without various siRNAs—siPKC ϵ , AllStars siNegative Control, AllStars Mouse siCell Death Control, siMAPK1, siPKC δ #4, and siPKC δ #5. All siRNAs are pre-made and verified from Qiagen (Valencia, CA). siPKC ϵ (Cat # SIO1388800) is a blend of four PKC ϵ gene target sequences: NM_011104, XM_989478, XM_989507, and XM_989534. siMAPK1 is a blend of four MAPK1 (ERK) target genes: NM_001038663, NM_002745, NM_011949, NM_138957. siPKC δ #4 (Cat # SIO1388744) has the target sequence (AGGGAAGACACTGGTACAGAA) and siPKC δ #5 (Cat # SIO2738197) the target sequence (CCGATTCAAGGTTTATAACTA). All of the above siRNAs were selected from a series of siRNAs from each target protein and verified in the CMs before experimentation. AllStars siNegative Control is the most thoroughly tested and validated negative control siRNA currently available. It has no homology to any mammalian gene and validated using Affymetrix GeneChip arrays and other assays to minimize nonspecific effects. AllStars Mouse siCell Death Control is a blend of highly potent siRNAs targeting ubiquitously expressed genes that are indispensable for cell survival as is used as a positive control for cell transfection. The siRNAs are prepared as follows. The samples were dissolved in RNase free water to a concentration of 20 μ M. The siRNA was diluted in NW media (serum-free media, Qiagen) to make a final concentration of 50 nM. HiPerfect Transfection Reagent (Qiagen) (12 μ l) was added to the diluted siRNA and mixed by vortexing. The samples were allowed to sit 5–10 min at room temperature to allow the formation of transfection complexes. The complexes were added dropwise onto the cells in the 35 mm dishes and gently swirled to ensure the uniform distribution of the transfection complexes. After 3 days in the incubator, the media was replaced with NW (serum-free media) for 3 h. NW media was then replaced with 1 ml NW media containing 1 μ M propanolol + 0.1 μ M rauwolscine and incubated for an additional 1 h at 37°C. To the dishes to be stimulated, phenylephrine was added at a final concentration of 100 μ M for 5 min. The media was discarded and the cells washed with ice-cold PBS. Lysis buffer containing protease/phosphatase inhibitors was added to the cells and incubated on ice for 15 min. The cells were scrapped into a microcentrifuge tube and boiled for 5 min and the lysate stored at –70°C until ready to be subjected to standard electrophoresis/blotting procedures.

Antibodies

All primary antibodies were from cell signaling technology with the following dilutions: STAT3 (1:1000, Cat # 9132); p-Tyr-705 STAT3 (1:500, Cat # 9131); p-Ser-727 STAT3 (1:500, Cat # 9143); SRC (1:1000, Cat # 2108); p-SRC (1:500, Cat # 2101); p38 (1:500, Cat # 9212); p-p38 (1:500, Cat # 9211); JAK2 (1:500, Cat # 3230); p-JAK2 (1:500, Cat # 3771); ERK (1:1000, Cat # 9102); p-ERK (1:1000, Cat # 9101); GAPDH (1:1000, Cat # 2118).

Statistical analysis

One-way ANOVA followed by the Bonferroni *post hoc* test was used for multiple data sets using GraphPad Prism 5.0 software. The data are presented as mean $\pm$ SEM. *P* values less than 0.05 were considered to be significant.

Results

α_1 -ARs stimulate STAT3 phosphorylation in primary cells of neonatal mouse cardiomyocytes (CMs)

Previous studies from our laboratory have demonstrated that activation of α_1 -ARs lead to the rapid and significant increase of phosphorylation at both Tyr-705 and Ser-727 of STAT3

in Rat-1 fibroblasts stably transfected with α_1 -ARs (10). To investigate if α_1 -ARs are able to stimulate STAT3 phosphorylation in primary heart cells, we used normal neonatal mouse cardiomyocytes (CMs). Similar to our observations with transfected Rat-1 fibroblasts, both Tyr-705 and Ser-727 phosphorylation of STAT3 increased in CMs when α_1 -ARs were activated with phenylephrine (Figure 1A). We found that the effect was not immediate but needed approximately 1–2 h of stimulation to achieve significant phosphorylation levels (Figure 1B).

