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α_{1A}-Adrenergic Receptors Regulate Cardiac Hypertrophy In Vivo Through Interleukin-6 Secretion

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ABSTRACT

The role of α_1-adrenergic receptors (ARs) in the regulation of cardiac hypertrophy is still unclear, because transgenic mice demonstrated hypertrophy or the lack of it despite high receptor overexpression. To further address the role of the α_1-ARs in cardiac hypertrophy, we analyzed unique transgenic mice that overexpress constitutively active mutation (CAM) α_{1A}-ARs or CAM α_{1B}-ARs under the regulation of large fragments of their native promoters. These constitutively active receptors are expressed in all tissues that endogenously express their wild-type counterparts as opposed to only myocyte-targeted transgenic mice. In this study, we discovered that CAM α_{1A}-AR mice in vivo have cardiac hypertrophy independent of changes in blood pressure, corroborating earlier studies, but in contrast to myocyte-targeted α_{1A}-AR mice. We also found cardiac hypertrophy in CAM α_{1B}-AR mice, in agreement with previous studies, but hypertrophy only developed in older mice. We also discovered unique α_1-AR–mediated hypertrophic signaling that was AR subtype-specific with CAM α_{1A}-AR mice secreting atrial naturietic factor and interleukin-6 (IL-6), whereas CAM α_{1B}-AR mice expressed activated nuclear factor-κB (NF-κB). These particular hypertrophic signals were blocked when the other AR subtype was coactivated. We also discovered that crossbreeding the two CAM models (double CAM $\alpha_{1A/B}$-AR) inhibited the development of hypertrophy and was reversible with single receptor activation, suggesting that coactivation of the receptors can lead to novel antagonistic signal transduction. This was confirmed by demonstrating antagonistic signals that were even lower than normal controls in the double CAM $\alpha_{1A/B}$-AR mice for p38, NF-κB, and the IL-6/glycoprotein 130/signal transducer and activator of transcription 3 pathway. Because $\alpha_{1A/B}$ double knockout mice fail to develop hypertrophy in response to IL-6, our results suggest that IL-6 is a major mediator of α_{1A}-AR cardiac hypertrophy.

Introduction

The sympathetic nervous system plays a crucial role in the regulation of cardiac function. Norepinephrine (NE) released from sympathetic neurons innervating the heart enhances cardiac contractility, hypertrophy, and blood flow, and protects from ischemic injury. The effects of NE are mediated by nine different adrenergic receptors (ARs) (α_{1A}-, α_{1B}-, α_{1D}-, α_{2A}-, α_{2B}-, α_{2C}, β_1-, β_2-, β_3-AR). These receptors are part of a larger superfamily of G protein-coupled receptors that mediate the effects of hormones and neurotransmitters.

Three different α_1-AR subtypes have been cloned (Cotecchia et al., 1988; Perez et al., 1991, 1994). The α_{1A}-AR and α_{1B}-AR are present in the myocyte (Michel and Insel, 1994; Michel et al., 1994). However, the lack of α_1-AR subtype-selective antagonists has made it difficult to identify the physiologic roles of α_1-AR subtypes in the heart. To circumvent this problem, several transgenic mouse models that either overexpress, knockout (KO), or heart-target the α_1-AR subtypes have been created and analyzed (Milano et al., 1994; Cavalli et al., 1997; Grupp et al., 1998; Wang et al., 2000; Lemire et al., 2001; Lin et al., 2001; Zuscik et al., 2001; Yun et al., 2003; O'Connell et al., 2006). Although most of these models agree that α_1-ARs are 3important for physiologic heart function, there is some variance on their roles in cardiac hypertrophy. Whereas previous cellular studies using mildly selective ligands suggest that the α_{1A}-AR is the mediator of hypertrophy in neonatal myocytes (Knowlton et al., 1993; Rokosh et al., 1996; Autelitano and Woodcock, 1998), the myocyte-targeted α_{1A}-AR transgenic mouse did not display hypertrophy (Lin et al., 2001) despite high levels of receptor overexpression. Most of the mouse models with the exception of one (Grupp et al., 1998) that overexpress or myocyte-target the α_{1B}-AR subtype demonstrated a mild, but significant cardiac hypertrophy.

We now further describe the role of the α_1-ARs in cardiac hypertrophy utilizing unique transgenic mice that overexpress CAM α_{1A}-ARs or CAM α_{1B}-ARs under the regulation of their isogenic promoters to achieve both myocyte and nonmyocyte expression. Not only did we find cardiac hypertrophy in both

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ABBREVIATIONS: ANF, atrial naturietic factor; AR, adrenergic receptor; BNP, brain naturietic peptide; BP, blood pressure; CAM, constitutively active mutation; ERK, extracellular signal-regulated kinase; gp130, glycoprotein 130; [^{125}I]-HEAT, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]-tetralone; HW/BW, heart to body weight ratio; IL, interleukin; IP_3, inositol-1,4,5-trisphosphate; KO, knockout; NE, norepinephrine; PE, phenylephrine; STAT3, signal transducer and activator of transcription 3.

mouse models in contradiction to previous studies, we also discovered unique α_1-AR–mediated hypertrophic signaling that was subtype-specific and focused on the IL-6 pathway for the α_{1A}-AR subtype. Of particular interest, the hypertrophy and associated signals were blocked when the other AR subtype was coactivated through agonism or through crossbreeding the two CAM models (double CAM $\alpha_{1A/B}$-AR).

Materials and Methods

Transgenic Mice and Cross-Mating. The generation of CAM α_{1A}-AR and CAM α_{1B}-AR mice has been described elsewhere (Zuscik et al., 2000; Rorabaugh et al., 2005). Normal littermates are used as controls. Tissue-specific distribution was achieved using large fragments of the mouse α_{1A}-AR or α_{1B}-AR promoters (Zuscik et al., 1999; O'Connell et al., 2001) to drive overexpression of cDNA that encodes the CAM receptors (Zuscik et al., 2000; Rorabaugh et al., 2005). All procedures on the mice conform to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and were approved through the institutional animal use committee (ARC 08906).

Radioligand Binding. The protocols used for membrane preparation and radioligand binding were previously described (Rorabaugh et al., 2005). Saturation binding was performed using the α_1-AR-selective radioligand 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]-tetralone ([^{125}I]-HEAT).

Measurement of Inositol-1,4,5-Trisphosphate. Heart tissue were weighed, chopped into small pieces, and incubated for 1 hour at 37°C in serum free Dulbecco's modified Eagle's medium containing 10 mM LiCl with or without 10 μM phenylephrine (PE). The inositol-1,4,5-trisphosphate (IP_3) was measured using a radioreceptor assay kit from PerkinElmer Life Sciences (Boston, MA) according to the manufacturer's protocol.

