# $\alpha_1$ Adrenergic Receptor Agonist, Phenylephrine, Actively Contracts Early Rat Rib Fracture Callus Ex Vivo

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**ABSTRACT:** Early, soft fracture callus that links fracture ends together is smooth muscle-like in nature. We aimed to determine if early fracture callus could be induced to contract and relax ex vivo by similar pathways to smooth muscle, that is, contraction via $\alpha_1$ adrenergic receptor ($\alpha_1$AR) activation with phenylephrine (PE) and relaxation via $\beta_2$ adrenergic receptor ($\beta_2$AR) stimulation with terbutaline. A sensitive force transducer quantified 7 day rat rib fracture callus responses in modified Krebs–Henseliet (KH) solutions. Unfractured ribs along with 7, 14, and 21 day fracture calluses were analyzed for both $\alpha_1$AR and $\beta_2$AR gene expression using qPCR, whilst 7 day fracture callus was examined via immunohistochemistry for both $\alpha_1$AR and $\beta_2$AR- immunoreactivity. In 7 day callus, PE ($10^{-6}$ M) significantly induced an increase in force that was greater than passive force generated in calcium-free KH ($n = 8$, mean 51% increase, 95% CI: 26–76%). PE-induced contractions in calluses were attenuated by the $\alpha_1$AR antagonist, prazosin ($10^{-6}$ M; $n = 7$, mean 5% increase, 95% CI: 2–11%). Terbutaline did not relax callus. Gene expression of $\alpha_1$ARs was constant throughout fracture healing; however, $\beta_2$AR expression was down-regulated at 7 days compared to unfractured rib ($p < 0.01$). Furthermore, osteoprogenitor cells of early fibrous callus displayed considerable $\alpha_1$AR-like immunoreactivity but not $\beta_2$AR-like immunoreactivity. Here, we demonstrate for the first time that early fracture callus can be pharmacologically induced to contract. We propose that increased concentrations of $\alpha_1$AR agonists such as noradrenaline may tonically contract callus in vivo to promote osteogenesis.  J Orthop Res 29:740–745, 2011

**Keywords:** fracture healing; smooth muscle; contraction; adrenergic receptors

We have previously shown that early, fibrous callus from fractured rat rib displays passive viscoelastic properties comparable to typical smooth muscle tissue.[1] Furthermore, cells of this callus in particular osteoprogenitor cells of early fibrous callus show significant expression of the essential contractile protein, alpha smooth muscle actin (αSMA),[2,3] which has recently been suggested as a possible marker for osteoprogenitor cells.[4] These characteristics suggest that early callus osteoprogenitor cells display a smooth muscle-like phenotype, like myofibroblasts. Myofibroblasts are specialized contractile fibroblasts characterized by their cytoskeletal αSMA content and are hence similar to smooth muscle.[5] Interaction of αSMA with other contractile myofilaments such as nonmuscle myosin II gives the myofibroblast a force generating capacity. This is most evident in healing of soft tissue injuries where myofibroblasts are responsible for contraction of granulation tissue.[6]

There are many similarities in repair of soft tissue injuries and early stages of bone fracture healing, particularly in composition of granulation tissue.[3,7] As early fibrous callus appears to consist of contractile smooth muscle-like osteoprogenitor cells, it is suggested that they too have a functional role for tissue contraction in the healing of bone fractures.[1]

We have previously isolated and quantified the passive viscoelastic phenomena of stress relaxation, reverse stress relaxation, and hysteresis in early callus ex vivo.[1] With this passive component isolated, it may be possible to identify any "active" smooth muscle-like contraction (i.e., force produced by myofilament cross-bridge cycling) in callus.[8] The contribution of active force development can be measured as force produced above preconditioned stable passive force.[8]

Contraction of smooth muscle and nonmuscle cells such as myofibroblasts is generally activated by pharmacomechanical coupling mechanisms, which, via different complex pathways, regulate the myosin light chain kinase/myosin phosphatase ratio and thus the degree of myosin and actin interaction.[9–12] Various smooth muscle effectors including the $\alpha_1$ adrenergic receptor ($\alpha_1$AR) agonist noradrenaline,[13] nerve growth factor,[14] galanin,[15] and nitric oxide[16] are up-regulated during early stages of fracture healing. It is suggested that these effectors may play a role in regulating the contractile state of early callus that may influence healing.[3]

