Published in final edited form as:

Biochem Biophys Res Commun. 2019 August 06; 515(4): 693–698. doi:10.1016/j.bbrc.2019.06.025.

Pro-remodeling peptides modulate collagen α1(I) promoter activity in rat cardiac myofibroblasts

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Abstract

Previous studies have extensively demonstrated the effect of endothelin-1 (ET-1), angiotensin II (Ang II), and TGF-β1 on the stimulation of collagen type I expression in cardiac myofibroblasts. However, the role of pro-remodeling peptides in the transcriptional regulation of the collagen promoter remains unclear. Thus, the purpose of this study was to investigate the net regulatory effects of pro-remodeling peptides on collagen type I promoter activity. Constructs of various lengths (300 bp, 1.1 kbp, 1.7 kbp, 2.3 kbp and 3.5 kbp) of the rat collagen α1(I) promoter were transfected into cardiac myofibroblasts in vitro and promoter activity was measured using chloramphenicol acetyl transferase (CAT) assays. Reduced promoter activity occurred across all treatments in myofibroblasts transfected with the 1.7 kbp construct. ET-1 was unable to increase promoter activity with constructs 300, 1.1, and 1.7 kbp, but induced promoter activity in cells with the 2.3 kbp construct. Additionally, while a combination of pro-remodeling peptides induced promoter activity across constructs, the resultant increase in the 2.3 and 3.5 kbp constructs were comparable to that observed from ET-1 treatment. Lastly, cells transfected with the entire promoter sequence had the lowest promoter activity. This data suggests that the collagen promoter is tightly regulated and that pro-remodeling factors produce an overall net effect on collagen expression, rather than additive.

Keywords

Collagen type I promoter (α1); chloramphenicol acetyltransferase (CAT); endothelin-1; angiotensin II; transforming growth factor (TGF)-β1; pro-remodeling peptides

Introduction

Collagen type I is a fibrillar collagen composed of two α1 chains and one α2 chain coiled around one another to form a triple helix. It is the most abundant protein of vertebrates and a major component of the extracellular matrices found in the bone, tendon, skin, ligaments,

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cornea, and many interstitial connective tissue [1]. Synthesized in large quantities by fibroblasts and osteoclasts, and to a lesser extent by nearly all other cell types, [2] type I collagen is important as an adhesive substrate for many cells. Additional roles of collagen type I include tissue and organ development, cell migration, proliferation and differentiation, wound healing, tissue remodeling, and maintenance of tissue homeostasis [1,3].

Responsible for the production of the structural proteins of the ECM of the heart are the cardiac fibroblasts, which lie in the interstitial matrix surrounding the cardiac myocytes. A wide variety of growth factors and vasoactive peptides can regulate cardiac fibroblast proliferation and synthesis of ECM constituents such as angiotensin II (AngII), endothelin-1 (ET-1), and transforming growth factor-β1 (TGF-β1). However, these same factors contribute to adverse cardiac remodeling and dysfunction and are also termed 'pro-remodeling factors' [4]. Cardiac cells that play a large role in cardiac remodeling, whether adverse or physiological, are the cardiac myofibroblasts. Myofibroblasts are phenotypically transformed fibroblast-like cells that test positive for α-smooth muscle actin and appear at the sites of injury. These cells play a major role in tissue remodeling following injury, as they are responsible for secretion of TIMPs and ECM proteins [5]. Previous studies have indicated that myofibroblasts are not only a local source of ET-1 and AngII [6-8], but they also express the receptors for these bioactive molecules [9,10].

Pro-remodeling factors used in this study include endothelin-1, angiotensin II, and transforming growth factor-β1 (TGF-β1). Members of the TGF-β superfamily are multifunctional cytokines that control various aspects of cell growth and differentiation and play essential roles in wound healing, immune system homeostasis, and organismal development [11,12]. TGF-β1 is the prototypic fibrogenic cytokine, enhancing gene expression of ECM constituents and down regulating matrix degrading enzymes, such as tissue inhibitors of matrix metalloproteinases (TIMP). In accordance to this, it has been shown that TGF-β1 induces type I collagen expression in human mesangial cells [13] and rat and human fibroblasts [14]. Furthermore, enhanced TGF-β1 expression precedes the increase in fibronectin and collagen types I and III in cardiac hypertrophy [15] and is often associated with adverse fibrotic states of tissues [16].

