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β 2 adrenergic activation induces the expression of IL-18 binding protein, a potent inhibitor of isoproterenol induced cardiomyocyte hypertrophy *in vitro* and myocardial hypertrophy *in vivo*

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Abstract

Both the sympathetic nervous system and the proinflammatory cytokine interleukin-18 (IL-18) play key roles in the pathophysiology of the hypertrophied failing heart. IL-18 binding protein (IL-18BP), a natural inhibitor of IL-18, counters its biological effects. β -AR stimulation induces IL-18 expression, but whether it also regulates IL-18BP is not known. Here we demonstrate that the β -AR agonist isoproterenol (ISO) increases steady state IL-18BP mRNA and protein levels in adult mouse cardiomyocytes in a β_2 -AR-dependent manner. We cloned mouse *Il18bp* 5' cis-regulatory region, and identified putative CREB and C/EBP β transcription factor-binding sites. Forced expression of mutant CREB or C/EBP β knockdown markedly attenuated ISO-induced *Il18bp* transcription and deletion or mutation of CREB and C/EBP motifs in the *Il18bp* promoter reduced ISO-induced promoter-reporter gene activity. ISO induced CREB and C/EBP β activation in cardiomyocytes via PI3K/Akt and ERK1/2. Importantly, ISO-induced hypertrophy *in vitro* was dependent on IL-18 induction as it was blunted by IL-18 neutralizing antibodies and forced expression of IL-18BP. Moreover, ISO-induced hypertrophy was markedly attenuated in IL-18 null and IL-18BP transgenic mice. These data support the novel concept that β -AR activation, in addition to inducing cardiomyocyte hypertrophy via IL-18, concomitantly induces a countering effect by stimulating IL-18BP expression, and that ISO-induced cardiomyocyte hypertrophy may result from a net effect of IL-18 and IL-18BP induction.

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Disclosures

None declared

Keywords

Signal transduction; transcription; cloning; molecular mechanism; adrenergic stimulation; heart failure

1. Introduction

Myocardial hypertrophy and failure are multi-factorial diseases. Sustained production of inflammatory cytokines plays a role in the initiation and progression of left ventricular hypertrophy to failure [1, 2]. Whereas low levels of cytokines may be protective for cardiomyocytes, chronically elevated levels appear to be detrimental [1–3]. Interleukin (IL)-18 is a pleiotropic cytokine belonging to the IL-1 family that is constitutively expressed as an inactive precursor in healthy cells, but during various immune, infectious, and inflammatory conditions, is converted to an active form by caspase-1 [4]. Processed (mature) IL-18 exerts its effects through the IL-18R receptor (IL-18R), a heterodimer of ligand-binding α and signal-transducing β subunits. Increased circulating IL-18 levels have been detected in patients with heart failure and a positive correlation has been demonstrated between serum IL-18 levels and the severity of myocardial dysfunction [5, 6], implying a pathophysiological role for this cytokine.

IL-18 binding protein (IL-18BP) on the other hand, is a naturally occurring, constitutively secreted inhibitor of IL-18, and a distinct gene product [7]. Differential splicing of human IL-18BP mRNA results in four isoforms: a, b, c and d. The ‘a’ isoform (IL-18BP_a) exhibits the greatest affinity for IL-18 with a rapid on-rate, a slow off-rate, and a dissociation constant of 0.399 nM (versus 2.94 nM for IL-18BP_c) [7], and both IL-18BP_a and IL-18BP_c neutralize human and mouse IL-18 by at least 95% at equimolar concentrations [7]. The b and d isoforms however, lacking a complete immunoglobulin (Ig) domain, fail to bind and neutralize IL-18, indicating that IL-18BP_a is the most potent isoform in man [7]. Of the four isoforms in mouse, only IL-18BP_c and IL-18BP_d have been shown to neutralize 95% of the IL-18 activity at equimolar concentrations [7].

IL-18BP binds IL-18 with higher affinity than IL-18 binds its receptor, and thus competitively blocks its activity [7]. The IL-18BP in the circulation of healthy humans is in at least 20-fold molar excess relative to IL-18. Thus under physiological conditions, a major portion of circulating IL-18 may be neutralized by binding to IL-18BP. In diseased states however, an active IL-18 ligand/receptor response axis may be elevated, and perhaps through more than one mechanism. For example, patients with heart failure have increased levels of IL-18 and IL-18R α , but significantly decreased IL-18BP [5]. Thus the combination of increased IL-18 and IL-18R α , and reduced IL-18BP may result in a positive amplification of IL-18 signaling. The therapeutic efficacy of IL-18BP_a has also been demonstrated in animal models of tissue injury [8, 9].

We and others have previously reported that IL-18 is potent pro-growth factor, inducing hypertrophy of isolated cardiomyocytes and H9c2 myoblasts [10, 11]. Neutralization of TNF- α , IL-1 and IFN- γ failed to modulate IL-18-mediated cardiomyocyte hypertrophy [10], suggesting the response to IL-18 was independent of other IL-18-induced cytokines. Studies from our laboratory as well as others have also shown that chronic administration of IL-18 induces myocardial hypertrophy with increased ANF expression [12, 13], and that pressure overload (transaortic constriction; TAC)-induced hypertrophy was markedly attenuated in IL-18 null mice [14].

β -AR stimulation induces myocardial and systemic elaboration of IL-18 [15]. ISO activated both basal and inducible *Il18* promoter reporter activities and stimulated IL-18 expression in cardiac microvascular endothelial cells [15]. These results indicate that ISO regulates IL-18 expression via increased gene transcription and mRNA stability, and suggest that IL-18 might contribute to chronic β -AR stimulation-induced hypertrophy and cardiac failure. However, whether β -AR stimulation also regulates IL-18BP expression is not known. Here we demonstrate for the first time that the β -AR agonist ISO upregulates IL-18BP expression in mouse cardiomyocytes in a β_2 -AR-dependent manner, and via PI3K/Akt and ERK1/2-dependent CREB and C/EBP β activation. Importantly, we also report that IL-18BP blunts ISO-induced cardiomyocyte hypertrophy *in vitro* and myocardial hypertrophy *in vivo*. Using IL-18BP transgenic (IL-18BP Tg) and IL-18 null mice, we further confirmed these observations. These data suggest that strategies that raise systemic levels of IL-18BP may have therapeutic potential in the treatment of hypertrophied failing heart, a diseased state characterized by sustained β -AR activation and IL-18 signaling.

2. Materials and methods

2.1. Materials

The materials used in this report are detailed in ‘Supplementary methods’ section.

2.2. Animals

All studies were performed in compliance with the NIH *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center at San Antonio, TX and Tulane University, New Orleans, LA. Male wild type C57Bl/6 mice and homozygous IL-18^{-/-} (IL-18 null C57Bl/6 mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-18 null mice have been previously described [14]. IL-18BP transgenic mice (IL-18BP Tg) that overexpress human IL-18BP isoform ‘a’ have also been previously described [16]. Non-transgenic littermates served as controls. All animals were used at ~3 months of age. Mice were subcutaneously implanted with mini-osmotic pumps (Alzet model 1007D) for continuous infusion of L-isoproterenol in 0.002% ascorbic acid at 15 mg/kg body wt/day [15]. Control mice were implanted with pumps that delivered vehicle (0.002% ascorbic acid) only. After 7 days, body weights were recorded. Blood was collected and serum separated and stored at -80°C. The heart was rapidly excised, rinsed in ice-cold physiological saline, and weighed. The atria were trimmed away, and the left (LV) and right ventricles separated. LV tissue was snap frozen in liquid nitrogen and stored at -80°C for biochemical and gene expression analysis. A portion of LV tissue was embedded in OCT for histo-morphometric analysis.

A subset of wild type male C57Bl/6 animals received the β_1 -AR antagonist betaxolol HCl (20 mg/kg. body wt), or the β_2 -AR antagonist ICI 118,551 (4 mg/kg. body wt) 1 h prior to ISO administration, and sacrificed after 3 h of ISO treatment, a time period at which maximal IL-18BP mRNA was detected. To further confirm that β_2 -AR stimulation induces myocardial IL-18BP expression, wild type male C57Bl/6 animals received the β_2 -AR agonist clenbuterol HCl (0.3 mg/kg. body wt, IP) for 3 h as previously described [15].

2.3. Adult and neonatal mouse cardiomyocytes

Calcium-tolerant adult mouse cardiomyocytes (ACM) were isolated from ~3 month-old male C57Bl/6 mice by a modified Langendorff perfusion and collagenase digestion technique, and has been previously described [17, 18]. Similarly, cardiomyocytes were isolated from IL-18 null and IL-18BP Tg mice. The yield, shape, and viability of cardiomyocytes from wild type, IL-18 null and IL-18BP Tg mice were similar (data not

shown). Since ACM are extremely difficult to transfect using standard transfection protocols, we used neonatal mouse cardiomyocytes (NMCM) for promoter-reporter assays. NMCM show relatively higher transfection efficiency (~58% with only 7% cell death) as determined using the pEGFP-N1 vector. NMCM were microporated (pulse voltage: 1700 volts, pulse width: 20 ms, pulse number: 1, and the tip type: 10 μ l) with 3 μ g of the vector using the Neon[®] transfection system (MPK-5000, Invitrogen). NMCM were isolated from 1- to 3-day-old neonatal mice (C57Bl/6 background) as previously described [17, 18].