Drug treatments and Measurement of Cardiac Hypertrophy. Six- to 8-month old CAM or normal mice were subjected to the following protocol. First, β-ARs were blocked in all experimental mice with propranolol (1 mg/kg body weight, i.p.). α_{1A}-ARs were stimulated in CAM mice using cirazoline (0.3 mg/kg i.p.). α_{1B}-ARs were stimulated in CAM mice using NE (1 mg/kg i.p.) and the α_{1A}-AR antagonist, 5-methylurapidil (10 μg/kg i.p.). In separate studies, mice were injected i.p. with IL-6 (0.1 ml, 40 ng). Control mice were injected with saline (0.9% NaCl). All mice were injected twice daily for 2 weeks. Mice were then weighed, anesthetized with 0.2 ml Nembutal (sodium pentobarbital; Lundbeck, Inc, Deerfield, IL), hearts removed, blotted free of blood five times, and weighed to determine heart to body weight ratio (HW/BW).

Echocardiography. Mice were subjected to echocardiographic analysis. The mice were anesthetized with isoflurane (0.2% v/v). Images were acquired using an echocardiographic machine Vevo 770 (Visual Sonics, Toronto, ON, Canada). The m-mode echocardiograms obtained from 9 to 10 beats allowed quantification of mean and S.E.M. for left ventricle size, anterior and posterior wall thickness, and left ventricle cavity dilation.

Blood Pressure. The measurement of the mean carotid artery blood pressure (BP) in conscious mice was performed as described previously (Zuscik et al., 2001). The mice were anesthetized with 0.1 mg/g ketamine and 2 μg/g acepromazine maleate. The recording began immediately after surgery and continued for a 7-hour period.

Fibrosis. Hearts were postfixed in ice-cold solution containing 2% paraformaldehyde, 75 mM lysine, 37 mM sodium phosphate, and 10 mM sodium peroxide, paraffin embedded, and processed for Masson's Trichrome staining to assess the extent of myocardial collagen deposition. Six 10-μm transverse (short-axis) sections at the level of the papillary muscles were analyzed from each animal for bright blue staining using the Image J analysis program (NIH, Bethesda, MD).

Serum IL-6 Levels. Mice were injected with 0.2 ml sodium pentobarbital solution (50 mg/ml) (Ovation Pharmaceutical, Deerfield, IL) and blood samples were collected through the tail vein and set at room temperature for 2 hours. Levels of IL-6 in serum were determined by enzyme-linked immunosorbent assay using the Quantikine mouse kit from R&D Systems (Minneapolis, MN) following the manufacturer's instructions.

Western Blot Analysis. Hearts were homogenized and processed as previously described (Gonzalez-Cabrera et al., 2003). After transfer, the blot was blocked and then incubated with one of the following primary antibodies overnight at 4°C: rabbit anti-Stat3 or glycoprotein 130 (gp130) at 1:1000, rabbit anti-p-Ser-Stat3 at 1:500, rabbit anti-p-Tyr-Stat3 at 1:500, mouse anti-phospho-IκBα at 1:1000, mouse anti-p38 or phospho-p38 at 1:1000, rat anti-IL-6 at 0.1 μg/ml, or goat antiglyceraldehyde 3-phosphate dehydrogenase at 1:1000 (Cell Signaling Technologies, Danvers, MA). The blots were incubated with the appropriate secondary antibody for 1 hour at room temperature (IgG horseradish peroxidase at 1:10,000; Jackson ImmunoResearch, West Grove, PA). The blots were washed before incubation with the Pierce SuperSignal chemiluminescent reagents and exposed using CL-Xposure film (Pierce, Rockford, IL).

Statistical Analysis. Analysis of variance and the Newman–Keuls post-test were used to compare functional and signaling parameters. A probability value <0.05 was considered statistically significant. Prism software (GraphPad, San Diego, CA) was used for all data analyses.

Results

Crossbreeding and Characterization of CAM Mice. CAM α_{1A}-AR and CAM α_{1B}-AR homozygous mice were crossbred and subsequent generations intercrossed to produce bi-transgenic mice that contained both CAM α_{1A}-AR and CAM α_{1B}-AR homozygous alleles as determined by Southern blot analysis (Fig. 1). We performed radioligand binding to determine the total density of α_1-AR receptors (Fig. 2A). In some tissue such as the heart, lung, and spleen, the α_1-AR density in CAM $\alpha_{1A/B}$-AR mice was additive. In the higher expressing tissue such as brain or liver, α_1-AR density was not additive in the CAM $\alpha_{1A/B}$-AR, suggesting that some regulatory mechanism was present in those organs or the result of crossover events that affected promoter activity. Although mouse liver is considered an α_{1B}-AR dominant tissue, the α_{1A}-AR is present in the liver vasculature, natural killer cells, and B lymphocytes as well as other immune cells in the liver sinusoids (Grisanti et al., 2011). To determine the levels of receptor activity and constitutive signaling in the heart, we analyzed the amount of IP_3 under basal and stimulated (PE, 10 υM) conditions. Although the basal IP_3 activity for the various CAM mouse models was significantly increased compared with normal hearts, the level of stimulated activity was greater in CAM than normal mice but plateaued between the transgenic mouse models (Fig. 2B).

Characterization of Cardiac Hypertrophy. CAM α_{1B}-AR mice were previously shown to have mild yet significant cardiac hypertrophy (Zuscik et al., 2001). To determine whether the other CAM mouse models also had cardiac hypertrophy, we assessed heart/body weight ratios in similarly aged (6–8 months) mice (Fig. 3A). Both the CAM α_{1A}-AR and CAM α_{1B}-AR mice had significantly increased HW/BW ratios compared with normal mice but the double CAM $\alpha_{1A/B}$-AR mice did not. A marker of maladaptive hypertrophy is fibrosis, which can be assessed through Masson Trichrome staining. Only the CAM α_{1B}-AR mice had significant fibrosis (Fig. 3B). We also determined mRNA expression of hypertrophy-associated fetal markers (Fig. 3C). Only CAM α_{1A}-AR had

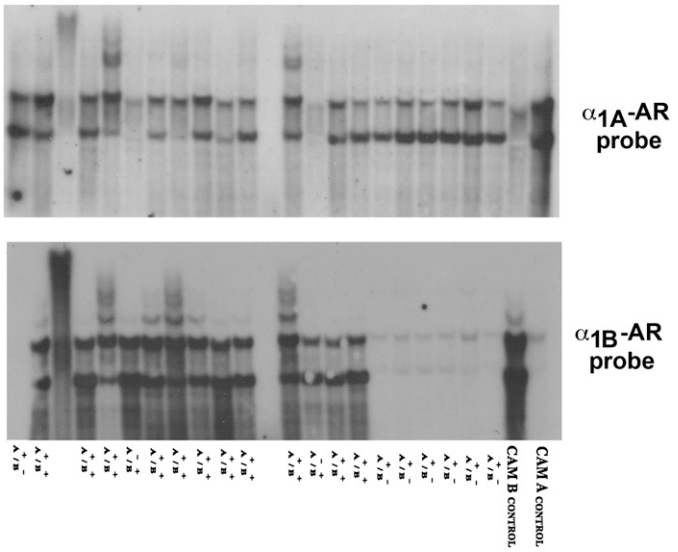

Fig. 1. Southern blot analysis of CAM α_{1A}-AR and CAM α_{1B}-AR cross-breeding to produce double CAM $\alpha_{1A/B}$-AR transgenic mice. Pups from CAM α_{1A}-AR × CAM α_{1B}-AR breeding were genotyped from tail DNA and subjected to Southern blot analysis. Each pup DNA was screened against an α_{1A}-AR–specific probe, designated as "A" on the blot (21) or an α_{1B}-AR specific probe, designated as "B" on the blot (14). Pup DNA that demonstrated positive results for both probes (A^+/B^+) were used as founders for the CAM $\alpha_{1A/B}$-AR mouse line and verified for homozygosity by back-breeding to wild-type mice.