Endothelin-1 (ET-1) is a potent vasoconstrictor initially thought to originate solely from endothelial cells [17]. Since then, it has been found that ET-1 is produced by several organs and tissues and possesses diverse physiological and pathophysiological functions [18,19]. Endothelin has been implicated in facilitating collagen synthesis in isolated coronary artery vascular smooth muscle cells [20] as well as in cardiac fibroblasts [10,21] and myofibroblasts [22]. A role for endothelin mediated collagen synthesis *in vivo* following myocardial infarction is supported by the findings that locally generated endothelin in the myocardium [23] coincides with the transient increase in collagen synthesis [5].

Thus, the production of type I collagen needs to be tightly regulated, and this regulation is suspected to occur mostly at the transcriptional level. As such, this study investigated whether individual, as well as combined, treatments of AngII, ET-1 and TGF-β1 results in differences in collagen promoter activity, and suggests *cis*-regulatory regions in the collagen α1(I) promoter that positively or negatively influence collagen α1(I) gene expression.

2. Materials and Methods

Cell cultures

Primary cell lines of rat heart myofibroblasts were generated as described earlier [6]. In brief, myofibroblasts were isolated under aseptic conditions from the border region of visible myocardial scars. Scars were generated by ligation of the left anterior descending coronary artery in adult male Sprague-Dawley rats. Myofibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (Life Technologies) and were used within passage 12.

Plasmid Construction

For transient transfection experiments, plasmids with different lengths of collagen promoter were constructed by cloning into pBasic CAT vectors (Promega), upstream of the CAT gene. The construct pCC 3.5 consists of 3520 bp of rat α1(I) collagen promoter, followed by 115 bp of the first exon of the rat α1(I) promoter upstream of the CAT gene. Smaller fragments of the promoter were made as shown in Fig. 1. Digestion of pCC 3.5 with HindIII resulted in pCC 2.3, which lacks a fragment between −3520 and −2296 bp. The construct pCC 1.7 was created by digesting pCC 3.5 with PstI. Lastly, pColCAT plasmids pCC 200, 300, and 1.1 were made utilizing the protocol as described earlier [24,25].

Transient transfections

Myofibroblasts were grown to sub-confluence as described previously [24]. Transient transfection experiments were carried out in 100 mm plates using 70% confluent cells that were kept in serum-free DMEM 12 hours prior to transfection. Four micrograms of plasmid DNA was transfected using Lipofectamine (Life Technologies) as the transfecting agent following manufacturer's instructions. Myofibroblasts were transfected with different pCC constructs and the effects of endothelin-1 alone, endothethin-1 in combination with Ang II plus TGF-β1, and various receptor antagonists and enzyme inhibitors on CAT activity were studied. Treatments lasted 24 hours post-transfection. Enzyme inhibitors were Lisinopril, an angiotensin converting enzyme (ACE) inhibitor, and [Phe22] Big ET-1, a known inhibitor of endothelin converting enzyme (ECE). The receptor antagonists used in this study were Losartan, an angiotensin type I receptor (AT1R) antagonist, PD123319, an angiotensin type II receptor antagonist (AT2R), and Bosentan, a non-specific antagonist of endothelin receptors, ET_A and ET_B. After removal of transfection mixture, the cells were kept in serum-free medium (AIM V medium, Gibco) during treatments to eliminate the influence of serum growth factors on the experimental conditions. An earlier preliminary study (data not shown) to select a suitable medium for the various treatments with bioactive peptides found that AIM medium provides better CAT expression as compared to DMEM containing 0.4% serum. Thus, AIM V medium was used in all subsequent experiments. Another set of experiments revealed that CAT expression was optimum at 24 hours post-transfection and was comparable to expression levels at 48 hours. Therefore, the treatments were carried out for 24 hours before harvesting the cells for CAT assays. Also, it was observed that 2 treatments at 12-hour intervals were sufficient to elicit the response by a growth factor instead of multiple treatments for a longer period of time. Lastly, TGF-β1 concentration for

treatments was at 1ng/ml medium as this concentration was sufficient to activate collagen promoter as compared to 10 ng/ml medium for all the constructs tested (data not shown).