STAT3 phosphorylation is enhanced in constitutively active α_{1A} -AR transgenic hearts

CAM α_{1A} -AR overexpressing hearts had increased phosphorylation levels of Tyr-705 and Ser-727 of STAT3 (Figure 2A, 2B). CAM α_{1B} -AR hearts did not demonstrate increased phosphorylation levels of Tyr-705 and Ser-727 of STAT3 consistent with previous results that indicate that gp130, the co-receptor for IL-6 type cytokines is downregulated in this mouse line (21).

α_1 -ARs stimulate c-SRC and JAK2 phosphorylation in CMs

Activation of type I angiotensin II receptor (AT₁), a calcium mobilizing GPCR that share similar signaling pathways as the α_1 -AR, has been shown to stimulate physical association of JAK2 and c-SRC to increase their kinase activities and to enhance the phosphorylation of STATs (22). To explore a similar coupling, time-course phosphorylation studies using CMs demonstrated that activation of α_1 -ARs significantly enhanced the phosphorylation of both JAK2 and c-SRC (Figure 3A, 3B) after 1 h of stimulation.

CAM α_{1A} -AR myocytes regulate Ser-727 but not Tyr-705 STAT3 phosphorylation through a PKC ϵ and ERK pathway

We focused on potential signaling pathways in neonatal myocytes isolated from the CAM α_{1A} -AR mice because this subtype is cardioprotective (reviewed in ¹) and stimulates the STAT3 pathway unlike the CAM α_{1B} mice (Figure 2B). We found that a chemical inhibitor of PKC ϵ reduced ERK and only Ser-727 STAT3 phosphorylation, while not affecting the phosphorylation status of SRC, JAK2 or Tyr-705 STAT3 (Figure 4A–4D). Inhibitors were used at a concentration that only affected their phosphorylated target (data not shown), while also not affecting the total protein levels of the targets (data not shown). Since PKC ϵ inhibition decreased ERK phosphorylation (Figure 4B) and both PKC ϵ and ERK inhibition decreased Ser-727 STAT3 phosphorylation (Figure 4D), PKC ϵ is likely upstream from ERK. PKC δ inhibition reduced SRC, JAK2 and affected only Tyr-705 STAT3 phosphorylation, while not modulating ERK or Ser-727 STAT3 phosphorylation. Interestingly, PKC inhibition from either isoform increased p38 phosphorylation while p38 inhibition did not affect any of the tested signals in the CAM α_{1A} -AR myocytes. We found that inhibition of either adenylate cyclase (cAMP levels) or caspase-8 also increased p38 phosphorylation (data not shown).

siRNA against PKC ϵ and ERK reduces p-Ser-727 STAT3 but not p-Tyr-705 STAT3 levels

Using siRNA against PKC ϵ and ERK in CAM α_{1A} -AR neonatal myocytes, we achieved significant reduction in p-Ser STAT3 levels without affecting SRC, JAK2 or p-Tyr STAT3 (Figure 5A–5D). siPKC ϵ decreased PKC ϵ protein levels over 50% compared to control without affecting PKC δ (Figure 5B). siPKC ϵ and siERK reduced ERK protein levels (Figure 5C) and p-Ser STAT3 (Figure 5D) without affecting p-Tyr STAT3 (Figure 5E). siCellDeath Control reduced all protein levels by at least half (Figure 5A–5E).

siRNA against PKC δ reduces p-SRC, p-JAK2 and both p-Tyr-705 STAT3 and p-Ser-727 STAT3 levels

Since PKC δ inhibition caused reductions in the phosphorylation levels of Src, Jak2, and Tyr STAT3, we utilized two different siRNAs against PKC δ 4 and 5 we achieved over 75% reductions in PKC δ protein levels (Figure 6B) without affecting PKC ϵ (Figure 6C). Both PKC δ siRNAs confirmed reductions in p-SRC (Figure 6D), p-JAK2 (Figure 6E) without changes in p-ERK (Figure 6A), which is different from the effects of siPKC ϵ (Figure 5). siPKC δ also reduced the levels of p-Tyr and p-Ser STAT3, which is consistent with the reductions in p-JAK2 (Figure 6F, G).