weak but significantly elevated levels of atrial naturietic factor (ANF) and only the double CAM $\alpha_{1A/B}$-AR mice displayed significantly increased brain naturietic peptide (BNP). This is consistent with our previous report that the CAM α_{1B}-AR mice did not display elevated ANF even though it had cardiac hypertrophy (Zuscik et al., 2001). To determine whether potential changes in BP affected hypertrophy, we measured both basal and induced BP with an indwelling catheter in the CAM α_{1A}-AR mice. Although basal BP in the CAM α_{1A}-AR mice was lower, it was not significantly different from controls and CAM α_{1A}-AR mice also had no significant changes in BP from normal control mice when stimulated with phenylephrine (Fig. 3D). We previously published that CAM α_{1B}-AR mice had decreased resting BP and pressure was blunted when stimulated by PE (Zuscik et al., 2001).

In addition to ANF and BNP levels, other hypertrophic signals previously associated with α_1-AR activation were analyzed, such as p38 (Zechner et al., 1997; Clerk et al., 1998; Nemoto et al., 1998) and nuclear factor-κB (NF-κB) (Hirotani et al., 2002). In Western blot analysis, we found that the level of phospho-IKBα that regulates NF-κB activity was substantially higher in the CAM α_{1B}-AR heart (Fig. 4), but was not elevated in the other mouse lines, even in the double CAM $\alpha_{1A/B}$-AR mice. We also measured p-extracellular signal-regulated kinase (ERK) and phospho-p38 levels (Fig. 4). Although phospho-p38 did not display any differences from normal mice in the single CAM mice, there was a significant decrease in phospho-p38 in the double CAM $\alpha_{1A/B}$-AR mice. In contrast, p-ERK levels were not different between any of the mouse lines. These results suggest that specific inhibitory signal transduction is occurring in the double CAM $\alpha_{1A/B}$-AR mice that may be associated with its inhibition of the cardiac hypertrophy response.

Echocardiography. To confirm cardiac hypertrophy in vivo in the CAM mouse models, we performed echocardiography at two different age ranges. In agreement with the HW/BW ratios, CAM α_{1A}-AR mice had significantly increased posterior wall dimensions at both 4–6 months and 11–12 months of age (Fig. 5, A and B). At older ages of 11–12 months, the CAM α_{1B}-AR mice displayed significantly increased wall thickness (Fig. 5, A and B). In chamber size, CAM α_{1A}-AR mice displayed increased left ventricular dimensions in both end systolic and end diastolic dimensions at both age ranges, whereas the CAM α_{1B}-AR mice only displayed increased chamber size at older ages and only for end diastole (Fig. 5, C–F). Double CAM $\alpha_{1A/B}$ -AR mice did not display any increase in wall thickness or chamber size at any age and actually displayed significantly smaller chamber size than normal mice. There were no significant differences between males and females in any of the mouse models.

Costimulation of α_1-AR Subtypes Decreases HW/BW Ratio. Since the double CAM $\alpha_{1A/B}$-AR mice did not display cardiac hypertrophy whereas the single receptor CAM mice did, we tested the theory that co-expression of the α_1-AR subtypes might lead to the repression of hypertrophy. First, normal mice were injected twice per day for 2 weeks with propranolol (to block β-AR effects) and either NE alone or NE in conjunction with the α_{1A}-AR antagonist 5-methylurapidil (to stimulate α_{1B}-ARs), or the α_{1A}-AR agonist cirazoline (to

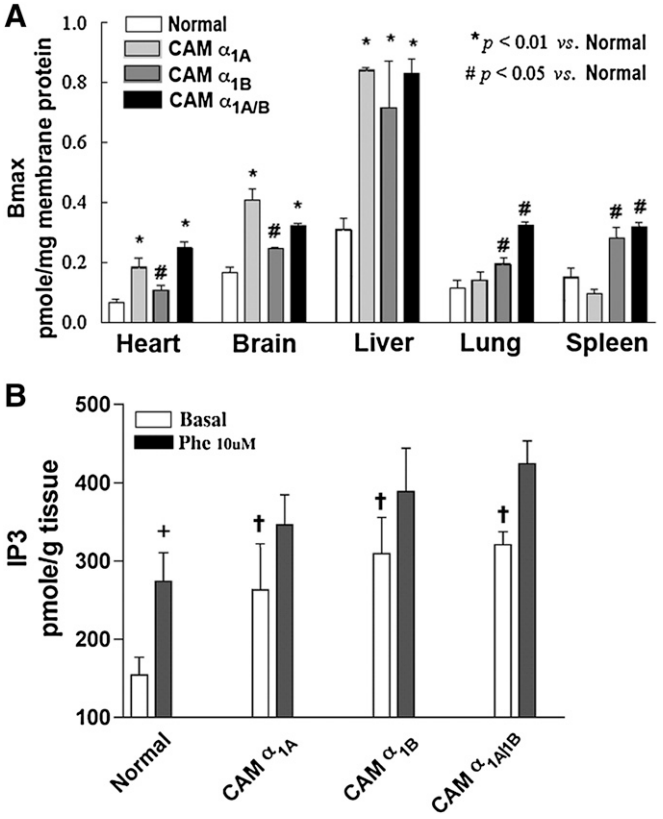

Fig. 2. Expression and constitutive activity of CAM $\alpha_{1A/B}$-AR. (A) Saturation binding was performed using [^{125}I]-HEAT to determine the density of α_1-ARs in hearts of transgenic and normal mice. $^*P < 0.01$; $^\#P < 0.05$ (significant difference compared with normal hearts). (B) IP_3 concentrations were measured in heart tissue from transgenic and normal mice and normalized to wet tissue weight. $^+$Significant activation of IP3 over nonstimulated tissue in normal hearts. †Significance of basal IP_3 over nonstimulated tissue. Data represent the mean ± S.E.M. of 4–8 mice of equal sexes.