2.4. Adenoviral infection and siRNA-mediated knockdown

Cardiomyocytes were infected with adenoviruses at the indicated multiplicities of infection (MOI) (Supplementary methods). The transfection efficiency with the adenoviral vectors was near 100%, and infection with the adenoviral vectors at indicated MOI had no significant effect on cardiomyocyte shape, adherence, or viability. C/EBP β was targeted by siRNA, and its knockdown was confirmed by immunoblotting. siRNA for GFP served as a control. Ad.CMV-IL-18BPc has been previously described [19]. Further details are provided in 'Supplementary methods' section.

2.5. Cell death analysis

To determine whether transduction of viral vectors, siRNA, or pharmacological inhibitors negatively affected cell viability, cell death was analyzed using the Cell Death Detection ELISA^{PLUS}. Cell viability was also tested by trypan blue dye exclusion and microscopic visualization of shape and for cells floating in the media. Details are provided in 'Supplementary methods' section.

2.6. Cardiomyocyte hypertrophy *in vitro* and *in vivo*

Cardiomyocyte hypertrophy *in vitro* was assessed by two independent methods: increased protein, but not DNA, synthesis and cell surface area. Details are provided in 'Supplementary methods' section. In addition to heart wt to body weight ratios in ISO-infused animals, hypertrophy was also assessed by quantifying diameter of cardiomyocytes in the region of the cell nucleus (100 cells/heart, 4 hearts/group, and 4 groups) in H&E stained cryosections. We also analyzed total and phosphorylated forms of the protein synthesis marker ribosomal S6 protein, and ANF gene expression.

2.7. *Il18bp* promoter-reporter assays

A 1964 bp fragment (p*Il18bp*-1964) of the 5'-flanking region of the mouse *Il18bp* gene was amplified from mouse genomic DNA using primers shown in Table 1. The sense primer contained an *Mlu*I restriction site at the 5' end, and the antisense primer contained an *Xho*I restriction site (lower case). The PCR product was cloned into pCR2.1-TOPO and subcloned into the pGL3-Luciferase reporter vector at the *Mlu*I/*Xho*I sites. The identity of the PCR product was confirmed by sequencing on both strands. *In silico* analysis of this region identified several putative transcription factor-binding sites (C/EBP, CREB, Sp1 and NF- κ B). Accordingly, deletion constructs were created using the antisense primers shown in Table 1. NMCM were transfected with 3 μ g of the reporter vectors, and cotransfected with 100 ng of the internal reference plasmid *Renilla* luciferase (pRL-TK) using the Neon transfection system described above. 48h after transfection, cells were treated with ISO. After 12 h cells were harvested for the dual-luciferase assay. Firefly luciferase data were normalized to that of corresponding *Renilla* luciferase activity. All plasmids were purified using EndoFree Plasmid Maxi kit.

2.8. Transcription factor activation

Nuclear extracts were prepared using Panomics Nuclear Extraction Kit, and is described in 'Supplementary methods' section. Lamin A/C and GAPDH served as loading and purity controls. CREB activation was analyzed by electrophoretic mobility shift assay (EMSA), and the oligonucleotide sequences are provided in Table 1. CREB activation was confirmed by a reporter gene assay using an adenoviral CRE-luciferase reporter vector (Ad.CRE-Luc). The empty adenoviral construct (Ad.MCS-Luc) served as a base-line control. Ad.pRL-TK served as an internal control. Firefly and *Renilla* luciferase activities were determined as described above. C/EBP activation was also analyzed by EMSA using gene-specific oligonucleotides (Table 1). In the gel supershift assay, the nuclear protein extract (5 µg) was preincubated for 40 minutes on ice with anti-C/EBP α or C/EBP β antibodies (0.2 µg) before the addition of ³²P-labeled C/EBP consensus double-stranded oligo as described in 'Supplementary methods' section. Respective normal IgG served as controls. Activation of NF- κ B was analyzed by EMSA.

2.9. Chromatin immunoprecipitation (ChIP) assay

ACM were treated with ISO (10^{-7} M) for 1 h. The ChIP assay was performed following the manufacturer's instructions using a kit (Millipore/Upstate Biotechnology, Inc.), and is detailed in 'Supplementary methods' section.

2.10. mRNA expression

IL-18BP, IL-18, ANF and EMMPRIN mRNA expressions were analyzed by Northern blotting as detailed in 'Supplementary methods' section. 28S rRNA served as a loading control. The autoradiographic signals were semiquantified by videoimage analysis.

2.11. Protein Extraction and Western Blot Analysis

Protein extraction from cardiomyocytes and left ventricular tissue, immunoblotting, detection of the immunoreactive bands by enhanced chemiluminescence, and densitometry are all detailed in 'Supplementary methods' section. α Tubulin and GAPDH served as loading controls. Activation of Akt, ribosomal S6 protein, and p70 S6 kinase was confirmed by immunoblotting using respective activation-specific antibodies.

2.12. Biological activity of rIL-18BP α -Fc, IL-18BP α and anti-IL-18 antibodies

The biological activity of IL-18BP α and IL-18 neutralizing antibodies on IL-18 induced NF- κ B was confirmed by EMSA. ACM were infected with Ad.IL-18BP α (MOI 50 for 24 h) or pre-treated with IL-18 neutralizing antibodies (10 µg/ml for 1 h) prior to IL-18 addition (5 ng/ml for 1 h). Ad.GFP and normal goat IgG served as controls. Both Ad.IL-18BP α and anti-IL-18 antibodies significantly inhibited IL-18 induced NF- κ B activation (Supplementary Figure S1-A). Secretion of IL-18BP α following Ad.IL-18BP α infection was confirmed by immunoblotting using TCA-precipitated culture supernatants (Supplementary Figure S1-B).

2.13. Statistical analysis

Comparisons between controls and various treatments were performed by analysis of variance with post hoc Dunnett's *t* tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E. Densitometric results were shown as ratios at the bottom of the panels whenever the results are less clear.

3. Results

3.1. Isoproterenol induces IL-18BP expression via β 2-AR in adult cardiomyocytes

We have previously shown that ISO induces IL-18 expression both *in vivo* and *in vitro* [15]. IL-18 is a powerful mediator of inflammation and its expression is tightly regulated through expression of IL-18 binding protein. Therefore we tested the hypothesis that ISO may also induce the expression of this neutralizing binding protein. Using Northern blot analysis, we demonstrate that ISO is a potent inducer of IL-18BP in adult mouse cardiomyocytes (ACM). ISO induced IL-18BP expression was both dose- (10^{-6} to 10^{-9} M; Fig. 1A) and time- (1–24 h; Fig. 1B) dependent, with peak levels detected at 10^{-7} M and 2 h. Therefore, in all subsequent studies, ISO was used at 10^{-7} M. Adult cardiomyocytes express both β 1 and β 2 receptors under basal conditions [20]. Therefore, we next determined the β -AR specificity in mediating the response to ISO. Our results indicate that treatment with the classic non-selective β 1- and β 2-AR blocker propranolol HCl markedly inhibits IL-18BP mRNA expression (Fig. 1C). Moreover, while treatment with the β 1-AR antagonist CGP 20712A failed to produce an effect, treatment with the β 2-AR antagonist ICI 118,551 significantly attenuated ISO-induced IL-18BP expression (Fig. 1C). Since ISO induces IL-18BP mRNA expression, we next investigated whether ISO regulates its expression via increased transcription or mRNA stability. The results show that while Actinomycin D (ActD) blunts, cyclohexamide (CHX) fails to affect ISO-induced IL-18BP mRNA expression (Fig. 1D). CHX also failed to modulate IL-18BP protein levels (Fig. 1E). ISO also induced IL-18BP protein expression via β 2-AR-dependent manner (Fig. 1F), and induced its secretion (Fig. 1G). These results indicate that ISO is a potent inducer of IL-18BP transcription in cardiomyocytes, and this effect is mediated by a β 2-AR-dependent signaling pathway (Fig. 1).