Chloramphenicol acetyl transferase (CAT) assays

Cells were harvested 48 hours after transfection in 1 ml of buffer containing 20mM Tris (pH 7.4) and 0.9% NaCl and collected by centrifugation. The pellet was resuspended in 0.25mM Tris (pH 8.0) and cells were disrupted by three cycles of freezing and thawing. After 5 min of centrifugation at 10K xg, the supernatants were tested for CAT activity. Total protein was measured by Biorad DC method as per the manufacturer's instructions. Aliquots of cell extract containing 50-100 μg of protein were analyzed for CAT activity as described previously [24]. The radioactivity in the acetylated and non-acetylated areas was removed from the thin-layer plates, placed into scintillation counting fluid, and measured in a Beckman scintillation counter. Percentage acetylation was calculated as (radioactivity in the acetylated areas)/(total radioactivity extracted from the thin-layer plate) × 100. All transfection experiments were done at least three times.

3. Results and Discussion

Studies using diverse experimental systems have indicated that transcriptional regulation of the type I collagen gene is species and tissue specific [26]. Several cis-acting elements as well as their trans-acting factors have been identified in the α1(I) and α2(I) genes [27,28]. For example, Kovacs et al. showed the presence of multiple factors that specifically bind to region −198 to −138 of the collagen α1(I) promotor in rat cardiac fibroblasts *in vitro* [25]. Thus, this study looked to investigate the effect of endothelin-1 as well as the combination of pro-remodeling factors on collagen α1(I) promoter regulation.

Endothelin-1 induces collagen promoter activity in pCC 2.3 constructs that is abrogated with Bosentan treatment

Our results show that ET-1 treatment induced the greatest upregulation (1.3-fold; Fig. 3B) of the collagen α1(I) promoter in myofibroblasts transfected with pCC 2.3. However, there was little to no increase in CAT activity seen in myofibroblasts transfected with pCC 300, 1.1, or 3.5 (Fig. 2A, B & D) and a surprising decrease (26% acetylation; Fig. 3B) seen in cells with the pCC 1.7 construct. These results suggest the presence of a positive regulatory element between base pairs −2300 and −1700 of the collagen α1(I) promoter. After showing the induction of the collagen promoter through ET-1, the next goal was to confirm the observed results through blocking ET-1 action. Since the action of endothelin-1 occurs through both endothelin receptors A and B, ET_A and ET_B, respectively, [29], the lab team utilized a non-selective endothelin receptor antagonist, Bosentan. Upon myofibroblast (pCC 2.3) treatment of ET-1 in conjunction with Bosentan, there was a decrease in collagen α1(I) promoter induction as seen by the decreased CAT activity compared to ET-1 treatment (Fig. 2D). Furthermore, if the positive regulatory element of the collagen promoter is to lie between −2300 and −1700, then there should be similar results seen in myofibroblasts transfected with pCC 3.5. Accordingly, this is what was observed, as these cells had slight increased CAT activity in response to ET-1 treatment that was diminished when treated in conjunction with Bosentan (Fig. 2E). Interestingly, Bosentan treatment was unable to abrogate the CAT

activity in myofibroblasts transfected with pCC 300, 1.1, and 2.3 (Fig. 2A, B, & D) when compared to ET-1 treatment. Instead, increased CAT activity was observed with pCC 1.1 when treated with Bosentan, which could be due to the absence of a sequence required for transcription factor binding between −1700 and −1100 bp.

Combination treatment of pro-remodeling factors induces collagen promoter activity in pCC 300, 1.1, and 2.3 constructs

Next, the study took aim at investigating the potential additive effects of ET-1, AngII, and TGF-β1 (combination treatment). It has been shown that ET-1, AngII, and TGF-β1 are all able to stimulate collagen expression in fibroblasts and myofibroblasts [21,30]. Additionally, studies have reported observations of increased proliferation in bronchial fibroblasts and increased collagen deposition with the *combined* treatment of ET-1, TGF-β1, and platelet-derived growth factor (PDGF) [31]. So, if the effects of these collagen-inducing cytokines and peptides are additive, then there should be a significant increase observed in CAT activity of pCC 3.5 (the entire promoter), at least. Contrary to this hypothesis, when treated with a combination of ET-1, AngII, and TGF-β1, there is no significant increase in the induction of the collagen α1(I) promoter with pCC 3.5 compared to ET-1 treatment alone (Fig. 2E). This suggests that the collagen α1(I) promoter is tightly regulated and various factors will increase this promoter expression, but only to a certain degree. Although each pro-remodeling factor independently increases collagen expression, these factors may be converging on similar signaling pathways that produce a *net* effect rather than an *additive* effect. The only constructs that showed increased promoter activity as a result of the combination treatment, when compared to ET-1 treatment alone, were pCC 300 and pCC 1.1 (Fig. 2A & B). As the previous results with ET-1 treatment alone suggested that ET-1 action may regulate the promoter between −2300 and −1700 bp, then it could be that the regulatory actions of AngII and TGF-β1 operate on sequences located before −1100 bp of the promoter or in the same region as ET-1 between −2300 and −1700.