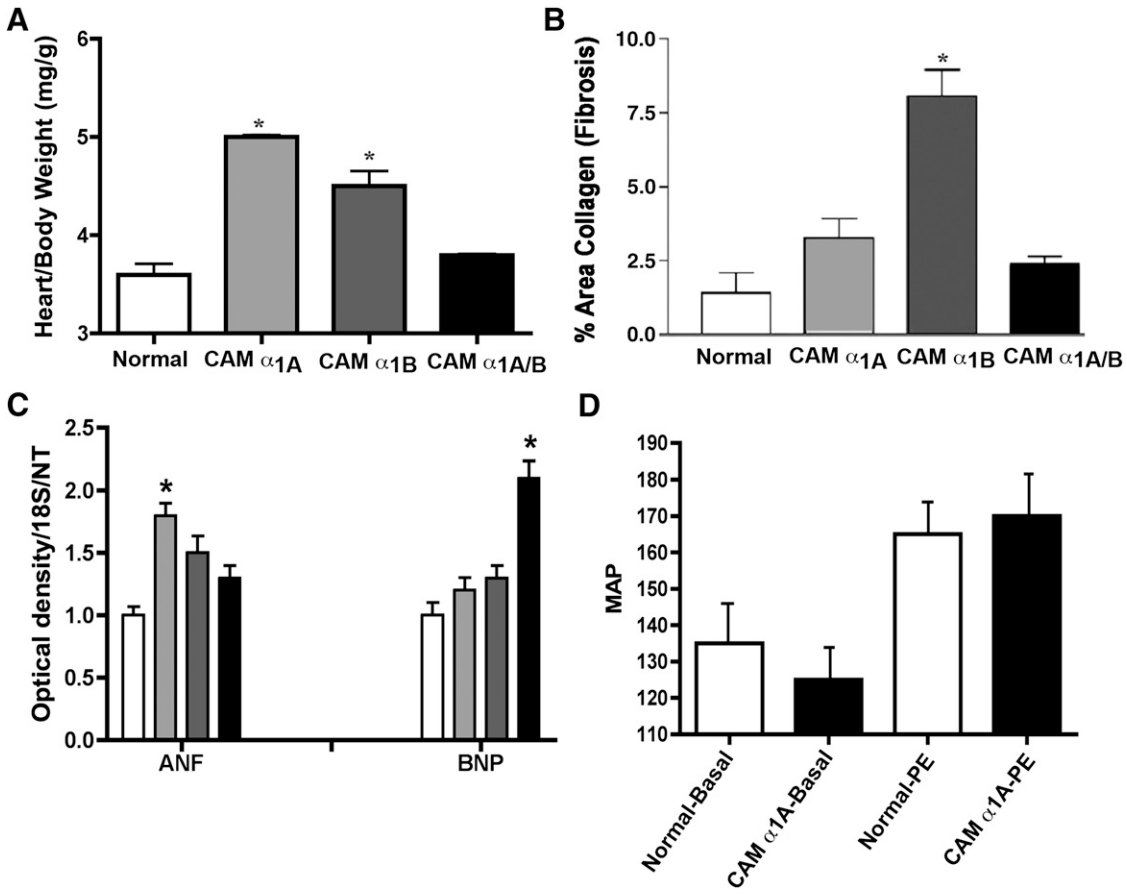

Fig. 3. HW/BW ratios (A), fibrosis (B), ANF/BNP levels (C), and BP (D). (A) The HW/BW ratio was determined in mice aged 6–8-months. (B) Hearts were subjected to Masson Trichrome staining and the amount of fibrosis determined through Image J analysis. (C) Total RNA from hearts were subjected to Northern blot analysis and probed for ANF and BNP mRNA. (D) Measurement of the mean carotid artery BP in conscious mice. BP studies in CAM α_{1B}-AR are published (Zuscik et al., 2001). Data represent the mean ± S.E.M. of 4–8 mice of equal sexes. $^*P < 0.05$ (significant difference compared with nontransgenic hearts).

stimulate α_{1A}-ARs). We found that normal mice induced cardiac hypertrophy to a similar degree with any subtype after α_1-AR stimulation (Fig. 6A). We next used the same protocol and injected CAM α_{1A}-AR or CAM α_{1B}-AR mice with either cirazoline or the α_{1B}-AR stimulation cocktail. We found that only costimulation of the opposite α_1-AR subtype significantly reduced the HW/BW ratio (Fig. 6B), whereas additional stimulation of the same α_1-AR subtype did not further increase hypertrophy. Finally, using the same protocol, we injected either cirazoline or the α_{1B}-AR stimulation cocktail into the double CAM $\alpha_{1A/B}$-AR mice and found that stimulation of either α_1-AR subtype increased cardiac hypertrophy (Fig. 6C).

IL-6 Levels. Since the IL-6/gp130/signal transducer and activator of transcription 3 (STAT3) pathway can mediate cardiac hypertrophy (Hirota et al., 1995; Kunisada et al., 1996) and we have previously shown that α_1-ARs can couple to this pathway and regulate the secretion of IL-6 in vitro (Gonzalez-Cabrera et al., 2003; Perez et al., 2009; Shi et al., 2012), we tested the level of IL-6 in the serum of the various mouse models. We found that only the CAM α_{1A}-AR mice had significant increased serum levels of IL-6 (Fig. 7A) whereas double CAM $\alpha_{1A/B}$-AR mice had levels similar to normal mice. These results suggest that IL-6 may be a prominent component of the hypertrophy response for the α_{1A}-AR and not for the α_{1B}-AR and may explain why myocyte-targeted transgenic mice for the α_{1A}-AR did not display cardiac hypertrophy.

IL-6 Signaling. Besides involvement in the secretion of IL-6, α_1-ARs can couple to the IL-6 signaling pathway independent of IL-6 through protein kinase 3/ERK signaling (Gonzalez-Cabrera et al., 2003; Perez et al., 2009; Shi et al., 2012). Therefore, we determined protein levels for gp130 and STAT3 in the various mouse models. We found that levels of gp130 as well as both phosphorylated forms of STAT3 in the hearts only from the double CAM $\alpha_{1A/B}$-AR mice were reduced compared with normal controls (Fig. 7B). These results suggest that the double CAM $\alpha_{1A/B}$-AR mice may be defective in gp130/STAT3 signaling.