Discussion

Canonical signaling pathways mediated by α_1 -ARs include coupling to Gq that leads to phospholipase C activation and production of inositol-1,4,5- trisphosphate and diacylglycerol. These molecules in turn lead to protein kinase C activation and intracellular Ca²⁺ release, respectively. Previous studies from our laboratory have demonstrated that activation of α_{1A} -ARs lead to the secretion of IL-6 and the rapid and significant increase of phosphorylation at both Tyr-705 and Ser-727 of STAT3 in Rat-1 fibroblasts stably transfected with α_1 -ARs (10). Besides Rat-1 fibroblasts, α_1 -ARs have also been shown to activate STAT3 in transfected PC12 cells, a type of undifferentiated neuronal cell line (23), in vascular smooth muscle cells (24) and recently shown to mediate cardiac hypertrophy in rat neonatal myocytes through EGFR transactivation (11) but the mechanism was not explored. We report here that the α_{1A} -AR couples to p-Ser STAT3 via PKC ϵ /ERK pathway without affecting p-Tyr STAT3, while phosphorylation of Tyr STAT3 appears to couple through PKC δ /SRC/JAK2 pathway. Adrenergic receptor mediated transactivation of EGFR can phosphorylate ERK (25). Therefore, our results are consistent with the report of Li et al. (11) but our results demonstrate the downstream signals that couple to STAT3. Activation of STAT3 by α_1 -ARs represented a non-canonical signaling pathway that was not mediated by IL-6 secretion since STAT3 phosphorylation could not be blocked by a neutralizing antibody to IL-6, whereas IL-6 mediated STAT3 phosphorylation was blocked by this same antibody (10). However, the mechanism of α_{1A} -AR coupling to STAT3 was previously not known and our current study demonstrates a novel coupling not described in any other GPCR pathway.

Besides cytokine receptors, early studies had shown that the GPCRs coupled to Gq, such as thrombin and angiotensin II (AT₁), can stimulate tyrosine phosphorylation of JAKs/STATs (22,26). These findings have now been substantiated and extended to a few other GPCRs such as bradykinin (27), platelet-activating factor (28), opioids (29), and the gastrin CCK2 receptor (30). These mechanisms involved either a direct coupling of JAK to Gq or receptor scaffolds, through reactive oxygen species, Rac, SRC or Tyk2 (16,17). It was shown in transfected PC12 cells that STAT3 activation could not occur with a mutation in the third intracellular loop of the α_{1A} -AR that uncouples it from G-proteins (31), suggesting that STAT3 activation does not use GRK/arrestin scaffolding. In addition, all of these previous studies were characterized in immortalized cell lines and may not represent physiological couplings. In the current study, we demonstrate for the first time a differential mechanism mediated through PKC isoforms by which α_{1A} -ARs couple to STAT3 phosphorylation (Figure 7). We found that α_{1A} -ARs coupled to p-Ser-727 STAT3 through a unique pathway not involving SRC or JAK2, as typically reported in GPCRs. This pathway, PKC ϵ /ERK, increased the phosphorylation status of Ser-727 (Figures 5 and 7), while PKC δ coupled to the previously described SRC/JAK2 pathway to affect p-Tyr-705 STAT3 (Figures 6 and 7).