3.2. Isoproterenol induces *Il18bp* transcription in neonatal cardiomyocytes

Since ISO-induced IL-18BP expression was regulated at the transcriptional level (Fig. 1D), we next cloned mouse *Il18bp* 5' *cis*-regulatory region 1964 basepairs upstream of the transcriptional start site (Supplementary Fig. S2-A). Analysis of the promoter region by the MatInspector Professional® software identified potential binding sites for various transcription factors, including one CREB, three C/EBP, two NF- κ B, and two Sp1 sites (Supplementary Fig. S2 and Fig. 2A). Since adult cardiomyocytes are difficult to transfect, we used neonatal mouse cardiomyocytes (NMCM) that show higher transfection efficiency. Transient transfection of NMCM with the full-length (1.964 kb) promoter-reporter construct showed a 2-fold increase in the reporter activity at basal conditions (*versus* the promoter-less pGL3-Basic), and treatment with ISO significantly increased its activity by an additional 4-fold (Fig. 2A). Progressive deletion of the promoter indicated that the construct (p*Il18bp*-1277) that still contained the putative CREB, C/EBP and Sp1 binding sites, showed the maximum activity following ISO treatment (Fig. 2A). Therefore, we used this construct to examine the contribution of these sites to the ISO response. Our results show that while forced expression of dnSp1 or pre-treatment with the Sp1 inhibitor mithramycin failed to modulate the response, forced expression of a Flag-tagged dominant negative mutant form CREB (Ad.KCREB; expression of Flag is confirmed by immunoblotting; upper right hand panel) and C/EBP knockdown (knockdown is shown in the lower right hand panel) blunted ISO-induced *Il18bp* promoter-dependent reporter gene activation (Fig. 2B). Of note, forced expression of dnp65 by adenoviral transduction failed to significantly modulate ISO-induced *Il18bp* promoter activity (Supplementary Fig. S2-B and C). We confirmed the role of the CREB and C/EBP sites by mutating these sites singly or together in the p*Il18bp*-1277 construct. While mutation of the middle C/EBP binding site had no significant effect, mutation of either the CREB or the remaining (distal) C/EBP sites markedly attenuated ISO-induced *Il18bp* promoter-reporter activity (Fig. 2C). These

inhibitory effects were more pronounced when both the proximal C/EBP and CREB sites were mutated. Moreover, forced expression of KCREB (left hand panel) or C/EBP β knockdown (right hand panel) inhibited ISO-mediated IL-18BP mRNA expression (Fig. 2D). Together, these results indicate that both CREB and C/EBP play critical roles in ISO-induced *Il18bp* transcription (Fig. 2).

3.3. Isoproterenol activates CREB in adult cardiomyocytes via cAMP, PI3K/Akt and ERK1/2

Since ISO stimulates *Il18bp* transcription and mRNA expression in part via CREB, we next investigated whether ISO activates CREB, and determined the underlying signal transduction pathways. Our results show that ISO induces CREB phosphorylation in a time-dependent manner, and this effect was significantly inhibited by the potent membrane permeant cAMP antagonist Rp-8-Br-cAMPS (Fig. 3A, left hand panel) and forced expression of a dominant negative mutant form of CREB (KCREB; Fig. 3A, right hand panel). Further, ISO stimulates CREB DNA binding activity (Fig. 3B) and CRE reporter gene activity (Fig. 3C), and CREB binding to the *Il18bp* promoter (Fig. 3D). Since CREB is a regulatory target of Akt [21], and Akt is an important intermediate in β -AR-mediated pro-growth and anti-apoptotic effects [22], we next investigated whether ISO activates Akt in cardiomyocytes. ISO induced time-dependent Akt activation (Fig. 3E), an effect that was significantly inhibited by the PI3K inhibitor wortmannin, the Akt inhibitor Akti-X, and by the adenoviral transduction of mutant PI3Kp85 (Ad.dnPI3Kp85) and Akt1 (Ad.dnAkt1) (Fig. 3F). Moreover, ISO activated ERK1/2, an effect that was markedly attenuated by wortmannin, Ad.dnPI3Kp85, U0126, Ad.dnMEK1 and PD98059 (Fig. 3G). Importantly, Rp-8-Br-cAMPS, Ad.dnPI3Kp85, Ad.dnAkt1, and Ad.dnMEK1 inhibited ISO-induced CREB activation (Fig. 3H) and IL-18BP mRNA expression (Fig. 3I). Of note, adenoviral transduction of constitutively active Akt (*myr*-Akt) induced CREB activation (Supplementary Fig. S3). These results indicate that ISO activates CREB *in vitro*, induces its binding to *Il18bp* promoter *in vivo*, and ISO-induced CREB activation and IL-18BP expression is cAMP, PI3K/Akt and ERK1/2 dependent (Fig. 3).

3.4. Isoproterenol also activates C/EBP β in adult cardiomyocytes via PI3K/Akt and ERK1/2

Our promoter studies also showed that the deletion construct lacking the proximal C/EBP is markedly less responsive to ISO (Fig. 2A). Mutation in the C/EBP sites also blunted *Il18bp* promoter-dependent reporter gene activity (Fig. 2C). Therefore, we next investigated whether ISO induces C/EBP activation, and determined the underlying signal transduction pathways. ISO potently induced C/EBP DNA binding activity *in vitro* (Fig. 4A), and the DNA/protein complex was supershifted by anti-C/EBP β antibodies (Fig. 4B). Further, ISO stimulated C/EBP β binding to *Il18bp* promoter *in vivo* (Fig. 4C). Similar to their inhibitory effects on CREB activation (Fig. 3H), Ad.dnAkt1, PD98059 and Ad.dnMEK1 all attenuated ISO-induced C/EBP β DNA binding activity (Fig. 4D), and C/EBP β knock down attenuated ISO-induced IL-18BP expression (Fig. 4E), indicating that ISO activates C/EBP β via PI3K/Akt and MEK/ERK1/2 (Fig. 4).

3.5. IL-18BP blocks isoproterenol/IL-18-mediated adult cardiomyocyte hypertrophy

IL-18 is a potent pro-hypertrophic factor, inducing cardiomyocyte hypertrophy *in vitro* [10] and myocardial hypertrophy *in vivo* [12] [13]. Since ISO induces IL-18 expression [15], we next investigated whether ISO-induced cardiomyocyte hypertrophy is IL-18 dependent. Confirming our earlier results in cardiac microvascular endothelial cells [15], ISO (10^{-7} M) stimulated time-dependent IL-18 mRNA expression (Fig. 5A, left hand panel), intracellular IL-18 protein levels (Fig. 5A, right hand panel), and IL-18 secretion (Fig. 5B) in adult cardiomyocytes. Further, unlike the positive control doxorubicin [23], ISO failed to induce cardiomyocyte death (Supplementary Figure S5-A). At this concentration, ISO also induced the phosphorylation of Bad, a pro-apoptotic protein (Supplementary Figure S5-B), rendering

it inactive. Notwithstanding this, ISO in fact induced cardiomyocyte growth; increased protein, but not DNA (Supplementary Figure S5-C) synthesis in ACM (Fig. 5C), an effect that was significantly attenuated by IL-18 neutralizing antibodies and IL-18BPc overexpression. Recombinant human IL-18BPc was equally effective in blunting ISO-induced cardiomyocyte hypertrophy (Fig. 5C). Increased cell surface area confirmed ISO's pro-hypertrophic effects (Supplementary Figure S5-D). Of note, similar to its effects in neonatal rat cardiomyocytes [10], IL-18 induced ACM hypertrophy, and its pro-growth effects were markedly attenuated by IL-18BPc, IL-18 neutralizing antibodies and rIL-18BPc (Supplementary Fig. S5-E). IL-18 also induced several hypertrophy-associated factors in ACM (Supplementary Fig. S5-F). Similar to IL-18, ISO induced phosphorylation of the protein synthesis marker p70 S6 kinase (Thr³⁸⁹; Fig. 5D), phosphorylation of ribosomal S6 protein (Fig. 5E), phosphorylation of the pro-survival factors Akt (Fig. 5F) and ERK1/2 (Fig. 5G), and the mRNA expression of the pro-hypertrophic marker ANF (Fig. 5H). Importantly, these effects were markedly attenuated by the adenoviral transduction of IL-18BPc and pretreatment with IL-18 neutralizing antibodies (Fig. 5, C-H). The effect of ISO on cell growth was also carried out on cardiomyocytes from IL-18 null and IL-18BP Tg mice. Although hypertrophic effects were seen on the cardiomyocytes from wild type and littermate control mice, it was markedly attenuated in cells from IL-18 null and IL-18BP Tg mice (Fig. 5I), indicating that ISO induces cardiomyocyte hypertrophy in part via IL-18 (Fig. 5).