Double CAM $\alpha_{1A/B}$-AR Mice Are Defective for IL-6–Mediated Cardiac Growth. We next determined whether the IL-6 signaling pathway is involved in α_1-AR mediated hypertrophy and whether that pathway is defective in the double CAM $\alpha_{1A/B}$-AR mice. We injected exogenous IL-6 into mice for 2 weeks and determined its effects on heart growth. Both normal and CAM α_{1B}-AR mice responded to IL-6 treatment by increasing the HW/BW ratio by 20–26%, whereas CAM α_{1A}-AR mice were unresponsive to IL-6 because they already possessed high IL-6 serum concentrations (Fig. 7C). Our results suggest that IL-6 is a contributing factor to the α_1-AR–mediated hypertrophic response. In addition, double

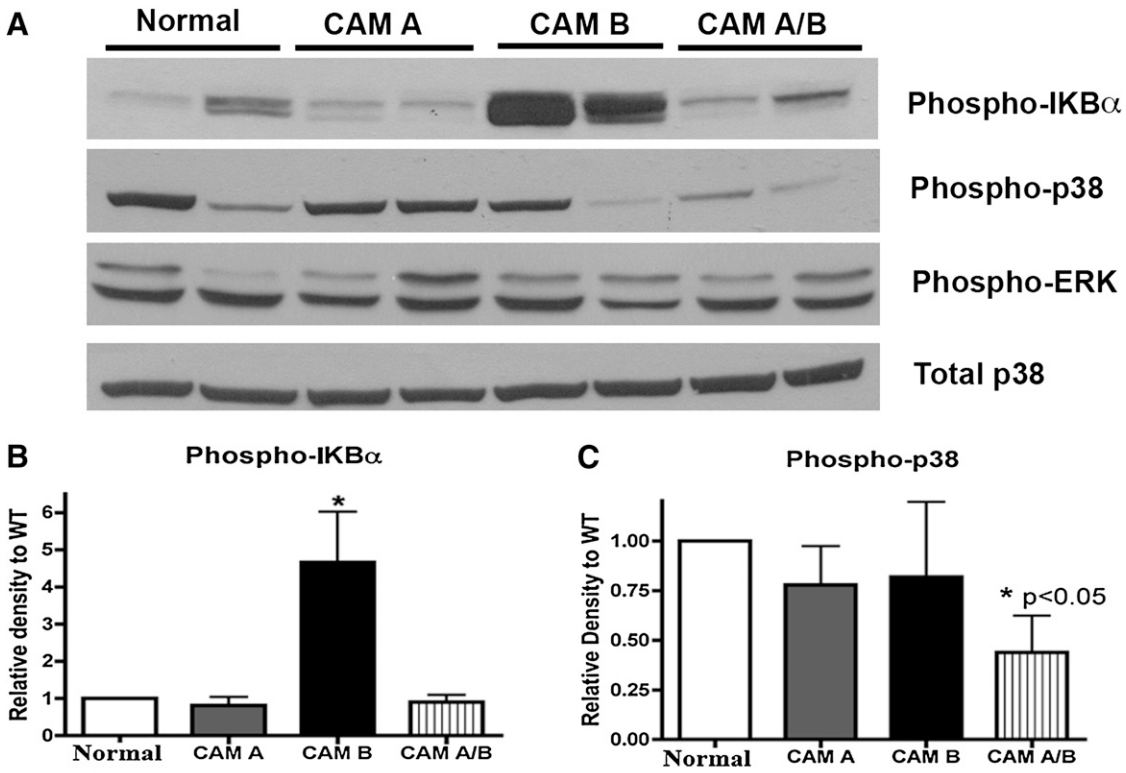

Fig. 4. Protein levels of phosphorylated p38 and IKBα. Hearts were homogenized from normal, CAM α_{1A}-AR (CAM A), CAM α_{1B}-AR (CAM B), and CAM $\alpha_{1A/B}$-AR (CAM A/B) mice and subjected to Western analysis. Phosphorylated proteins were normalized to total protein and glyceraldehyde 3-phosphate dehydrogenase. Data represent the mean ± S.E.M. of 4–6 mice of equal sexes. *$P < 0.05$ (significant difference compared with control).

CAM $\alpha_{1A/B}$-AR mice was unresponsive to IL-6, confirming that the IL-6 pathway was defective and at least part of the mechanism for the inhibition of cardiac hypertrophy.

Discussion

Early studies (Simpson, 1983) demonstrated that incubation of myocytes with catecholamines caused cellular hypertrophy by activation of α_1-ARs. Although many pathways have been shown to affect α_1-AR mediated hypertrophy, several of these pathways merge into the mitogen-activated protein kinase pathways (Zechner et al., 1997; Clerk et al., 1998; Nemoto et al., 1998) but have not been previously associated with IL-6/gp130/STAT3 signaling. We recently showed that α_1-AR–mediated protein kinase 3 and mitogen-activated protein kinase pathways activation can affect the phosphorylation status of STAT3 independent of IL-6 (Shi et al., 2012) and that α_1-AR–mediated p38 and NF-κB activation can regulate the expression and secretion of IL-6 (Gonzalez-Cabrera et al., 2003; Perez et al., 2009).

Although previous studies suggest that the α_{1A}-AR subtype mediated hypertrophy in neonatal myocytes (Knowlton et al., 1993; Rokosh et al., 1996; Autelitano and Woodcock, 1998), myocyte-targeted mouse models suggested otherwise, independent from expression levels (Lin et al., 2001). In this study, we show for the first time that a mouse model of the α_{1A}-AR subtype can mediate cardiac hypertrophy in vivo similar to CAM α_{1B}-AR mice (Zuscik et al., 2001). The α_{1A}-AR appears to mediate hypertrophy not through direct effects on the myocyte, consistent with the myocyte-targeted studies of Lin et al. (2001), but on secreted factors in the blood from noncardiac tissue, prominent of which is IL-6 (Fig. 8). As the native promoter in our transgenic mice allows systemic expression, α_1-ARs are expressed in other cell types that may be required for secretion of paracrine factors that ultimately affect the myocyte, such as IL-6 (Fig. 8). IL-6 is secreted from various cell types regulated through α_1-ARs (Loppnow and Libby, 1990; Yamauchi-Takihara et al., 1995; Hirasawa et al., 1996; Tayebati et al., 2000; Faber et al., 2001; Jensen et al., 2010; Grisanti et al., 2011), such as smooth muscle cells (Loppnow and Libby, 1990) and fibroblasts (Faber et al., 2001). IL-6 appears to play a prominent role in α_{1A}-AR–mediated hypertrophy since both normal and CAM α_{1B}-AR mice still respond to exogenous IL-6 (Fig. 7C), but not the CAM α_{1A}-AR mice, which were already saturated due to high serum levels (Fig. 7A). In addition, norepinephrine failed to initiate hypertrophy in IL-6 KO mice (Meier et al., 2009) and IL-6 failed to initiate a hypertrophic response in $\alpha_{1A/B}$ KO mice (Fig. 7C), suggesting that IL-6 is a prominent factor in α_1-AR–mediated cardiac hypertrophy.