Upon cytokine receptor and gp130 receptor activation, STAT3 phosphorylation at Tyr 705 is mediated by JAK1 or JAK2. This activation of STAT3 is required for dimerization with

itself or other STATs, nuclear translocation, and DNA binding (reviewed in ⁽³²⁾). A second phosphorylation site at Ser 727 can be regulated by serine kinases and influences the functional transcriptional efficacy of STAT3 (33–35). PKC ϵ has been previously linked to STAT3 Ser-727 phosphorylation in various human cancer cell lines (36), being responsible for anti-apoptotic survival pathways commonly found in cancers. In the heart cardioprotective effects of late preconditioning can be activated through the PKC ϵ /ERK pathway (9,12,13). On the other hand, PKC δ has been reported to mediate cardiac maladaptive effects such as apoptosis (reviewed in ⁽³⁷⁾). In contrast, α_1 -ARs are reported to use PKC δ to modulate the increase in sarcolemmal ATP-sensitive potassium channel current needed in ischemic preconditioning (15). STAT3 activity promotes compensatory CM hypertrophy, survival, and protects against cardiovascular stress (6–9). Therefore, our results are consistent with the theory that α_1 -ARs may use both PKC ϵ and PKC δ to mediate STAT3 cardioprotective effects.

We focused on potential signaling pathways in neonatal myocytes isolated from the CAM α_{1A} -AR mice because this subtype is cardioprotective and neuroprotective (1,14,20,38) and stimulates the STAT3 pathway. This is in contrast to the CAM α_{1B} mice (Figure 2B) which down-regulates the gp130 receptor protein level and mRNA in whole hearts (21) and in α_{1B} -AR transfected Rat-1 fibroblasts (10) resulting in non-significant stimulated levels of STAT3 phosphorylation. CAM α_{1B} mice display cardiac maladaptation (19) as well as neurodegeneration (18). Our current results are consistent with our previous work showing that CAM α_{1A} -AR mice are inherently preconditioned through a staurosporine-sensitive, chelerythrine-insensitive (i.e. PKC-like) pathway (20).

Conclusion

We conclude that the α_{1A} -AR couples to p-Ser STAT3 via PKC ϵ /ERK pathway without affecting p-Tyr STAT3, while phosphorylation of Tyr STAT3 appears to couple through PKC δ /SRC/JAK2 pathway. This cross talk would link adrenergic GPCR signal transduction to cytokine and gp130 mediated pathways and demonstrates for the first time a differential mechanism mediated through PKC isoforms by which GPCRs couple to STAT3 phosphorylation. Our results also suggest that the PKC ϵ /ERK/Ser-727 STAT3 pathway and the PKC δ /SRC/JAK2/Tyr-705 STAT3 pathway may be responsible for at least part of the cardioprotective effects seen in the CAM α_{1A} -AR mice and with α_{1A} -AR agonists (39) as the STAT3 pathway is cardioprotective (9,12,13), promotes compensatory hypertrophy and protects against apoptosis and cardiac stress (6,7).

Acknowledgments

This work was supported by a NIH RO1HL098279 (to DMP), a Grant-in-Aid from the American Heart Association (to DMP, Great Rivers Affiliate) and a Beginning-Grant-In-Aid (to TS, Great Rivers Affiliate).

References

1. Perez DM, Doze VA. Cardiac and neuroprotection regulated by α_1 -adrenergic receptor subtypes. *J Recept Signal Transduct Res.* 2011; 31:98–110. [PubMed: 21338248]
2. Cotecchia S, Schwinn DA, Randall RR, Lefkowitz RJ, Caron MG, Kobilka BK. Molecular cloning and expression of the cDNA for the hamster α_1 -adrenergic receptor. *Proc Natl Acad Sci USA.* 1988; 85:7159–7163. [PubMed: 2845398]
3. Perez DM, Piascik MT, Graham RM. Solution-phase library screening for the identification of rare clones: isolation of an α_{1D} -adrenergic receptor cDNA. *Mol Pharmacol.* 1991; 40:876–883. [PubMed: 1661838]