Inhibition of endogenous angiotensin II and endothelin-1 production on collagen promoter activity

Since myofibroblasts are known to secrete endogenous ET-1 and AngII [6,8], it was imperative to investigate if the effects observed from the treatment of exogenous ET-1 and AngII were truly resultant of these treatments. Thus, [Phe22] Big ET-1 and Lisinopril were utilized to inhibit the endogenous formation of ET-1 and AngII, respectively. It was expected that CAT activity in myofibroblasts would be similar or less than that observed in the untreated myofibroblast groups. However, myofibroblasts transfected with pCC 2.3 and pCC 3.5 both resulted in increases in promoter activity, with the latter resulting in a significant increase (Fig. 2D and E).

Interestingly, the largest increase in promoter activity seen in cells with pCC 3.5 was observed with the Lisinopril plus [Phe22] Big ET-1 treatment (Fig. 2E). As only endogenous AngII and ET-1 were inhibited in this treatment group, this still allows endogenous TGF-β1 to induce activation of the collagen α1(I) promoter. Additionally, although Lisinopril plus [Phe22] Big ET-1 treatment induced the greatest activation of the collagen α1(I) promoter within the myofibroblasts transfected with pCC 3.5, the CAT activity seen in this treatment

group between all pCC constructs was the lowest for pCC 3.5 and the greatest for pCC 1.1 (Fig. 2B & E). However, a study conducted by Tzanidis et al. showed that treatment of AngII and ET-1 receptor blockers resulted in a reduction in collagen type I deposition in rat hearts post-myocardial infarction [32]; contrary to what the results of this study suggest. On the other hand, a study by Fang et al. reported that while Lisinopril reduced collagen I secretion by 3T3 fibroblast cells, conjunction treatment of Lisinopril and TGF-β1 still resulted in collagen deposition greater than untreated 3T3 fibroblasts [33]. However, the Lisinopril and AngII treatment [33] is missing the ET-1 inhibitor that this study included. Nevertheless, this again suggests that the site of regulation for TGF-β1 may reside earlier within the collagen α1(I) promoter, and that the entire promoter has many sites of regulation mediating collagen type I expression.

Combination of receptor antagonists and enzyme inhibitors (BLPZP) does not completely abrogate collagen promoter activity

Further investigation into the effect of combination treatment (ET-1, AngII, and TGF-β1) commenced with treatment of transfected myofibroblasts with a combination of receptor antagonists and enzyme inhibitors. The receptor antagonists used were PD12319 and Bosentan to target angiotensin type II receptor (AT2R) and both ET_A and ET_B receptors, respectively. Enzyme inhibitors used included ZK, Lisinopril, and [Phe22] Big ET-1 to inhibit TGF-β action, ACE, and ECE, respectively. Interestingly, upon blocking the activities of ET-1, AngII, and TGF-β1 together, there was still enhanced CAT activity observed with pCC 300, and 1.1, and 3.5 (Fig. 2A & B). Nonetheless, Kovacs et al. in 1996 performed a study suggesting a positive regulatory element in the collagen α1(I) promoter between base pairs −200 and −140, supporting the observations of increased CAT activity in pCC 300 [25]. Contrary to what was observed in this study, transfections of collagen promoter constructs performed in 1997 by Dhalla et al. suggested there is a negative regulatory element of the collagen α1(I) promoter from −400 to −325 bp [24]. However, there was reduced CAT activity within the pCC 1100 construct with no treatment compared to pCC 300 (Fig. 3A). The increased activity upon treatment of the combination of receptor antagonists and enzyme inhibitors could be due to effects from other pathways affected by the inhibition of ACE. For example, kinins, such as bradykinin and kallikrein, are endogenous vasoactive peptides that are degraded by ACE [34]. Inhibition of ACE would then cause an accumulation of kinin peptides that could be catalyzed into des-Arg^9-bradykinin and Lysdes-Arg^9-bradykinin, natural agonists for the bradykinin B_1 receptor [34]. Bradykinin B_1 receptor has been shown to be expressed in cardiac myofibroblasts [35] and has been implicated in playing a role in the progression of cardiac fibrosis [36]. Thus, induction through bradykinin B1 receptor could explain why even in the presence of the combination of ET_A, ET_B, and AT2R receptors as well as ACE and ECE inhibitors there was still an observed increase in collagen α1(I) promoter activity.