Interestingly, the signals associated with hypertrophy are different and unique in the two mouse models. The CAM α_{1A}-AR mice expressed ANF (Fig. 3C) and secreted IL-6 into the bloodstream (Fig. 7A). CAM α_{1B}-AR mice, while not secreting IL-6, robustly activated the NF-κB (Fig. 4) hypertrophic pathway in the heart (Hirotani et al., 2002) and displayed fibrosis (Fig. 3). Although both IL-6 and NF-κB are associated with hypertrophy, they have not been previously associated with α_1-AR cardiac signaling. The selectivity of ANF expressing in the CAM α_{1A}-AR mice is not unexpected because

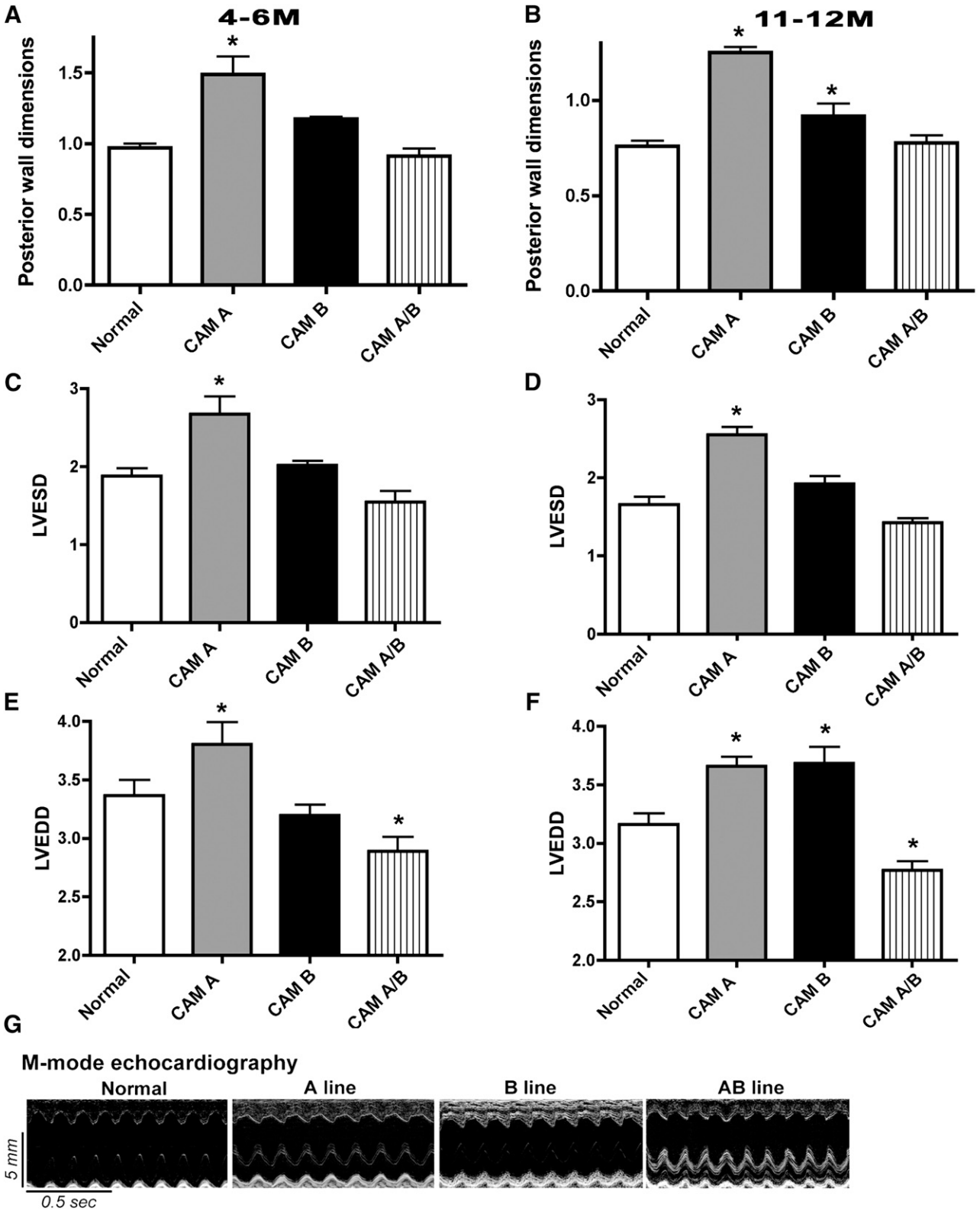

Fig. 5. Echocardiographic analysis of posterior wall dimensions and chamber size at ages 4–6 months and 11–12 months. Mice were subjected to echocardiographic analysis and anesthetized with isoflurane (0.2% v/v). M-mode echocardiograms (G) obtained from 9 to 10 beats per mouse allowed direct measurement (mean ± S.E.M.) of posterior wall thickness (A and B) and ventricular dimensions end systolic dimensions (C and D), and left ventricular end diastolic dimensions (E and F). $*P < 0.05$ (significance compared with age-matched normal controls). n = 6–8 mice of equal sexes.