4. Perez DM, Piascik MT, Malik N, Gaivin R, Graham RM. Cloning, expression, and tissue distribution of the rat homolog of the bovine α_{1C} -adrenergic receptor provide evidence for its classification as the α_{1A} subtype. *Mol Pharmacol*. 1994; 46:823–831. [PubMed: 7969068]
5. Graham RM, Perez DM, Hwa J, Piascik MT. α_1 -adrenergic receptor subtypes. Molecular structure, function, and signaling. *Circ Res*. 1996; 78:737–749. [PubMed: 8620593]
6. Negoro S, Kunisada K, Tone E, Funamoto M, Oh H, Kishimoto T, Yamauchi-Takahara K. Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction. *Cardiovasc Res*. 2000; 47:797–805. [PubMed: 10974228]
7. Jacoby JJ, Kalinowski A, Liu MG, Zhang SS, Gao Q, Chai GX, Ji L, Iwamoto Y, Li E, Schneider M, Russell KS, Fu XY. Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. *Proc Natl Acad Sci USA*. 2003; 100:12929–12934. [PubMed: 14566054]
8. Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, Sack MN. Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. *Cardiovasc Res*. 2004; 63:611–616. [PubMed: 15306216]
9. Hilfiker-Kleiner D, Hilfiker A, Fuchs M, Kaminski K, Schaefer A, Schieffer B, Hillmer A, Schmiedl A, Ding Z, Podewski E, Podewski E, Poli V, Schneider MD, Schulz R, Park JK, Wollert KC, Drexler H. Signal transducer and activator of transcription 3 is required for myocardial capillary growth, control of interstitial matrix deposition, and heart protection from ischemic injury. *Circ Res*. 2004; 95:187–195. [PubMed: 15192020]
10. Gonzalez-Cabrera PJ, Gaivin RJ, Yun J, Ross SA, Papay RS, McCune DF, Rorabaugh BR, Perez DM. Genetic profiling of α_1 -adrenergic receptor subtypes by oligonucleotide microarrays: coupling to interleukin-6 secretion but differences in STAT3 phosphorylation and gp-130. *Mol Pharmacol*. 2003; 63:1104–1116. [PubMed: 12695539]
11. Li Y, Zhang H, Liao W, Song Y, Ma X, Chen C, Lü Z, Li Z, Zhang Y. Transactivated EGFR mediates α_1 -AR-induced STAT3 activation and cardiac hypertrophy. *Am J Physiol Heart Circ Physiol*. 2011 Aug 19. [Epub ahead of print].
12. Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Messing RO, Bolli R. Role of the protein kinase C-epsilon-Raf-1-MEK-1/2-p44/42 MAPK signaling cascade in the activation of signal transducers and activators of transcription 1 and 3 and induction of cyclooxygenase-2 after ischemic preconditioning. *Circulation*. 2005; 112:1971–1978. [PubMed: 16172266]
13. Budas GR, Mochly-Rosen D. Mitochondrial protein kinase Cepsilon (PKCepsilon): emerging role in cardiac protection from ischaemic damage. *Biochem Soc Trans*. 2007; 35:1052–1054. [PubMed: 17956277]
14. Huang Y, Wright CD, Merkwand CL, Baye NL, Liang Q, Simpson PC, O'Connell TD. An α_{1A} -adrenergic-extracellular signal-regulated kinase survival signaling pathway in cardiac myocytes. *Circulation*. 2007; 115:763–772. [PubMed: 17283256]
15. Turrell HE, Rodrigo GC, Norman RI, Dickens M, Standen NB. Phenylephrine preconditioning involves modulation of cardiac sarcolemmal K(ATP) current by PKC delta, AMPK and p38 MAPK. *J Mol Cell Cardiol*. 2011; 51:370–380. [PubMed: 21740910]
16. Pelletier S, Duhamel F, Coulombe P, Popoff MR, Meloche S. Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. *Mol Cell Biol*. 2003; 23:1316–1333. [PubMed: 12556491]
17. Wu EH, Lo RK, Wong YH. Regulation of STAT3 activity by G16-coupled receptors. *Biochem Biophys Res Commun*. 2003; 303:920–925. [PubMed: 12670499]
18. Zuscik MJ, Sands S, Ross SA, Waugh DJ, Gaivin RJ, Morilak D, Perez DM. Overexpression of the α_{1B} -adrenergic receptor causes apoptotic neurodegeneration: multiple system atrophy. *Nat Med*. 2000; 6:1388–1394. [PubMed: 11100125]
19. Zuscik MJ, Chalothorn D, Hellard D, Deighan C, McGee A, Daly CJ, Waugh DJ, Ross SA, Gaivin RJ, Morehead AJ, Thomas JD, Plow EF, McGrath JC, Piascik MT, Perez DM. Hypotension, autonomic failure, and cardiac hypertrophy in transgenic mice overexpressing the α_{1B} -adrenergic receptor. *J Biol Chem*. 2001; 276:13738–13743. [PubMed: 11278430]