3.6. Isoproterenol-induced myocardial IL-18BP expression *in vivo* is β 2-AR dependent

We have demonstrated that ISO is a potent inducer of IL-18BP *in vitro* in cardiomyocytes (Fig. 1). Therefore, we next investigated whether ISO induces IL-18BP expression *in vivo* in myocardium, and determined the receptor specificity. Supporting the *in vitro* data, ISO induced IL-18BP protein expression in myocardium *in vivo* in a time-dependent manner, with peak levels detected after 3 h (Fig. 6A; densitometric analysis of n=4/group is summarized in panel B), and this effect was markedly attenuated by the β 2-AR antagonist ICI 118,551 (Fig. 6C; densitometric analysis of n=4/group is summarized in the lower panel). Further, clenbuterol HCl, a β 2-AR agonist, induced IL-18BP protein expression *in vivo* (Fig. 6D; densitometric analysis of n=3/group is summarized in the right hand panel), indicating that ISO induces IL-18BP *in vivo* in β 2-AR dependent manner (Fig. 6).

3.7. Isoproterenol-induced myocardial hypertrophy in wild type mice is characterized by increased IL-18BP and IL-18 expression

Since ISO-induced cardiomyocyte hypertrophy *in vitro* is characterized by enhanced expression of IL-18BP and IL-18 (Figs. 1 and 5), we next determined the expression levels IL-18BP and IL-18 in ISO-induced myocardial hypertrophy *in vivo*. Indeed, continuous infusion of wild type mice with ISO for one week induced myocardial hypertrophy, as evidenced by increased heart to body weight ratios (Fig. 7A; histology of heart is shown on the right) and induction of hypertrophy-associated ANF gene expression (Fig. 7B). Further, IL-18BP mRNA expression was markedly elevated (Fig. 7C), its protein levels (Fig. 7D) were modestly elevated at 7 days. ISO also upregulated IL-18 protein expression (Fig. 7E). Confirming the *in vitro* data, ISO induced CREB phosphorylation/activation (Fig. 8A), CREB (Fig. 8B) and C/EBP (Fig. 8C) DNA-binding activities, and Akt (Fig. 8D) and ERK1/2 (Fig. 8E) activation. Unlike the positive control doxorubicin HCl [23], ISO at the indicated concentration and duration, failed to induce cardiomyocyte death *in vivo* (data not shown). These results indicate that ISO-induced myocardial hypertrophy is characterized by enhanced IL-18BP and IL-18 expression (Figs. 7 and 8).

3.8. Isoproterenol-induced myocardial hypertrophy is blunted in IL-18BP Tg and IL-18 null mice

Since IL-18 and IL-18BP are both induced by ISO, but have opposite effects on myocardial hypertrophy, we further dissected their roles in ISO-induced hypertrophy using gene-altered mouse models. Infusion of ISO into the overexpressing IL-18BP Tg mice resulted in significantly less hypertrophy compared to littermate, non-Tg controls (Fig. 9A). Further, cardiomyocyte diameter (Fig. 9B), protein levels of phospho-ribosomal S6 protein (Fig. 9C, upper panel) and ANF mRNA expression (Fig. 9C, lower panel) were markedly attenuated in ISO-infused IL-18BP Tg mice. This is consistent with our *in vitro* studies that ISO-induced IL-18 contributes to cardiomyocyte hypertrophy and this is blocked by IL-18BP (Fig. 5A–C). Furthermore, it would be expected that in IL-18 null mice, ISO-induced myocardial hypertrophy and hypertrophy-associated markers should also be reduced compared to matched controls, and this was indeed our observation (Fig. 9D–F). Our studies indicate that β -AR stimulation induces IL-18 expression, which is pro-hypertrophic, but also induces IL-18BP, which counters the IL-18 effects. As ISO ultimately promotes cardiomyocyte and myocardial hypertrophy, the concomitant induction of IL-18BP may act as a natural negative regulatory mechanism to limit the effects of IL-18 on this process (Fig. 9).

4.0 Discussion

Myocardial hypertrophy and heart failure are characterized by sustained β -AR activation and chronically elevated expression of proinflammatory cytokines [1, 2]. IL-18 is a proinflammatory cytokine that modulates cardiomyocyte growth *in vitro* and myocardial hypertrophy *in vivo* [10, 13]. Increased levels of IL-18 are associated with hypertrophy and failure in humans.

We have previously demonstrated that the β -AR agonist ISO is a potent inducer of IL-18 expression in the mouse heart *in vivo* [15]. Here we show that ISO is also a potent inducer of IL-18 in cardiomyocytes *in vitro*. Importantly, we also demonstrated for the first time that ISO induces the expression of IL-18BP, a neutralizing IL-18 binding protein, via a β 2-AR-dependent signaling. ISO activates CREB and C/EBP β in a PI3K/Akt and ERK1/2-dependent manner, and induces *Il18bp* transcription in part via CREB and C/EBP β (Fig. 9G). Moreover, continuous infusion of ISO induces myocardial hypertrophy, and is associated with enhanced CREB and C/EBP β DNA-binding activity, Akt and ERK1/2 activation, and IL-18BP expression, indicating that ISO-induced myocardial hypertrophy is paradoxically accompanied by increased IL-18BP expression *in vivo*. Importantly, our data suggest that ISO-induced IL-18BP expression, at least initially, provides a negative regulatory mechanism to attenuate IL-18's pro-hypertrophic effects both *in vivo* and *in vitro*. Interestingly, however, while ISO-induced IL-18BP mRNA expression in cardiomyocytes reached near basal levels by 24 h, induction of IL-18 was rapid, and persisted at high levels throughout the 24 h study period, suggesting that down-regulation of IL-18BP and sustained IL-18 expression due to impaired β AR signaling may contribute to myocardial hypertrophy in chronically stressed heart.

Myocardial hypertrophy and failure are characterized by sustained activation of β -AR stimulation [24]. β 2-AR is a G protein-coupled receptor, and signals via both stimulatory (Gs) and inhibitory (Gi) G proteins [25]. Stimulation of Gs signaling activates adenylyl cyclase (AC), cAMP generation, and activation of protein kinase A (PKA). In contrast, Gi signaling attenuates AC, inhibits cAMP synthesis and PKA activation [25]. Intriguingly, our results showed that ISO-induced IL-18BP expression is both CREB and C/EBP β -dependent, and treatment with pertussis toxin, a Gi inhibitor, while enhancing CREB activation, moderately inhibited IL-18BP expression (data not shown), suggesting the critical role of C/

EBP, and that ISO-induces IL-18BP expression via both Gi-dependent and independent mechanisms. Of note, chronic ISO treatment can lead to desensitization of β -AR signaling, a Gi-mediated function [26–28]. Therefore, it is possible that low levels of IL-18BP detected in cardiomyocytes at 24 h following ISO treatment may be due to desensitization of the β 2-AR signaling.

Our results demonstrate that CREB and C/EBP β are both critical to ISO-mediated *Il18bp* transcription in mouse cardiomyocytes. Of note, human *Il18bp* has also been shown to be a C/EBP β -responsive gene [29]. Interestingly, C/EBP β itself is a CREB-responsive gene. The C/EBP promoter contains two CRE sites, and activated CREB induces C/EBP β transcription [30], suggesting that ISO-induced CREB not only induces *Il18bp* transcription, but will positively regulate *C/EBP*, thus potentiating its effects on IL-18BP expression. Interestingly, CREB has also been shown to play a role in the induction of various genes associated with myocardial hypertrophy and failure. We also found that ISO upregulates ANF, and ANF is CREB responsive [31]. Significantly, CREB has been shown to positively regulate *ADRB2* (adrenergic, beta-2-, receptor, surface; β 2-AR) transcription [32], suggesting that ISO-mediated CREB activation upregulates not only *Il18bp*, but also *ADRB2* and various other hypertrophy associated genes.

In addition to CREB and C/EBP β , we also identified potential binding sites for NF- κ B and Sp1 in the mouse *Il18bp* promoter. However, despite the fact that ISO activated NF- κ B and induced IL-18 expression via NF- κ B (Supplementary Fig. S2-B), overexpression of dnp65 mutant failed to significantly modulate ISO-mediated *Il18bp* transcription in cardiomyocytes (Supplementary Fig. S2-B), suggesting that mouse *Il18bp* may not be a κ B-responsive gene in cardiomyocytes treated with ISO. Previous reports suggest that following activation, phospho-CREB competes with p65 for the limited amounts of the transcriptional cofactor CREB-binding protein (CBP)/p300 available [33]. In contrast, since the catalytic subunit of protein kinase A (PKAc) phosphorylates p65 [34], the possibility exists that phospho-p65 and CBP interaction may potentiate ISO-induced CREB activation and *Il18bp* transcription. In fact, in a recent report the antioxidant PDTTC was shown to inhibit TNF- α -induced IL-18BP expression in human rheumatoid arthritis synovial fibroblasts [35], implying a role for NF- κ B in human IL-18BP expression. Conceivably, the regulation of *Il18bp* transcription is not only stimulus-specific, but also species and cell type dependent. Alternatively, NF- κ B may associate with C/EBP β , and their cross coupling might synergistically induce IL-18BP expression. In support of this hypothesis, it has been previously reported that NF- κ B and C/EBP β physically associate with each other via the Rel homology domain of NF- κ B and the bZIP region of C/EBP β , and this cross coupling results in inhibition of promoters with κ B enhancer motifs and in the synergistic stimulation of promoters with C/EBP binding sites [36]. Of note, crosstalk between NF- κ Bp65 and C/EBP β has also been described in the induction of several inflammatory genes, including IL-6 and IL-8 [37, 38]. Whether such an interaction plays a role in IL-18BP expression in mouse cardiomyocytes is not known.