4. Conclusion

In summary, our results support earlier observations regarding the suggestion of a negative regulatory element from −400 to −325bp of the collagen α1(I) promoter sequence [24] and of a net positive regulatory element between −200 and −140 bp [25]. Additionally, this study

suggests the presence of an additional negative regulatory element between −1700 and −1100 bp as well as a positive regulatory element upstream of the promoter between −2300 and −1700. Furthermore, the inability of the combination treatment of ET-1, AngII, and TGF-β1 to induce greater promoter activity than ET-1 alone suggests that effects of multiple fibrogenic factors are not additive.

Further studies are necessary to delineate the specific sequences involved in the regulation of the collagen α1(I) promoter by ET-1 and other pro-remodeling factors known to induce collagen α1(I) expression. Collagen α1(I) expression appears to be tightly regulated, as is suggested by the greatly reduced CAT activity found in myofibroblasts transfected with pCC 3.5 across all treatments (Fig. 3A-D) compared to the other constructs. Nonetheless, elucidating the regulatory mechanisms involved in collagen α1(I) expression can open the door to treatments concerned with manipulating these elements at the genetic level; rather than at the macromolecular level where abrogating certain pathways can enhance other compensatory pathways and bypass the intention of the treatment. While much is known about peptides and cell signaling pathways involved in the induction of the collagen α1(I) gene, gaps of knowledge remain regarding the intricate and complex regulation of the collagen α1(I) promoter to produce the net gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the National Institute of Health grant, HL-R01-60047 (LCK). The authors would like to thank Drs. Devi M. Nair and Vishnu Chintalgattu for their contributions to earlier experiments, as well as Dr. Jagan C. Kandala for his contribution in creating the constructs necessary for this study.

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- −2300 to −1700 sequence greatly enhanced endothelin-1 activation of collagen promoter
- Endothelin-1 increased promoter activity comparable to combination treatment
- Pro-remodeling peptides produce net effect on collagen promoter rather than additive
- Presence of −1700 to −1100 sequence reduces promoter activity across all treatments
- Tight regulation of full collagen promoter compared to deletion constructs

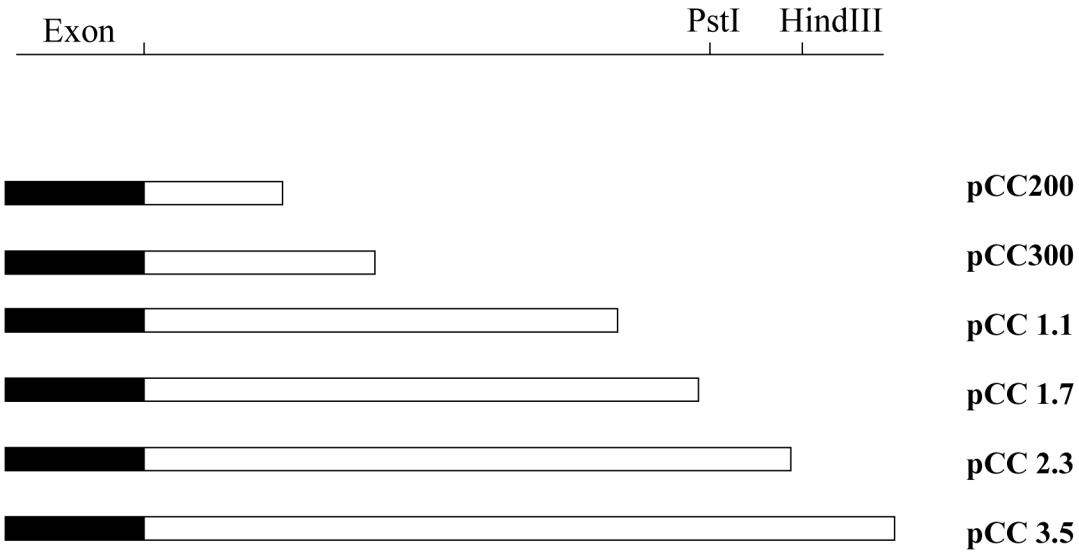

Fig. 1.