20. Rorabaugh BR, Ross SA, Gaivin RJ, Papay RS, McCune DF, Simpson PC, Perez DM. α_{1A} - but not α_{1B} -adrenergic receptors precondition the ischemic heart by a staurosporine-sensitive, chelerythrine-insensitive mechanism. *Cardiovasc Res.* 2005; 65:436–445. [PubMed: 15639483]
21. Yun J, Zuscik MJ, Gonzalez-Cabrera P, McCune DF, Ross SA, Gaivin R, Piascik MT, Perez DM. Gene expression profiling of α_{1B} -adrenergic receptor-induced cardiac hypertrophy by oligonucleotide arrays. *Cardiovasc Res.* 2003; 57:443–455. [PubMed: 12566117]
22. Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature.* 1995; 375:247–250. [PubMed: 7746328]
23. Zhong H, Murphy TJ, Minneman KP. Activation of signal transducers and activators of transcription by α_{1A} -adrenergic receptor stimulation in PC12 cells. *Mol Pharmacol.* 2000; 57:961–967. [PubMed: 10779380]
24. Sasaguri T, Teruya H, Ishida A, Abumiya T, Ogata J. Linkage between α_1 adrenergic receptor and the Jak/STAT signaling pathway in vascular smooth muscle cells. *Biochem Biophys Res Commun.* 2000; 268:25–30. [PubMed: 10652206]
25. Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, Lefkowitz RJ, Luttrell LM. The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem.* 2000; 275:9572–9580. [PubMed: 10734107]
26. Rodríguez-Liñares B, Watson SP. Phosphorylation of JAK2 in thrombin-stimulated human platelets. *FEBS Lett.* 1994; 352:335–338. [PubMed: 7925997]
27. Ju H, Venema VJ, Liang H, Harris MB, Zou R, Venema RC. Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signalling proteins in plasmalemmal caveolae. *Biochem J.* 2000; 351:257–264. [PubMed: 10998369]
28. Lukashova V, Asselin C, Krolewski JJ, Rola-Pleszczynski M, Stanková J. G-protein-independent activation of Tyk2 by the platelet-activating factor receptor. *J Biol Chem.* 2001; 276:24113–24121. [PubMed: 11309383]
29. Yuen JW, So IY, Kam AY, Wong YH. Regulation of STAT3 by mu-opioid receptors in human neuroblastoma SH-SY5Y cells. *Neuroreport.* 2004; 15:1431–1435. [PubMed: 15194868]
30. Ferrand A, Kowalski-Chauvel A, Bertrand C, Escrieut C, Mathieu A, Portolan G, Pradayrol L, Fourmy D, Dufresne M, Seva C. A novel mechanism for JAK2 activation by a G protein-coupled receptor, the CCK2R: implication of this signaling pathway in pancreatic tumor models. *J Biol Chem.* 2005; 280:10710–10715. [PubMed: 15640156]
31. Lee D, Robeva A, Chen Z, Minneman KP. Mutational uncoupling of α_{1A} -adrenergic receptors from G proteins also uncouples mitogenic and transcriptional responses in PC12 cells. *J Pharmacol Exp Ther.* 2003; 306:471–477. [PubMed: 12724349]
32. Levy DE, Lee CK. What does Stat3 do? *J Clin Invest.* 2002; 109:1143–1148. [PubMed: 11994402]
33. Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell.* 1995; 82:241–250. [PubMed: 7543024]
34. Wierenga AT, Vogelzang I, Eggen BJ, Vellenga E. Erythropoietin-induced serine 727 phosphorylation of STAT3 in erythroid cells is mediated by a MEK-, ERK-, and MSK1-dependent pathway. *Exp Hematol.* 2003; 31:398–405. [PubMed: 12763138]
35. Decker T, Kovarik P. Serine phosphorylation of STATs. *Oncogene.* 2000; 19:2628–2637. [PubMed: 10851062]
36. Aziz MH, Manoharan HT, Church DR, Dreckschmidt NE, Zhong W, Oberley TD, Wilding G, Verma AK. Protein kinase Cepsilon interacts with signal transducers and activators of transcription 3 (Stat3), phosphorylates Stat3Ser727, and regulates its constitutive activation in prostate cancer. *Cancer Res.* 2007; 67:8828–8838. [PubMed: 17875724]
37. Churchill EN, Mochly-Rosen D. The roles of PKCdelta and epsilon isoenzymes in the regulation of myocardial ischaemia/reperfusion injury. *Biochem Soc Trans.* 2007; 35:1040–1042. [PubMed: 17956273]
38. Knudson CA, Carr PA, Perez DM, Doze VA. α_{1A} -adrenergic receptor overexpression protects hippocampal interneurons. *FASEB J.* 2007; 21:A1209(896.6).