Although Sp1 is a transcriptional regulator of several cardiac genes and genes involved in cell growth [39], in our studies pretreatment with the Sp1 inhibitor mithramycin failed to affect *Il18bp* transcription. However, confirming our earlier results [18], the Sp1 inhibitor mithramycin inhibited IL-18-induced EMMPRIN expression (Supplementary Figure S2-C). Since cAMP deglycosylates Sp1, and targets the hypoglycosylated Sp1 for rapid proteolytic degradation [40], it needs to be investigated whether ISO-treatment results in Sp1 deglycosylation and whether overexpression of wild type Sp1 by itself induces *Il18bp* transcription.

Myocardial hypertrophy and heart failure are complex disease processes [2]. It is known that a number of proinflammatory cytokines play critical roles in the initiation and progression of myocardial hypertrophy and transition to heart failure [1, 2]. Previously we demonstrated that ISO upregulates cardiac IL-1 β , IL-6 and TNF- α [41, 42]. However, it appears to do so without significantly altering their systemic levels [41, 42], suggesting that it is the local expression of these cytokines that promotes the underlying processes responsible for cardiac hypertrophy following β -AR stimulation. IL-18 is a proinflammatory cytokine that is expressed by all myocardial constituent cells, and its induction is characterized by increases in both local and systemic levels. Since the locally generated and the systemic IL-18 can further amplify inflammation via induction of other cytokines and chemokines, our studies identify it as a critical mediator, and thus a potential therapeutic target, of cardiac hypertrophy. Here we show that IL-18BP is also induced in cardiomyocytes by ISO treatment, and that both *in vitro* and *in vivo* it acts to limit the prohypertrophic effects of IL-18. Therefore IL-18BP may have potential use in the treatment of the hypertrophied failing heart.

Research highlights

- The β -AR agonist isoproterenol induces cardiomyocyte hypertrophy *in vitro* and *in vivo* via IL-18.
- Isoproterenol induces the expression of IL-18BP, a neutralizing IL-18 binding protein via β 2-AR.
- Isoproterenol induces IL-18BP expression via PI3K/Akt and ERK1/2-dependent CREB and C/EBP β activation.
- Isoproterenol induced cardiomyocyte hypertrophy is blunted in IL-18BP Tg and IL-18 null mice.
- IL-18BP may have therapeutic potential in the treatment of hypertrophied failing heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACM	adult mouse cardiomyocytes
ADRB2	adrenergic, beta-2-, receptor, surface
ANF	atrial natriuretic factor
β-AR	beta adrenergic receptor
bZIP	the basic leucine zipper domain
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
C/EBP	CCAAT/enhancer-binding protein
CMV	cytomegalo virus
CRE	cAMP-response element

CREB	CRE-binding protein
db-cAMP	dibutyl-cAMP
DMSO	dimethylsulfoxide
dn	dominant negative
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated protein kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
Gi	inhibitory G proteins
IL	Interleukin
IL-18BP	IL-18 binding protein
ISO	isoproterenol
Luc	luciferase
KCREB	dominant negative mutant form CREB
MEK	mitogen-activated protein kinase kinase
NF-κB	nuclear factor κ B
NMCM	neonatal mouse cardiomyocytes
nt	nucleotide
null	homozygous knockout
PDTC	pyrrolidine dithiocarbamate
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PTx	pertussis toxin
siRNA	small interference RNA
Sp1	specificity protein 1
TAC	transverse aortic constriction
TNF	tumor necrosis factor
Tg	transgenic
wt	wild type

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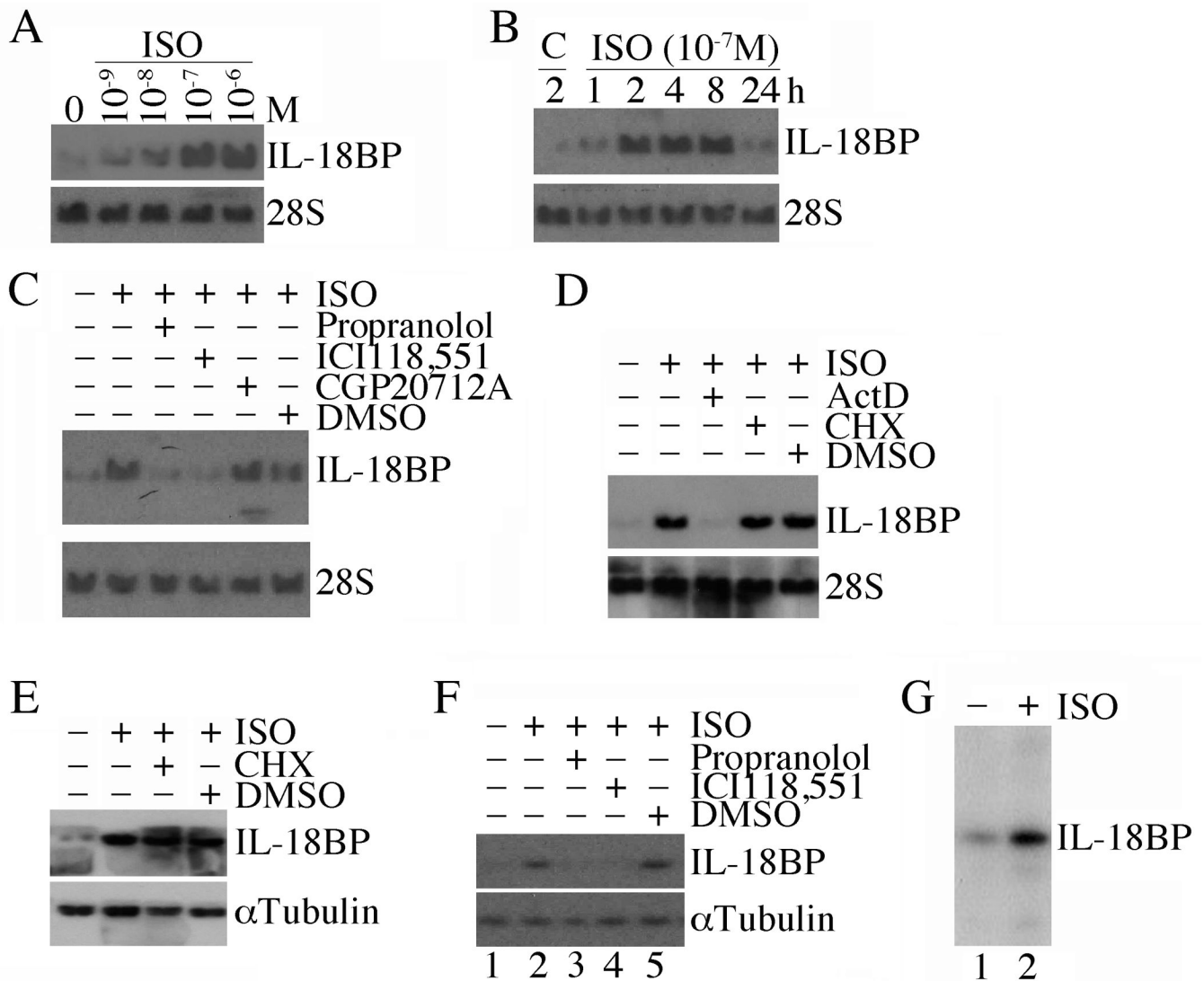


Fig. 1. Isoproterenol (ISO) induces IL-18 binding protein (IL-18BP) expression in adult mouse cardiomyocytes (ACM)

A, Dose-dependent effects of ISO on IL-18BP mRNA expression. ACM were cultured for 12 h in complete medium, then in medium containing 0.5% BSA for an additional 12 h (quiescence). Quiescent ACM (2.5×10^5) were treated with ISO for 2 h. 20 μ g of DNA-free total RNA was then analyzed for IL-18BP expression by Northern blotting. 28S rRNA served as a loading control. A representative of three independent experiments is shown. B, Time-dependent effects of ISO on IL-18BP mRNA expression. Quiescent ACM were treated with ISO (10^{-7} M). At the indicated times, IL-18BP mRNA expression was analyzed by Northern blotting as described in A ($n=3$). C, ISO induces IL-18BP expression via β -AR. Quiescent ACM were treated with the β 1- and β 2-AR blocker propranolol HCl (25 μ M), the β 1-AR antagonist CGP 20712A (300 nM in DMSO), β 2-AR antagonist ICI 118,551 (300 nM in water), or DMSO for 1 h prior to ISO (10^{-7} M for 2 h) addition. IL-18BP mRNA expression was analyzed as in A ($n=3$). D, ISO-induced IL-18BP is transcriptionally regulated. Quiescent ACM were treated with ISO (10^{-7} M for 2 h) followed by the addition of the translational inhibitor cycloheximide (CHX, 20 μ g/ml) or the transcriptional inhibitor actinomycin D (ActD, 5 μ g/ml) for 2 h. IL-18BP mRNA expression

was analyzed as in *A* (n=3). *E*, Cyclohexamide (CHX) fails to affect ISO-induced IL-18BP protein expression. ACM treated as in *D* were analyzed for IL-18BP expression by immunoblotting (n=3). *F*, ISO-induced IL-18BP protein expression is β 2-AR dependent. Quiescent ACM treated as in *C* were analyzed for IL-18BP protein levels at 2 h by immunoblotting (n=3). *G*, ISO stimulates IL-18BP secretion. Culture supernatants from ACM treated with ISO (lanes 1 and 2 in *F*) were analyzed for IL-18BP levels by immunoblotting following TCA precipitation.