Schematic map of constructs generated for use in the transfection experiments. The pCC 200 construct was not used in myofibroblast transfection. Relevant restriction site positions are indicated above. pCC, pColCAT.

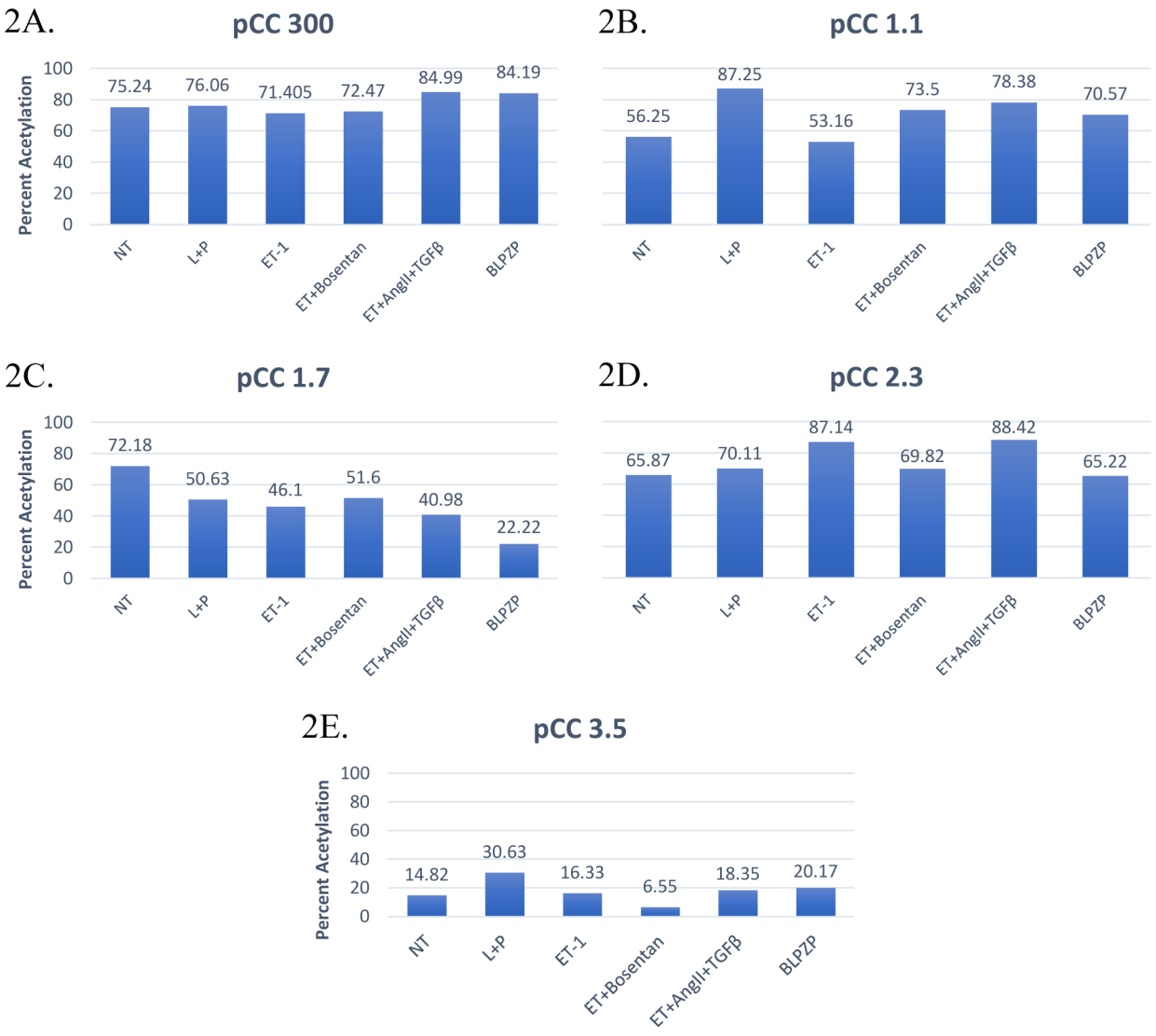

Fig. 2.