39. Jensen BC, O'Connell TD, Simpson PC. α_1 -adrenergic receptors: targets for agonist drugs to treat heart failure. *J Mol Cell Cardiol.* 2011; 51:518–528. [PubMed: 21118696]

Figure 1.

α_1 -AR activation by phenylephrine (PE) stimulates STAT3 phosphorylation at both Tyr-705 and Ser-727 in normal neonatal mouse cardiomyocytes. Cells were pretreated with the β -AR blocker propranolol (1 μ M) and the α_2 -AR blocker rauwolscline (0.1 μ M) for 30 min, followed by treatment with PE (100 μ M) for various time periods as indicated. Total protein was extracted from cells and levels of phosphorylated and total STAT3 were assessed by western blot. (A) Representative blot of 3 independent experiments. (B) Relative expression levels of p-Tyr and p-Ser STAT3 levels normalized to total STAT3. Blots were scanned and analyzed using Image J software. One-way ANOVA followed by the Bonferroni *post hoc* test was used. * $p < 0.05$ vs. time 0 min.

Figure 2.

Levels of phosphorylated STAT3 in *ex vivo* hearts of CAM α_{1A} -AR and CAM α_{1B} -AR transgenic mice. (A) Whole hearts were isolated and homogenized from normal non-transgenic (NTG), CAM α_{1A} -AR (CAM α_{1A}), CAM α_{1B} -AR (CAM α_{1B}) adult mice and proteins extracted as indicated in the methods. Levels of phosphorylated and total STAT3 were assessed by western blots. Each lane represents an individual heart from age 6–8 Mo. adult mice and 7 hearts were analyzed in total. (B) Relative expression levels of p-STAT3 normalized to total STAT3 and then to NTG hearts. Blots were scanned and analyzed using Image J software. One-way ANOVA followed by the Bonferroni *post hoc* test was used. **p* 0.05 vs. NTG or wild-type control.

Figure 3.

Phosphorylation of JAK2 and SRC by α_1 -ARs in normal neonatal mouse cardiomyocytes. (A) Cells were pretreated with β -AR blocker propranolol (1 μ M) and the α_2 -AR blocker rauwolscine (0.1 μ M) for 30 min, followed by treatment with PE (100 μ M) for various times as indicated. Total protein was extracted from cells and levels of phosphorylated and total JAK2 and SRC were assessed by western blots. (B) Relative expression levels of p-JAK and p-SRC at various times normalized to total JAK and SRC, respectively, then normalized to time 0 min. Blot is representative of 3 independent experiments. One-way ANOVA followed by the Bonferroni *post hoc* test was used. * p < 0.05 vs. time 0 control.

Figure 4.