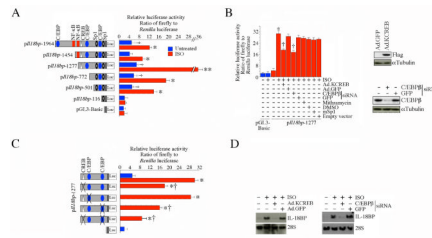


Fig. 2. ISO induces *Il18bp* promoter-reporter activity via CREB and C/EBP

A, Neonatal mouse cardiomyocytes (NMCM) were transfected with the indicated mouse *Il18bp* promoter-reporter vectors (3 μ g) using the Neon® transfection system. Cells were co-transfected with the internal reference plasmid *Renilla* luciferase (pRL-TK; 100 ng). 48 h after transfection, cells were treated with ISO (10^{-7} M for 12 h), and harvested for the dual-luciferase assay. † $P < 0.05$ versus pGL3-Basic; * $P < \text{at least } 0.05$, ** $P < 0.001$ versus corresponding Untreated (n=12). **B**, ISO-induced *Il18bp* promoter-reporter activity is C/EBP and CREB-dependent. NMCM were transfected with p*Il18bp*-1277 promoter-reporter vector (3 μ g) along with C/EBP β siRNA (60 nM), infected 24 h later with Flag-tagged mutant CREB (KCREB, MOI 50), or mutant Sp1 (MOI 10; [18]). Forty-eight hours later, cells were treated with ISO (10^{-7} M for 12 h). pRL-TK served as transfection control as before. Expression of Flag (upper right hand panel) and knockdown of C/EBP β (lower right hand panel) were confirmed by immunoblotting. * $P < 0.001$ versus untreated p*Il18bp*-1277, † $P < 0.01$ versus ISO (n=12). **C**, Mutations in C/EBP or CREB blunt ISO-induced *Il18bp* promoter-reporter activity. NMCM were transfected with wild type p*Il18bp*-1277 or p*Il18bp*-1277 with mutations in C/EBP, CREB, or both as described under A. * $P < 0.001$ versus untreated p*Il18bp*-1277, † $P < 0.01$ versus p*Il18bp*-1277 + ISO (n=12). **D**, ISO induces IL-18BP mRNA expression via CREB and C/EBP β . NMCM infected with Ad.KCREB (MOI 50 for 24 h) or transfected with C/EBP β -specific siRNA prior to ISO addition (10^{-7} M for 2 h) were analyzed for IL-18BP mRNA expression by Northern blotting (n=3).

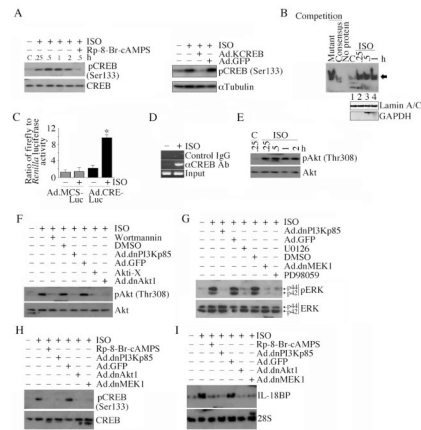


Fig. 3. ISO induces IL-18BP expression via CREB

A, Time-dependent effects of ISO on CREB activation. Quiescent ACM were treated with the cAMP antagonist Rp-8-Br-cAMPS (100 μ M in water for 1 h) or infected with Ad.KCREB (right hand panel) prior to ISO addition (10^{-7} M for 30 min). Total CREB, phospho-CREB (Ser133) and α Tubulin were quantified by immunoblotting (n=3). **B**, ISO induced CREB DNA-binding activity. ACM treated with ISO (10^{-7} M) for up to 1 h were analyzed for CREB DNA-binding activity by EMSA using gene-specific primers and nuclear protein extracts. Specificity of DNA binding, indicated by an arrow, was verified in competition experiments as described in 'Materials and methods'. Unbound labeled probe at the bottom of gel is not shown. Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls, and are shown in the bottom panel. **C**, ISO-mediated CREB activation was confirmed by a reporter assay. * $P < 0.001$ versus Ad.MCS-Luc + IL-18. **D**, ISO-stimulated CREB binding to *Il18bp* promoter *in vivo* was confirmed by the ChIP assay (n = 3). **E**, ISO induced Akt activation. ACM treated with ISO were analyzed by immunoblotting using phospho-Akt (Thr308) and Akt antibodies (n=3). **F**, ISO-induced Akt activation is attenuated by PI3K and Akt inhibitors and adenoviral transduction of dnPI3Kp85 and dnAkt1 (n=3). **G**, ISO induces ERK1/2 activation via PI3K, Akt, and MEK1/2. ACM were pre-treated with specific inhibitors of PI3K, Akt, MEK1/2 and ERK1/2, or transduced with adenoviral mutant vectors prior to ISO addition. Total and phospho-ERK1/2 were analyzed by immunoblotting (n=3). **H**, ISO activates CREB via PI3K, Akt, and MEK1/2. ACM treated as in **A** and **G** were analyzed for phospho-CREB levels (n=3). **I**, ISO induces IL-18BP mRNA expression via PI3K, Akt, and MEK1/2. ACM treated as in **A** and **G** were analyzed for IL-18BP mRNA expression by Northern blotting (n = 3).

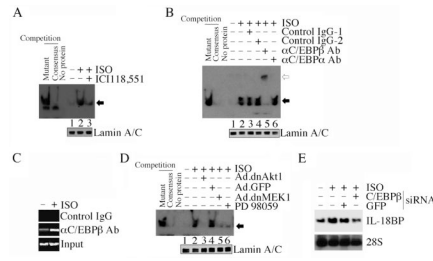


Fig. 4. ISO induces IL-18BP mRNA expression via C/EBP

A, ISO induces C/EBP DNA binding activity. ACM treated with ISO and/or the β 2-AR antagonist ICI 118,551 were analyzed for C/EBP DNA binding activity by EMSA using gene-specific primers and nuclear protein extracts as described under Fig. 3B (n=3). Lamin A/C served as a loading control, and is shown in the bottom panel. **B**, ISO-induced C/EBP DNA binding activity is super-shifted (open arrow) by anti-C/EBP β , but not C/EBP α antibodies. The nuclear protein extracts from ISO-treated ACM were preincubated with anti-C/EBP α , C/EBP β or respective control IgG prior to the addition of the labeled probe (n=3). Lamin A/C served as a loading control, and is shown in the bottom panel. **C**, ISO-stimulated C/EBP β binding to *Il18bp* promoter *in vivo* was confirmed by the ChIP assay (n = 3). **D**, ISO induces C/EBP DNA binding activity via Akt, MEK1/2 and ERK1/2. ACM were infected with Ad.dnAkt1, Ad.MEK1 or pretreated with the ERK1/2 inhibitor PD98059 prior to ISO addition. C/EBP DNA binding activity was analyzed as in A (n=3). Lamin A/C served as a loading control, and is shown in the bottom panel. **E**, ISO induces IL-18BP mRNA expression via C/EBP β . ACM were transfected with C/EBP β -specific siRNA (60 nM for 48 h) prior to ISO addition (10^{-7} M for 2 h). IL-18BP mRNA was analyzed by Northern blotting (n=3).