Graphs represent CAT activity within pCC constructs, (A) pCC 300, (B) pCC 1.1, (C) pCC 1.7, (D) pCC 2.5, or (E) pCC 3.5, in response to various treatments. Cardiac myofibroblasts were transfected with plasmid constructs of varying length, presented with various treatments, and then assayed for CAT activity. CAT activity was represented as percent acetylation and calculated as (radioactivity in acetylated areas) / (total radioactivity extracted from the thin-layer plate) × 100. NT: no treatment; L + P: Lisinopril and [Phe22] Big ET-1; ET-1: endothelin-1; ET + Bosentan: endothelin-1 and Bosentan; ET + AngII + TGFβ1: endothelin-1, angiotensin II, and transforming growth factor-β; BLPZP: Bosentan, Lisinopril, [Phe22] Big ET-1, Losartan, PD123319, and ZK.

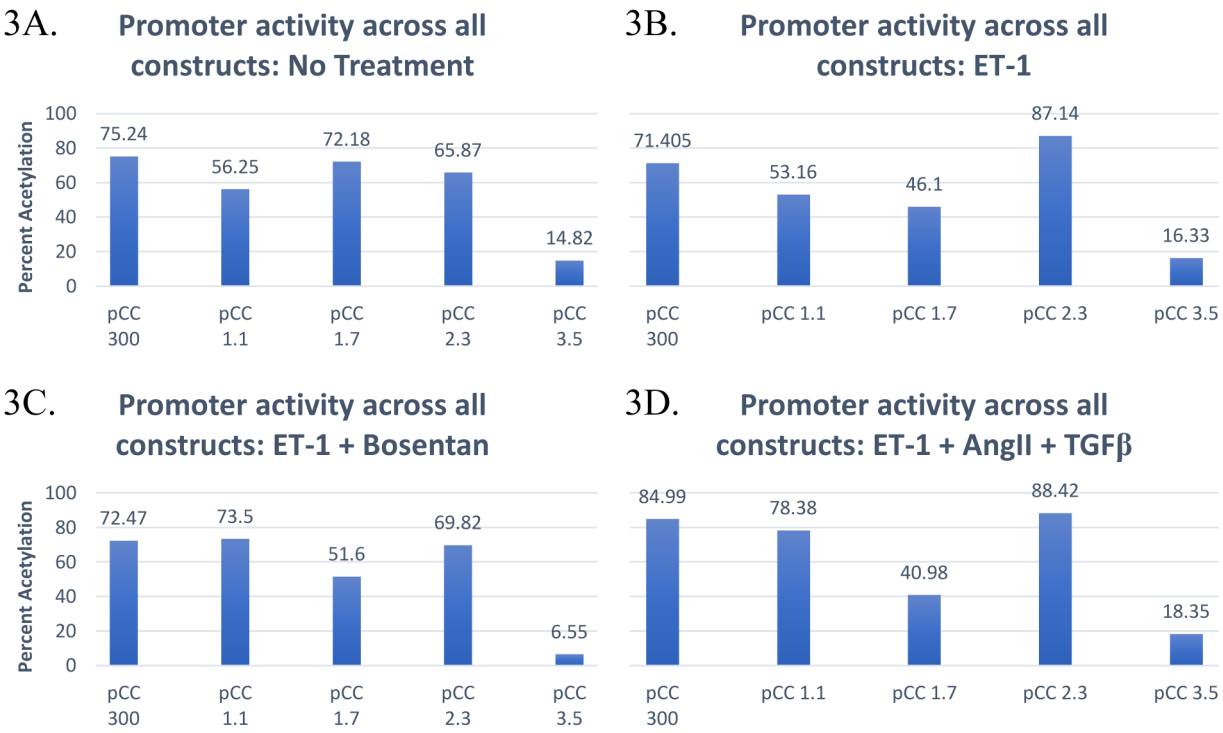

Fig. 3.

Graphs represent CAT activities between pCC construct groups in response to the treatments (A) No Treatment, (B) Endothelin-1, (C) Endothelin-1 and Bosentan, (D) Endothelin-1, Angiotensin II, and TGFβ1. Cardiac myofibroblasts were transfected with plasmid constructs of varying length, presented with various treatments, and then assayed for CAT activity. CAT activity was represented as percent acetylation and calculated as (radioactivity in acetylated areas) / (total radioactivity extracted from the thin-layer plate) × 100.