Differentiation of smooth muscle cells into cells that display an osteoblast-like phenotype is supported by static conditions.[17] Furthermore, the differentiation pathway of osteoprogenitor cells seems to be mechanically controlled; in that generation of tension via cytoskeletal contraction increases osteoblastogenesis and inhibits chondrogenesis.[18,19] Thus, we propose that contraction of callus smooth muscle-like osteoprogenitor cells in vivo should augment their differentiation into an osteoblastic phenotype to enhance osteogenesis and thereby improve fracture healing. In contrast, relaxation of callus smooth muscle-like cells would likely decrease callus strain and in so doing, reduce phenotypic change to impair healing.

Accordingly, the aims of the present study were to (i) determine if early callus can be induced to tonically contract ex vivo via an established mechanism of smooth muscle contraction, that is, $\alpha_1$AR activation by addition of phenylephrine (PE); (ii) establish if any PE-induced contraction can be prevented by prior addition of the $\alpha_1$AR antagonist, prazosin; (iii) determine if early callus

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can be stimulated to actively relax via addition of the $\beta_2$AR agonist, terbutaline; (iv) investigate both $\alpha_1$AR and $\beta_2$AR gene expression in unfractured rib as well as callus at 7, 14, and 21 days post-fracture; and (v) attempt to localize $\alpha_1$ARs and $\beta_2$ARs in callus via immunohistochemistry.

## METHODS

### Animals and Rib Fracture Surgery

This project was approved by the La Trobe University Animal Ethics Committee (04/11 v3). A rib from each rat was fractured as described previously.[3] Seven day calluses were used for force measurement as such calluses are fibrous in nature and ideal for measurement of active force production.[3] For gene expression and immunohistochemical analyses, groups of rats were killed at 7, 14, and 21 days post-fracture. Chemicals for all the following procedures were obtained from Sigma Chemical Co. (St. Louis, MO, AR grade) except where otherwise stated.

### Force Measurement

#### *Dissection*

Calluses were dissected in calcium-free Krebs–Henseleit (KH) solution. Each callus had surrounding skeletal muscle trimmed away prior to connection to a sensitive isometric force transducer (SensoNor AE 801), capable of measuring forces as small as 1 μN.[3]

#### *Callus Bathing Solutions*

Calluses were immersed in the following modified physiological solutions[20]: (i) calcium-free KH solution (0-$Ca^{2+}$KH), (ii) normal KH solution ([$Ca^{2+}$] = 1.2 mM) containing $\alpha_1$AR agonist, PE ($10^{-6}$ M), (iii) normal KH solution containing $\alpha_1$AR antagonist, prazosin ($10^{-6}$ M) and $\alpha_1$AR agonist, PE ($10^{-6}$ M) added 30 min later; and (iv) normal KH solution containing $\beta_2$AR agonist terbutaline ($10^{-6}$ M). Previous investigations have used similar or higher concentrations of agonists and the antagonist to display submaximal contractile responses or inhibition of contraction, respectively, in either smooth muscle or granulation tissue preparations.[21–24] KH solution had the following composition (mM): NaCl 135; KCl 5; $MgCl_2$ 3; $NaH_2PO_4$ 0.8; HEPES 10; calcium ($Ca^{2+}$) 1.2; glucose 5. Solution temperature was 22°C and its stable pH was stable at 7.4 for a period of at least 6 h.