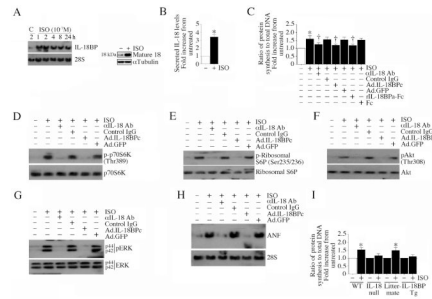


Fig. 5. ISO induces cardiomyocyte hypertrophy in part via IL-18

A, B, ISO induces IL-18 expression. ACM were treated with ISO (10^{-7} M) for up to 24 h (IL-18 mRNA, **A**, left hand panel), 1 h (intracellular IL-18 protein; **A**, right hand panel) or 24 h (secreted IL-18). IL-18 mRNA was analyzed by Northern blotting ($n=3$), protein by immunoblotting ($n=3$), and secreted IL-18 by ELISA (**B**). **B**. $*P < 0.001$ versus control ($n=6$). **C**, Neutralization of IL-18 blunts ISO induced cardiomyocyte hypertrophy. ACM were incubated with rIL-18BP α -Fc (10 μ g/ml for 1 h), anti-IL-18 neutralizing antibodies (10 μ g/ml for 1 h), or infected with Ad.IL-18BP α (MOI 50 for 24 h) prior to ISO addition (10^{-7} M for 48 h). Hypertrophy was assessed by increased protein synthesis, but not DNA content, as described in ‘Materials and methods’. $*P < 0.001$ versus control, $\dagger P < 0.05$ versus ISO ($n=6$). **D–H**, Neutralization of IL-18 attenuates ISO induced p70S6K (**D**), Ribosomal S6 protein (**E**), Akt (**F**), and ERK1/2 (**G**) activation and ANF mRNA expression (**H**). ACM treated as in **C**, but for 2 h (**D–G**) or 6 h (**H**) following ISO, were analyzed by immunoblotting (**D–G**) or Northern blotting (**H**) ($n=3$). **I**, ISO fails to induce hypertrophy of ACM isolated from IL-18BP Tg and IL-18 null mice. Cardiomyocytes from IL-18BP Tg and IL-18 null mice were incubated with ISO (10^{-7} M for 48 h) and analyzed for cell growth as in **C**. $*P < 0.001$ versus control, $\dagger P < 0.05$ versus ISO ($n=6$).

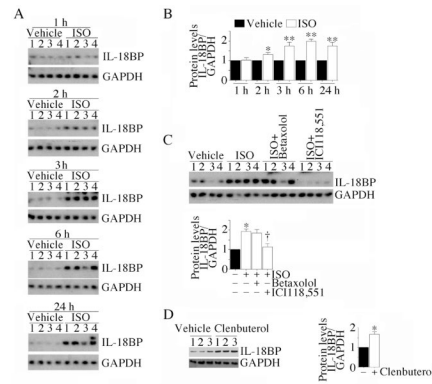


Fig. 6. ISO induces myocardial IL-18BP expression via β 2-AR

A, Time-dependent induction of IL-18BP *in vivo*. Male C57Bl/6 mice (n=4/group) were continuously infused with ISO (15 mg/kg body wt/day in 0.002% ascorbic acid) via miniosmotic pumps. 0.002% ascorbic acid served as a control (vehicle). At the indicated time periods, left ventricular tissue was analyzed for IL-18BP protein levels by immunoblotting. GAPDH served as a loading control. Each lane represents an individual animal (numbered at top). **B**, Densitometric analysis. The intensity of IL-18BP-specific band was semiquantified by densitometric analysis, and results from 4 animals at each time point were normalized to respective GAPDH levels, and presented as fold increase from vehicle-treated controls. * $P < 0.05$, ** $P < 0.01$ versus control. **C**, ISO-induced IL-18BP expression *in vivo* is β 2-AR dependent. Wild type mice received the β 1-AR antagonist betaxolol HCl (20 mg/kg. body wt) or the β 2-AR antagonist ICI 118,551 (4 mg/kg. body wt) 1 h prior to ISO administration, and sacrificed after 3 h. Densitometric analysis is shown in the lower panel. * $P < 0.01$ versus vehicle, † $P < 0.05$ versus ISO (n=4/group). **D**, Administration of the β 2-AR agonist clenbuterol HCl induces myocardial IL-18BP mRNA expression. Wild type mice received clenbuterol HCl (0.3 mg/kg. body wt, IP) for 3 h. Myocardial IL-18BP levels were analyzed by immunoblotting. Densitometric analysis is shown in the right hand panel. * $P < 0.05$ versus control (n=3/group).

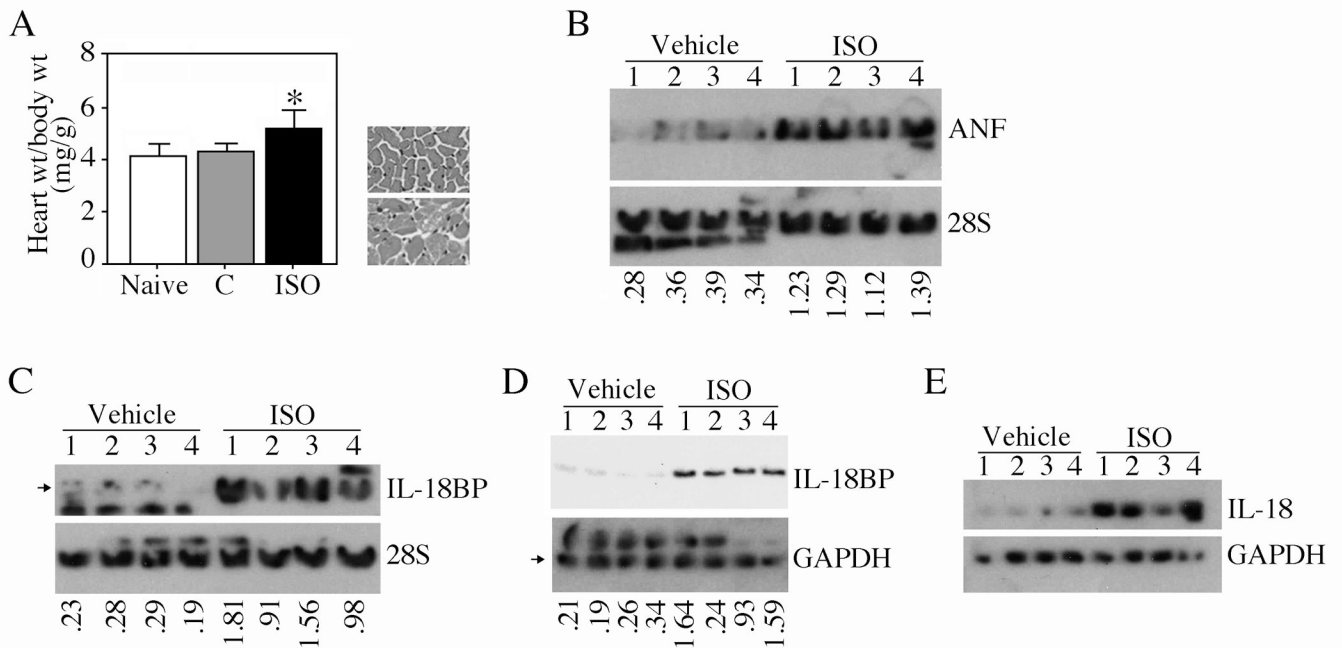


Fig. 7. ISO induces myocardial hypertrophy *in vivo*

A, ISO induces myocardial hypertrophy. Male C57Bl/6 mice were continuously infused with ISO (15 mg/kg body wt/day in 0.002% ascorbic acid) via miniosmotic pumps. 0.002% ascorbic acid served as a control (C). Heart and body weights were recorded after 7 days. Histology of the heart is shown in the right panel. * $P < 0.01$ versus naïve or control (n=6/group). B–E, ISO induces ANF mRNA (B; ratios of ANF mRNA to respective 28S rRNA are shown at the bottom), IL-18BP mRNA (C; ratios of IL-18BP [indicated by an arrow] to respective 28S rRNA are shown at the bottom), IL-18BP protein (D; ratios of IL-18BP to respective GAPDH [indicated by an arrow] are shown at the bottom) expression and mature IL-18 protein levels (E) in left ventricular tissue from 4 animals randomly selected from vehicle and ISO-infused animals.