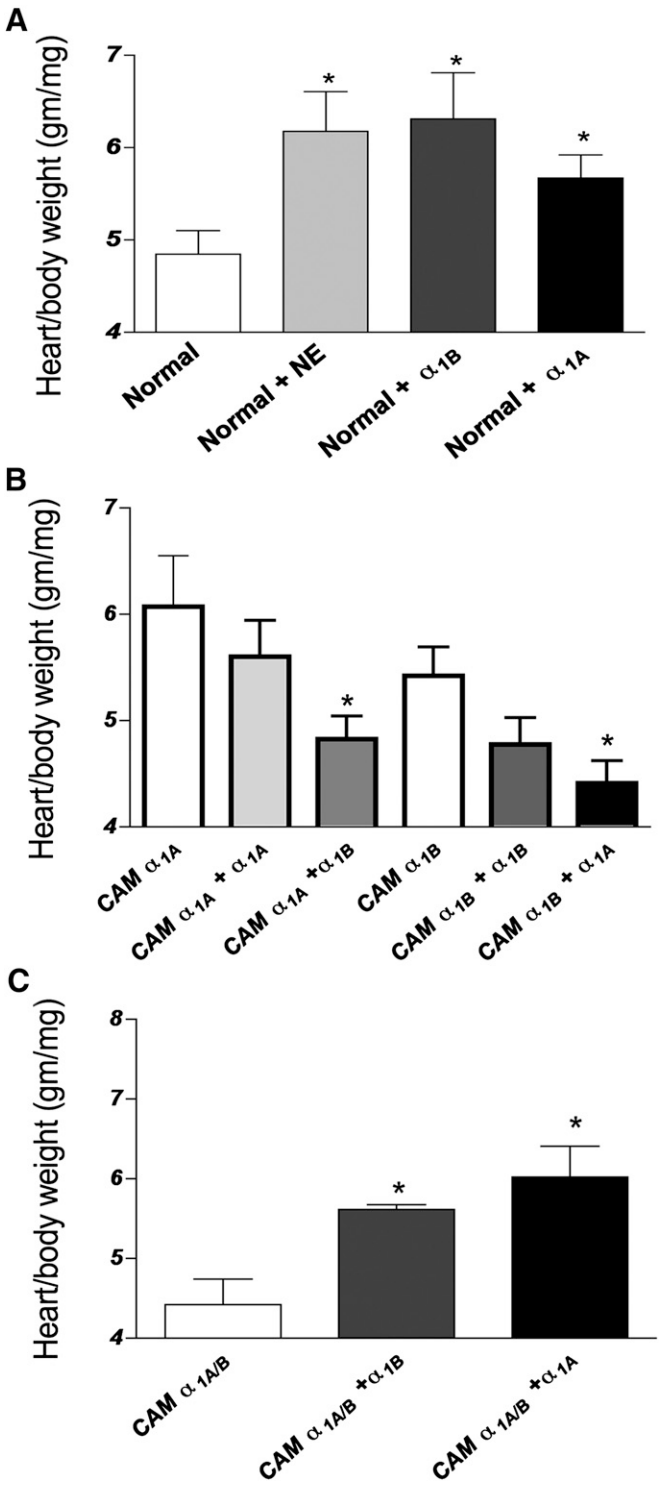

Fig. 6. α_1-AR subtype induced cardiac hypertrophy and suppression by coactivation. Normal or CAM mice were subjected to i.p. injections of various α_1-AR agonists and antagonists. α_{1A}-ARs were stimulated using cirazoline (0.3 mg/kg i.p.). α_{1B}-ARs were stimulated using NE (1 mg/kg i.p.) and the α_{1A}-AR antagonist, 5-methyurapidil (10 μg/kg i.p.). Control mice were injected with saline (0.9% NaCl). All mice were injected twice daily for 2 weeks and HW/BW ratios determined. Data represent the mean ± S.E.M. of 6–8 mice of equal sexes. *$P < 0.05$ (significant difference compared with control).

several studies suggested that ANF transcriptional activity is α_{1A}-AR driven (Knowlton et al., 1993; Autelitano and Woodcock, 1998; McWhinney et al., 2000). BNP was only expressed in the double CAM $\alpha_{1A/B}$-AR (Fig. 3C). Although BNP is often associated as a marker of hypertrophy and heart failure, exogenous and endogenous application of BNP is antihypertrophic, antifibrotic, and cardioprotective [reviewed in Ritchie et al. (2009)], consistent with the phenotype of the double CAM $\alpha_{1A/B}$-AR mice and is also a novel signal produced through coactivation of the two α_1-AR subtypes.

Our data suggest that both the CAM α_{1A}-AR and CAM α_{1B}-AR mice develop eccentric hypertrophy (Fig. 5) with both increased posterior wall thickness and chamber dilation, although this takes a longer time to develop in the CAM α_{1B}-AR mice and the effect is much milder. Eccentric hypertrophy is often seen with volume and not pressure overload (Spotnitz and Sonnenblick, 1973). Cardiac hypertrophy initially has beneficial effects in terms of muscular economy by normalizing wall stress (i.e., adaptive hypertrophy). However, several studies have demonstrated that chronic hypertrophy can be associated with a significant increase in the risk of heart failure, ischemic heart disease, and apoptosis [i.e., maladaptive hypertrophy; reviewed in Selvetella et al. (2004)]. Several studies have suggested that activation of the α_{1A}-AR but not the α_{1B}-AR subtype can be cardioprotective, which indicates a different involvement of the α_1-AR subtypes in the progression of adaptive to maladaptive hypertrophy [reviewed in Perez and Doze (2011) and Jensen et al. (2011)]. Because IL-6–mediated hypertrophy is also adaptive and cardioprotective (Kunisada et al., 2000; Jacoby et al., 2003; Hilfiker-Kleiner et al., 2004; Butler et al., 2006), our results suggest that IL-6 may be partially responsible for cardioprotection seen in the CAM α_{1A}-AR mouse. In addition, collagen synthesis is an indication of fibrosis, a condition of maladaptive hypertrophy, and only the CAM α_{1B}-AR mice displayed increased collagen deposition (Fig. 3B). Because collagen synthesis is decreased when STAT3 is inhibited (Mir et al., 2012), this may also explain why the double CAM $\alpha_{1A/B}$-AR mouse inhibited collagen deposition.

Surprisingly, double CAM $\alpha_{1A/B}$-AR transgenic mice did not develop hypertrophy as did the single CAM receptor transgenic mice (Figs. 3 and 5) and hypertrophy was repressed when the opposite α_1-AR subtype was coactivated in the CAM single receptor mouse models (Figs. 6 and 8). The double CAM $\alpha_{1A/B}$-AR mouse also showed depressed hypertrophic signals for p38, NF-κB, gp130, and p-STAT3 (Figs. 4, 7B, and 8), even less than normal receptors. However, hypertrophy developed in the double CAM $\alpha_{1A/B}$-AR mouse when either receptor subtype was further stimulated (Fig. 6C), suggesting that the regulation of hypertrophy was through signaling per se and not any permanent defect or artifact in the mouse model. Indeed, the inhibition of hypertrophy in the double CAM $\alpha_{1A/B}$-AR seems resultant of the antagonistic hypertrophic signaling changes caused by co-expression and coactivation of the α_{1A}- and α_{1B}-ARs. The co-expression of CAM α_{1B}-AR essentially blocked the ability of CAM α_{1A}-AR mice to secrete IL-6 (Fig. 7A). Likewise, the co-expression of the CAM α_{1A}-AR blocked the ability of the CAM α_{1B}-AR mice to activate NF-κB (Fig. 4). Whereas inhibition of particular signals has been previously shown to reverse hypertrophy, this is the first report of co-receptor activation mediating the same effect.

Mechanistically, inhibition of p38 and NF-κB signaling in the double CAM $\alpha_{1A/B}$-AR likely downregulated IL-6 since we have shown that α_1-AR–mediated IL-6 expression is regulated through p38 in myocytes (Fig. 8) (Perez et al., 2009). In