#### *Adjustments to Preparation Tension*

All callus responses were measured from a known reference tissue tension, deemed "basal tension." To achieve this, callus preparations were firstly manually set to absolute zero tension by a micromanipulator on which the force transducer was mounted. Once force had stabilized, the preparation was stretched by 50 μN and then monitored, with the eventual stabilized force measurement designated as the basal tension for force measurement throughout the experiment. This low basal tension was desirable, as it allowed for bilateral changes in tension without placing a restrictive external tension on the tissue. From basal tension, callus was preconditioned with cyclic stretching and releasing by 50 μN. Preconditioning was necessary as callus displays a degree of hysteresis.[1] Once a steady force had been reached, tension was reduced from the designated basal tension to 0 and force was recorded. Importantly, although reductions in tension to reach this point varied between callus samples, changes in tension between solutions were always equal. This allowed for comparison of intra-callus responses between solutions and accounted for some variance of inter-callus force responses. Release of tension typically produces passive reverse stress relaxation.[1] In eight callus preparations, PE was added to KH solution to make a final concentration of $10^{-6}$ M PE and after 30 min tension was reduced from basal tension to absolute 0 and recorded until a steady-state force was produced. This force was compared to the passive response preceding PE addition, with additional force following PE exposure deemed to be due to "active" contraction. In six separate preparations the $\alpha_1$AR antagonistic effect of prazosin was analyzed. This was achieved by adding PE to the organ bath to make a final concentration of $10^{-6}$ M PE, 30 min after $10^{-6}$ M prazosin exposure. As in other solutions, force responses were measured from 0 external tension after 30 min of exposure to PE. Relaxation responses were similarly measured from basal force, with calluses stretched and responses recorded until steady state was reached. This stretch typically produces passive stress relaxation[1]; further relaxation would be considered active relaxation.

### qPCR

Unfractured ribs and fracture calluses at 7, 14, and 21 days post-fracture were extracted and stored in RNAlater (Ambion, Austin, TX) at −80°C. To extract RNA, calluses were crushed using a frozen metal probe in an Eppendorf tube. Total RNA was extracted from each unfractured rib or callus using the RNA isolation reagent, RNAwiz, according to manufacturer's instructions (Ambion, Austin, TX). All consumables and equipment were sterilized and RNase free. Quantity and purity of RNA were assessed by absorbance ratios at $A_{260/280}$.

Reverse transcription was performed using SuperScript First-Strand Synthesis System according to manufacturer's instructions (Invitrogen, Mulgrave, Victoria). Each target gene sequence was located using PubMed genome sequence search (GenBank®, http://www.ncbi.nlm.nih.gov/genbank/). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.

Beacon Designer 2.0 program (Biosoft International, Palo Alto, CA) was used to design optimal forward and reverse primer sequences (Table 1), which were prepared commercially (GeneWorks Pty Ltd., Adelaide, SA, Australia).

Expression of $\alpha_1$ARs and $\beta_2$ARs in unfractured ribs and calluses was analyzed using the iCycler iQ Multi-Colour Real-Time PCR detection system (Bio-Rad, Hercules, CA). Each reaction well contained 12.5 μl of SYBR Green mix (Bio-Rad), 1.5 μl of forward and reverse primers, 5.0 μl of cDNA ($10^{-2}$ M) and 2.5 μl of RNase free $H_2O$. PCR was run for 55 cycles and a melt curve analysis was performed post-cycling to establish the specificity of DNA products. Using the $2^{-\triangle\triangle CT}$ (Livak) method, levels of $\alpha_1$AR and $\beta_2$AR expression were normalized to GAPDH relative to unfractured rib.

### Immunohistochemistry

Seven day calluses ($n = 4$) and intact rib were placed in 4% paraformaldehyde fixative in 0.1 M sodium cacodylate buffer for 48 h, after which tissues were washed in 3 min × 30 min changes of the cacodylate buffer containing 7% sucrose. Tissues were then cryosectioned at a thickness of 10 μm. Sections were immunostained using a similar protocol to that described previously.[3] Briefly, a 1:500 dilution of rabbit anti-$\alpha_1$AR (Abcam, Cambridge, UK) or rabbit anti-$\beta_2$AR (Biotechnology Inc., Santa Cruz, CA) overnight at 4°C was used. Sections were then incubated in a 1:200 dilution of goat anti-rabbit antiserum Cy3 (indicated by red fluorescence) for 1 h, washed again and nuclear counterstained with DAPI ( indicated by blue