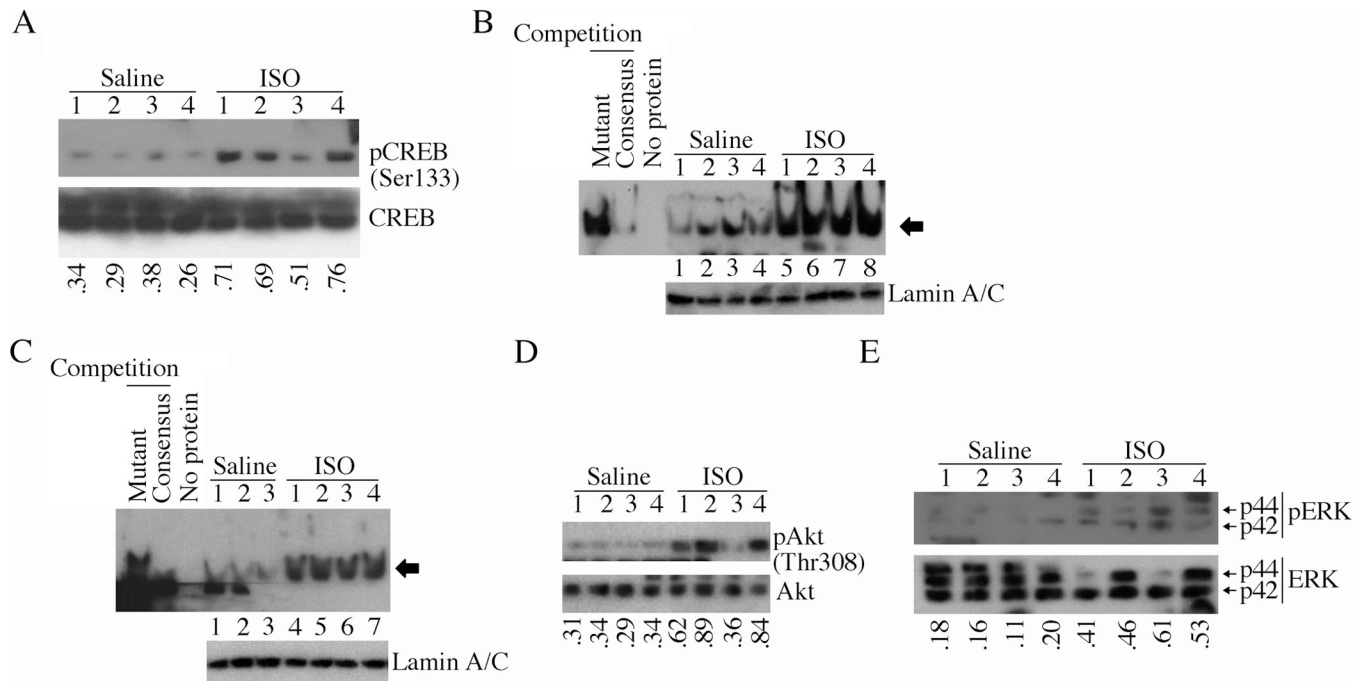


Fig. 8. ISO activates CREB and C/EBP in heart *in vivo*

A–E, Mice administered with ISO (n=4/group) as in 6A were analyzed for CREB activation (A; ratios of pCREB to respective total CREB are shown at the bottom), CREB DNA binding activity (B; Lamin A/C served as a loading control, and is shown in the bottom panel), C/EBP DNA binding activity (C; Lamin A/C served as a loading control, and is shown in the bottom panel), activation of Akt (D; ratios of pAkt to respective total Akt is shown at the bottom) and ERK1/2 (E; ratios of pERK1/2 to respective total ERK1/2 are shown at the bottom) by immunoblotting using activation-specific antibodies (A, D, E) and EMSA using gene-specific primers and nuclear protein extracts (B, C).

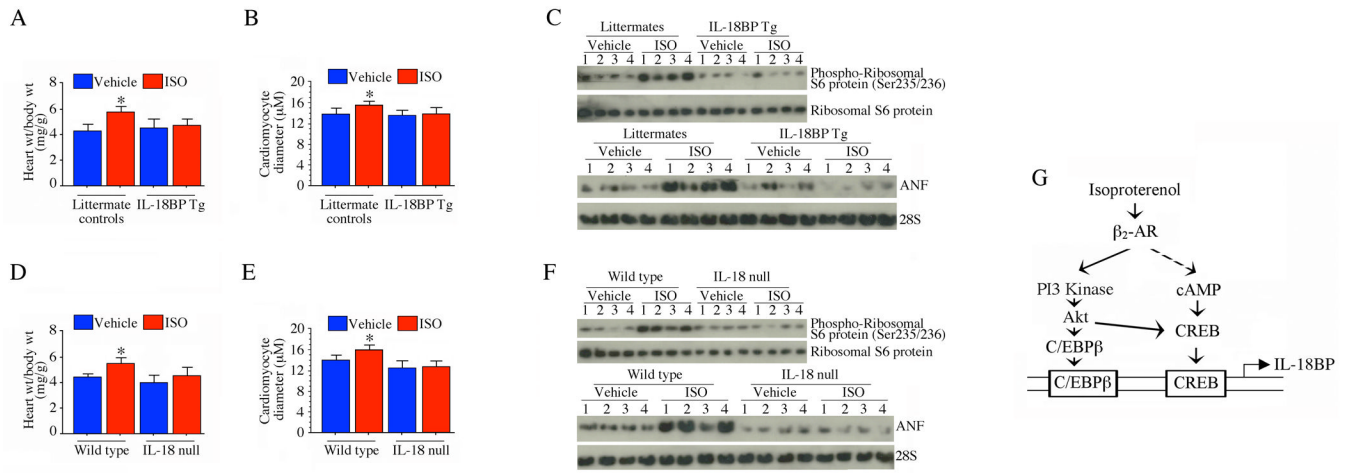


Fig. 9. ISO-induced myocardial hypertrophy is markedly attenuated in IL-18BP transgenic (Tg) and IL-18 null mice

A, Three month-old male IL-18BP Tg and IL-18 null mice were continuously infused with ISO (15 mg/kg body wt.) via subcutaneously implanted osmotic minipumps. Heart and body weights were measured after 7 days. * $P < 0.05$ versus corresponding vehicle-administered animals ($n=6$ /group). **B**, Hypertrophy was also assessed by quantifying diameter of cardiomyocytes from IL-18BP Tg and littermates in the region of the cell nucleus in H&E stained cryosections'. * $P < 0.01$ versus corresponding vehicle-administered animals (100 cells/heart, 4 hearts/group). **C**, The pro-hypertrophic markers p70 S6 kinase and ANF mRNA expression were markedly attenuated in IL-18BP Tg mice. LV tissue from 7 day ISO-infused IL-18BP Tg and littermates ($n=4$ /group) were analyzed for phospho-p70S6K by immunoblotting (upper panel) and ANF mRNA expression by Northern blotting (lower panel). **D**, ISO-induced myocardial hypertrophy is attenuated in IL-18 null mice. Wild type and IL-18 null mice treated with ISO were analyzed for myocardial hypertrophy as in **A**. **E**, Hypertrophy was also assessed by quantifying diameter of cardiomyocytes from wild type and IL-18 null mice as in **B**. * $P < 0.01$ versus corresponding vehicle-administered animals (100 cells/heart, 4 hearts/group). **F**, The pro-hypertrophic markers p70 S6 kinase ANF mRNA expression were markedly attenuated in IL-18 null mice. LV tissue from 7 day ISO-infused wild type and IL-18 null ($n=4$ /group) were analyzed for phospho-p70S6K by immunoblotting (upper panel) and ANF mRNA expression by Northern blotting (lower panel). **G**, Schema showing the signal transduction pathways involved in ISO induced IL-18BP expression. The β_2 -AR agonist ISO induces IL-18BP expression via β_2 -AR-dependent PI3K/Akt-mediated C/EBP β and CREB activation.

Table 1

Primers used in mouse IL-18BP analysis.

Promoter	
Sense 1	5'-ggt acc CTAGGCCTAACCTTGCTGAACA-3'
Sense 2	5'-ggt acc CCTTCATGGAAGTGTGACATGT-3'
Sense 3	5'-ggt acc CCTAGGTAGGCTTGGTCATTTGT-3'
Sense 4	5'-ggt acc CCACAACCTCAGCCTGTTCTCTTA-3'
Sense 5	5'-ggt acc CTAGAGATCCTGGATTCTGAG-3'
Sense 6	5'-ggt acc GGCTATTGGGGTCAGCTCTC-3'
Antisense	5'-aag ctt CCAGGCAGCTATCCTGGCTGAGCC-3'
EMSA	
C/EBP	Sense, 5'-GAACAGGGT TGGGCA AAGCTGCG-3'
Mutant C/EBP	Sense, 5'-GAACAGGG ACTAGTC AGCTGCG-3'
CREB	Sense, 5'-CAGGC TGACGTTA AGAATGGTGA-3'
Mutant CREB	Sense, 5'-CAGGC TGTCATA AGAATGGTGA-3'
ChIP assay	
c/EBP	Sense, 5'-CTAGGCCTAACCTTGCTGAACA-3' Antisense, 5'-TCTGGCACCCGTCTGAGCTCT-3' 203nt
CREB	Sense, 5'-CACAGAGCAGACAGACTAATG-3' Antisense: 5'-AAGGGGACTTCTGAGCAGGC-3' 208nt
ORF	Sense, 5'-TCTGCACCTCAGACAACCTGCCA-3' Antisense, 5'-TCAATGAAGGAACCATTGCCAG-3' 221nt
Northern blot analysis (398 bp)	
	Sense, 5'-TCTGCACCTCAGACAACCTGC-3'
	Antisense, 5'-TGGGCCAGAATGATGTGATA-3'