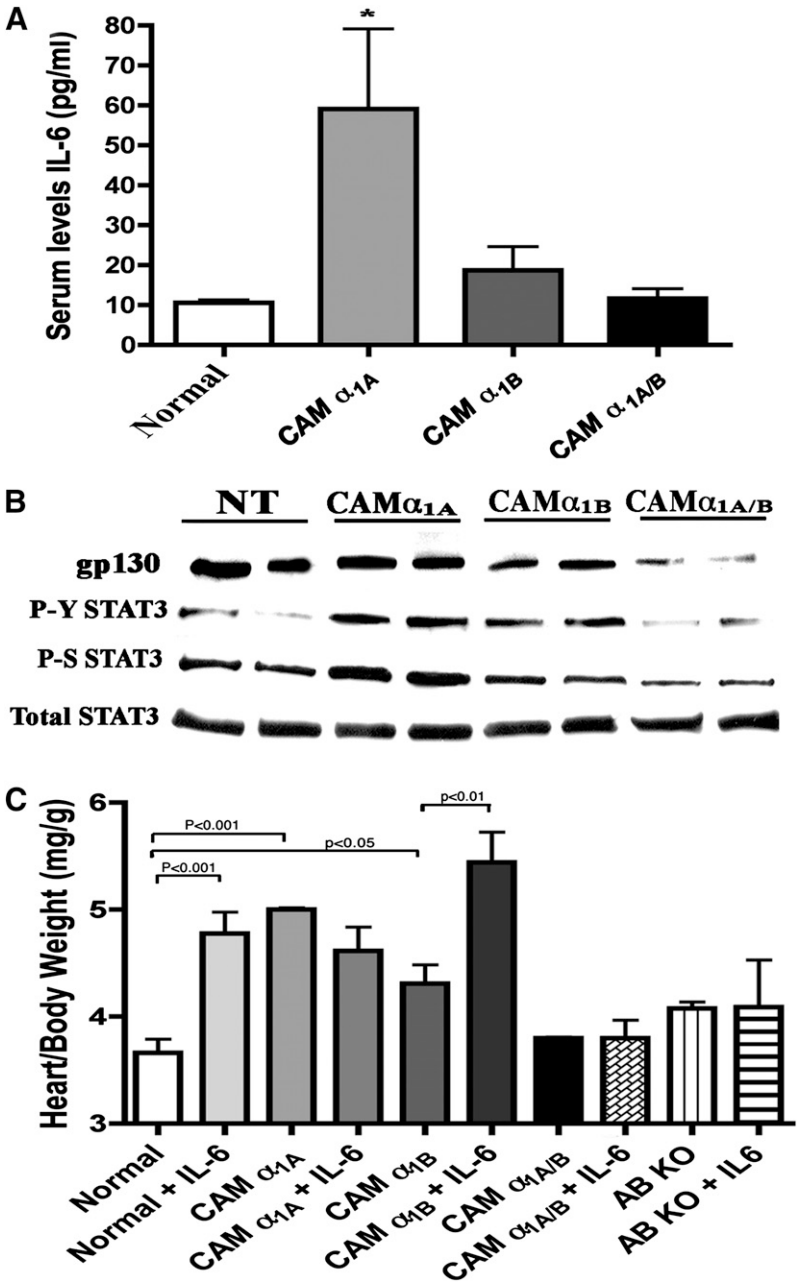

Fig. 7. IL-6/gp130/STAT3 levels and mediated hypertrophy in CAM mice. (A) Serum IL-6 was determined using the Quantikine mouse kit following the manufacturer's instructions. (B) Levels of gp130, phosphorylated, and total STAT3 as assessed by Western blot. (C) Mice were injected daily for 2 weeks i.p. with IL-6 (0.1 ml, 40 ng) and HW/BW ratios determined. Data represent the mean ± S.E.M. of 4–6 mice of equal sexes. $^*P < 0.05$ (significant difference compared with nontransgenic mice).

fact, both p38 and NF-κB regulate IL-6 expression and release in myocytes (Craig et al., 2000). Gp130 may downregulate through α_1-AR signaling due to gp130 phosphorylation by CaM kinases that target Ser782 to increase its internalization (Gibson et al., 2005).

One intriguing possibility is that heterodimer signaling of the α_1-AR subtypes is the initial step that suppresses hypertrophic signals (Fig. 8). There is precedence for this paradigm in various G protein-coupled receptor heterodimers that allow either mutually opposite, decreased signaling, or promoted novel signaling pathways (Jordan and Devi, 1999; Jordan et al., 2003; Stanasila et al., 2003; Hague et al., 2006; Rediger et al., 2011). α_{1A}- and α_{1B}-ARs have been shown to form heterodimers (Stanasila et al., 2003) and novel functional activities (Hague et al., 2006). Under physiologic conditions, the heart contains a disproportionate ratio of the α_1-AR subtypes. The rodent and human heart expresses approximately a 70/30 ratio in receptor density for the α_{1B}- and α_{1A}-AR subtypes (5–6) that may allow endogenous catecholamines to induce hypertrophy in vivo via a single α_1-AR subtype.

Our results are consistent with the theory that there are different signals mediating cardiac hypertrophy between the α_{1A}-AR and α_{1B}-AR. There is a prominent role of IL-6 in mediating α_{1A}-AR hypertrophy. Coactivation of α_{1A}- and α_{1B}-ARs results in antagonistic hypertrophic signaling for p38, NF-κB, gp130, and STAT3 (Fig. 8) that besides verifying the importance of the IL-6 pathway in α_1-AR–mediated hypertrophy, may offer an alternative therapeutic strategy for heart failure once sufficiently selective α_1-AR agonists are developed.

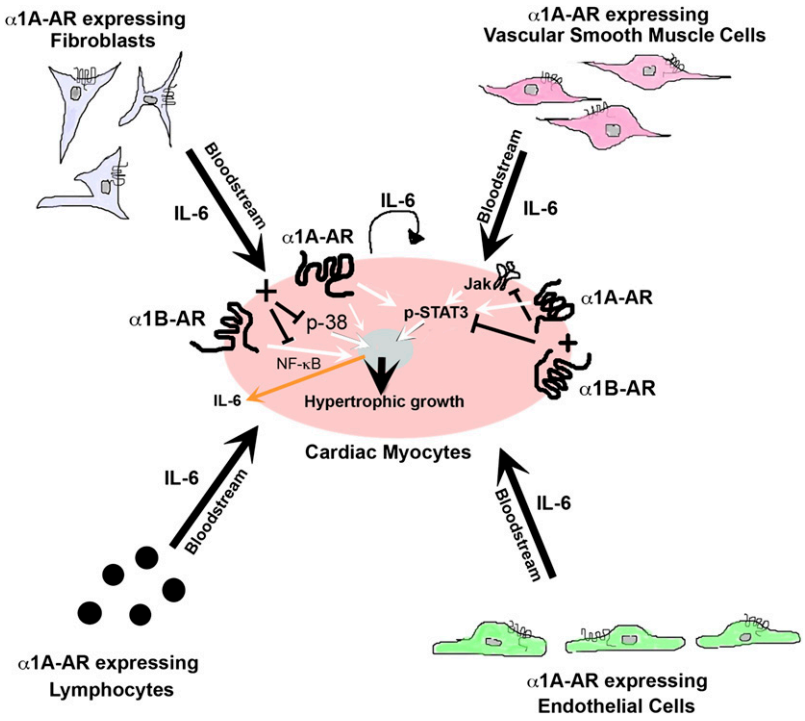

Fig. 8. Schematic of α_{1A}-AR–mediated cardiac hypertrophy and antagonistic hypertrophic signaling initiated with coactivation with the α_{1B}-AR. α_{1A}-ARs mediate the secretion of IL-6 into the bloodstream from various cell types such as myocytes, vascular smooth muscle cells, fibroblasts, lymphocytes, and endothelial cells. The secreted IL-6 acts on the myocyte to mediate cardiac hypertrophy through STAT3 nuclear signaling. α_{1A}-ARs also phosphorylate STAT3 independent of IL-6 secretion. α_{1B}-ARs mediate hypertrophic NF-κB signaling. When α_{1A}- and α_{1B}-ARs are coexpressed and coactivated, hypertrophic signals through p38, NK-κB, and STAT3 are inhibited. Inhibition of both p38 and NF-κB downregulate the expression and secretion of IL-6 from the myocyte.

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Authorship Contributions

Participated in research design: Papay, Shi, Piascik, Naga Prasad, Perez.

Conducted experiments: Papay, Shi, Piascik, Naga Prasad.

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Wrote or contributed to the writing of the manuscript: Papay, Shi, Naga Prasad, Perez.

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