**Table 1.** Primer Sequences, Melt Temperature ($T_m$), and GC% Used in the Experiment

| Oligonucleotide | NCBI reference | | Sequence (5'–3') | Length (bp) | $T_m$ (°C) | GC % |
|---|---|---|---|---|---|---|
| GAPDH | NM_017008.3 | Sense | AGTTCAACGGCACAGTCAAGG | 21 | 58.3 | 52.4 |
| | | Antisense | ACATACTCAGCACCAGCATCAC | 22 | 58.1 | 50.1 |
| $\alpha_{1a}$AR | NM_017191.2 | Sense | AGAAGGCGGCGGAGTCAG | 18 | 58.1 | 66.7 |
| | | Antisense | CAGCAGAGGACGAAGCAACC | 20 | 58.3 | 60 |
| $\beta_2$AR | AY057896.1 | Sense | CACGACATCACTCAGGAAC | 19 | 52.5 | 52.6 |
| | | Antisense | AACTTGGCAATGGCTGTG | 18 | 52.6 | 50 |

fluorescence) for 5 min. Positive controls were rat trabecular bone osteoblasts for $\alpha_1$AR-like immunoreactivity (LI) and rat lung bronchioles for $\beta_2$AR-LI. Negative controls consisted of incubations omitting primary antibodies. Immunostained sections were examined using a Leica DRB microscope with appropriate fluorescent filters for Cy3 and DAPI. High-resolution digital images were captured using a Leica DC 300, 7.3 megapixel digital camera (Leica, Microsystems, Heerbrugg, Switzerland).

### Statistical Analysis

A 95% confidence interval (CI) was used to analyze mean differences in isometric force responses between force production in solutions i–iv. A Mann–Whitney test was used to assess differences between 7, 14, and 21 day callus and unfractured rib gene expression ratios. All data were expressed as mean ± SE. A $P$-value of $<0.05$ was considered statistically significant. Statistics were performed using GraphPad InStat 3 software package for Windows (GraphPad Software Inc., La Jolla, CA).

## RESULTS

### Force Measurement

All eight calluses in 0-$Ca^{2+}$KH responded to 0 external tension with an instant, prolonged passive reverse stress relaxation to near maximal force, followed by a sustained period of force maintenance (Fig. 1). Active contractile responses were observed in all eight calluses, with PE producing a mean peak force 51% greater than the passive component (95% CI: 22–76; Figs. 1 and 2A). The nature of this active contractile force was consistently rapid with no evidence of fatigue in callus responses to PE. Prazosin consistently attenuated the active contractile force of calluses when added 30 min prior to PE

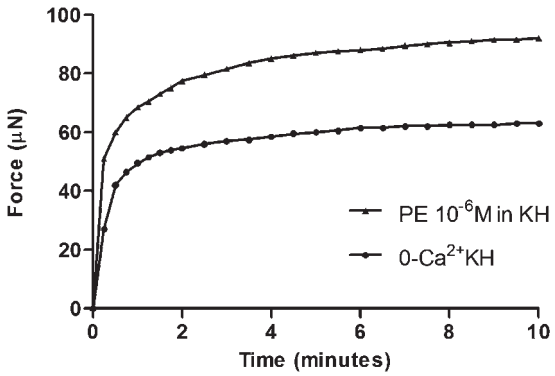


**Figure 1.** Typical callus force response to 0-$Ca^{2+}$KH and $10^{-6}$ M PE in KH. Time 0 indicates the point where tension in the preparation was manually set to 0 from a set basal tension.