Regulation of STAT3 phosphorylation in CAM α_{1A} -AR myocytes. (A) Neonatal cardiomyocytes were isolated from CAM α_{1A} -AR pups and were untreated (Control) or pre-incubated with the various inhibitors as indicated for 30 min, then followed by treatment with phenylephrine (Phe, 100 μ M) for 30 min. Levels of various phosphorylated and total protein for STAT3, SRC, p38, JAK2, and ERK were analyzed by western blot. Inhibitors were used at a concentration that only affected their phosphorylated target (data not shown), while not affecting the total protein levels of the targets (data not shown). Experiments were repeated 3–4 times with separate preparations of myocytes and a representative blot shown. Blots were scanned and analyzed as followed using Image J software. (B) Relative levels of p-ERK normalized to total ERK levels and unstimulated control. (C) Relative expression of p-Tyr-STAT3 normalized to total STAT3 and control. (D) Relative expression of pSer-STAT3 normalized to total STAT3 and control. One-way ANOVA followed by the Bonferroni *post hoc* test was used; [#] $p < 0.05$ Phe vs. Control; ^{*} $p < 0.05$ vs. Phe; ^{**} $p < 0.01$ vs. Phe.

Figure 5.

Regulation of pSer-STAT3, p-SRC and p-ERK through siPKC ϵ and siERK in CAM α_{1A} -AR myocytes. (A) Neonatal cardiomyocytes were isolated from CAM α_{1A} -AR pups and were unstimulated (Control) or pre-incubated with the various siRNAs as indicated for 3 days, followed by treatment with phenylephrine (Phe, 100 μ M) for 30 min. Levels of various phosphorylated and total protein for STAT3, JAK, SRC and ERK were assessed by western blot. Shown is a representative blot from a total of 4 independent experiments. Blots were scanned and analyzed as followed using Image J software and normalized to both total protein and GAPDH levels. (B) Relative levels of PKC ϵ with various siRNAs compared to controls. (C) Relative levels of p-ERK with various siRNAs compared to controls. (D) Relative levels of pSer-STAT3 with various siRNAs compared to controls. (E) Relative levels of p-Tyr STAT3 with various siRNAs compared to controls. One-way ANOVA followed by the Bonferroni *post hoc* test was used; # $p < 0.05$ Phe vs. unstimulated control; * $p < 0.05$ versus Phe stimulation; ** $p < 0.05$ versus unstimulated controls.

Figure 6.

Regulation of p-SRC, p-JAK2 and p-Tyr 705 STAT3 through siPKC δ in CAM α_{1A} -AR myocytes. (A) Neonatal cardiomyocytes were isolated from CAM α_{1A} -AR pups and were unstimulated (Control) or treated with phenylephrine (Phe, 100 μ M) for 30 min. Some cells were also pre-incubated with the various siRNAs (siControl, PKC δ #4, PKC δ #5) as indicated for 3 days prior to stimulation. Levels of various phosphorylated and total protein for STAT3, JAK, SRC and ERK were assessed by western blot. Shown is a representative blot from a total of 4 independent experiments. Blots were scanned and analyzed as followed using Image J software and normalized to both total protein and GAPDH levels. (B) Relative levels of PKC δ with various siRNAs compared to controls. (C) Relative levels of PKC ϵ with various siRNAs compared to controls. (D) Relative levels of p-SRC with various siRNAs compared to controls. (E) Relative levels of p-JAK2 with various siRNAs compared to controls. (F) Relative levels of p-Tyr STAT3 compared to controls. (G) Relative levels of p-Ser STAT3 compared to controls. One-way ANOVA followed by the Bonferroni *post hoc* test was used; # p < 0.05 Phe vs. unstimulated control; * p < 0.05 versus Phe.

Figure 7.

Schematic of signaling pathways regulating STAT3 phosphorylation in CAM α_{1A} -AR myocytes. Stimulation of CAM α_{1A} -AR myocytes activates PKC ϵ that affects p-ERK that modulates the phosphorylation status of Ser-727 of STAT3. PKC δ mediates p-SRC and p-JAK2 to affect Tyr-705 phosphorylation of STAT3. There was no effect of p38 inhibition on any STAT3 phosphorylation and inhibition of PKC ϵ increased p-p38 levels.