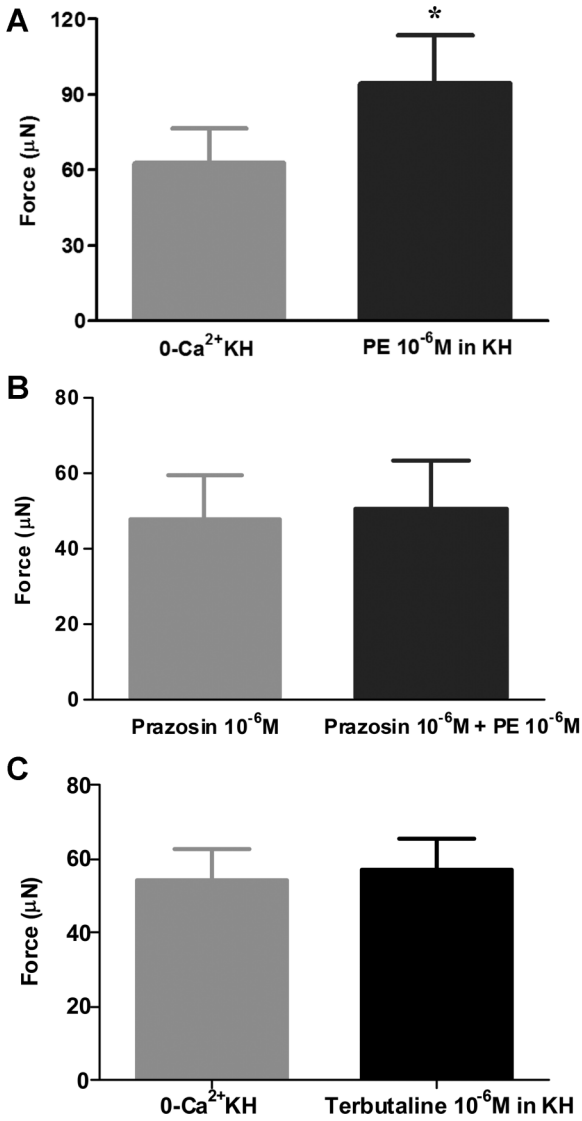


**Figure 2.** (A) Mean force produced in 0-$Ca^{2+}$KH and $10^{-6}$ M PE in KH. Data for eight individual calluses were pooled to produce mean forces developed by each solution. PE induced a mean force of 51% greater than 0-$Ca^{2+}$KH (*significantly different; 95% CI: 26–76%). (B) Mean force produced by $10^{-6}$ M prazosin in KH and $10^{-6}$ M PE in KH with prazosin added 30 min prior. Data for seven individual calluses were pooled to produce mean forces developed in each solution. No statistically significant difference between groups was found (mean 5% increase, 95% CI: 2–11%). (C) Mean force produced in 0-$Ca^{2+}$KH and $10^{-6}$ M terbutaline in KH. Data for seven individual calluses were pooled to produce mean forces developed in each solution. No statistical difference was observed between groups.

(Fig. 2B, $n = 7$, mean 5% increase in force, 95% CI: 2–11%). Terbutaline did not induce relaxation of calluses (Fig. 2C, $n = 7$, mean 1% decrease in force, 95% CI: 7–9%).

### qPCR

There was no difference in $\alpha_1$AR gene expression in calluses at 7 days compared to unfractured ribs (Fig. 3A; $p = 0.29$). Expression of $\alpha_1$ARs in calluses remained relatively constant throughout the healing period and was similar to unfractured ribs. There was a decrease in $\beta_2$AR mRNA expression in calluses at 7 days post-fracture compared to unfractured ribs ($p < 0.05$), although by 14 and 21 day post-fracture expression levels were back towards prefracture levels (Fig. 3B).

### Immunohistochemistry

There was intense $\alpha_1$AR-LI in osteoprogenitor cells in fibrous regions of 7 day callus, although a number of cells did not display $\alpha_1$AR-LI in this tissue (Fig. 4A). Osteoblasts on trabecular bone exhibited intense $\alpha_1$AR-LI (results not shown). Osteoprogenitor cells and osteoblasts on newly formed bone, showed minimal to no $\beta_2$AR-LI, however, chondrocytes within callus displayed moderate $\beta_2$AR-LI (Fig. 4B). Rat lung bronchioles displayed intense $\beta_2$AR-LI (results not shown).

## DISCUSSION

In 7 day callus, PE induced a significant force over and above the passive component, thereby suggesting that

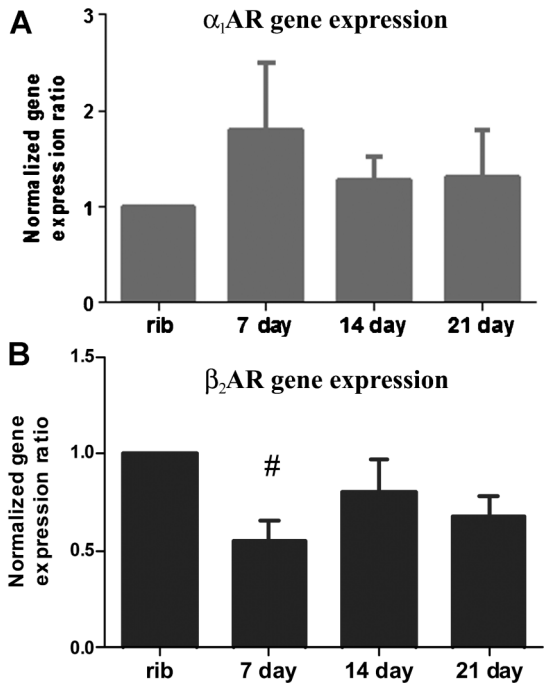


**Figure 3.** (A) $\alpha_1$AR gene expression relative to GAPDH in unfractured rib and 7, 14, and 21 day fracture calluses ($n = 7$ for each group). No statistical difference was observed between groups. (B) $\beta_2$AR gene expression in unfractured rib as well as 7, 14, and 21 day fracture calluses ($n = 7$ for each group), expressed relative to GAPDH. $\beta_2$AR expression was down-regulated 7 days after fracture ($^{\#}p > 0.01$) but returned towards unfractured rib levels by 14 days post-fracture.

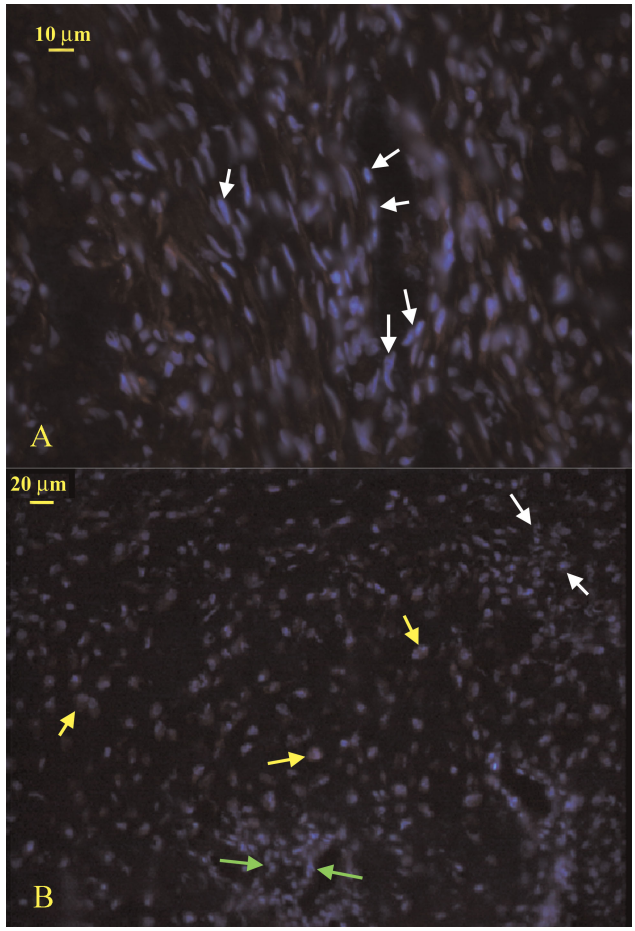


**Figure 4.** (A) Immunohistochemistry for $\alpha_1$AR-LI of 7 day fibrous callus. Osteoprogenitor cells display intense $\alpha_1$AR-LI (red Cy3 fluorescence), however a number of cells lack $\alpha_1$AR-LI (white arrows). Nulcei blue: stained with DAPI. Original magnification ×400. (B) Immunohistochemistry for $\beta_2$AR-LI. Seven-day-old fibrous callus osteoprogenitor cells (white arrows) exhibit no apparent $\beta_2$AR-LI (indicated by red, Cy3 fluorescence). Chondrocytes display moderate $\beta_2$AR-LI (yellow arrows). Osteoblasts on newly formed bone lack $\beta_2$AR-LI (green arrows). Nulcei blue: stained with DAPI. Original magnification ×200.

PE stimulated active contraction of this tissue. Contractions of this nature are deemed to be sustained tonic contractions.[25] Various studies on smooth muscle also report that PE induces large, prolonged tonic contractions.[25–28] In smooth muscle following an initial phase of tonic contraction, force is able to be maintained with a relatively lower level of myosin light chain phosphorylation and thus cross-bridge cycling.[29] This phase of steady force is commonly termed the "latch state."[29,30] However, in typical smooth muscle this maintained level of contraction fatigues after some time.[31] In contrast, we observed no evidence of fatigue in callus responses to PE.

The mechanism of PE action on callus appeared to be via $\alpha_1$AR activation. Both immunohistochemistry and gene expression analyses provided additional evidence that 7 day fracture callus contains $\alpha_1$ARs. Specifically, $\alpha_1$AR-LI was localized to osteoprogenitor cells in fibrous regions of early callus. Further, pretreatment with the $\alpha_1$AR antagonist, prazosin completely inhibited the

active contractile response to PE in KH. Studies on smooth muscle have found that prazosin administered at similar concentrations completely blocked PE-induced contractions.[23] Active contractions induced by PE and the inhibitory action of prazosin, together with the intense $\alpha_1$AR-LI on osteoprogenitor cells of fibrous callus signify the likely existence of $\alpha_1$ARs in contractile cells of callus. It is possible that sustained tonic contractions by $\alpha_1$AR activation may also occur in vivo, as concentrations of the $\alpha_1$AR agonist noradrenaline increase in early fracture healing.[13]

Although $\alpha_1$AR activation seems to have initiated contraction, we were not able to define the exact intracellular pathway through which contraction occurred. Measurements of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) concentrations throughout the period of tonic contraction would provide an insight of the intracellular pathways that are possibly involved.[25] Crowley et al.[25] used $[Ca^{2+}]_i$ imaging by confocal microscopy to determine that PE induced a transient rise in $[Ca^{2+}]_i$ only during development of tonic contraction in saphenous veins of humans. They suggested maintenance of PE-induced tonic contraction was most likely by signals other than $Ca^{2+}$, such as Rho kinase, that cause smooth muscle myofilaments to become sensitized to $Ca^{2+}$. However, while Rho kinase is known to cause $Ca^{2+}$ sensitization,[12] the concept of $\alpha_1$AR activation initiating the Rho-Rho kinase pathway in smooth muscle is yet to be confirmed.[32] For these reasons, we are unable to speculate the exact intracellular pathway that is initiated by $\alpha_1$AR activation in callus.

Although smooth muscle-like active contraction was apparent, we did not observe active relaxation in response to the $\beta_2$AR agonist terbutaline. Further, there was a down-regulation of $\beta_2$AR gene expression in callus tissue at 7 days post-fracture and little to no $\beta_2$AR immunoreactivity in callus osteoprogenitor cells. Taken together, these results suggest contraction (rather than relaxation) may be a preferential mechanism during fracture healing.

Differentiation of smooth muscle cells to an osteoblast-like phenotype appears to be mechanically controlled, that is, inhibited in the presence of cyclic-strain and enhanced under static-strain.[17] In our previous study, we proposed that the passive viscoelastic phenomena of stress relaxation and reverse stress relaxation may promote maintenance of such static conditions.[1] The present study found PE generated prolonged tonic contractions significantly above the passive component. This maintained tonic contraction is a form of static-strain,[33] and may therefore stimulate differentiation of callus smooth muscle-like osteoprogenitor into cells displaying an osteoblast-like phenotype (Fig. 5). Furthermore, studies have shown that actively contracted smooth muscle tissue has an increased resistance to stretch, thus providing an additional mechanism to promote the desired static conditions in early callus.[34]

This study has described for the first time that early callus of fractured rib of rat can be pharmacologically induced to actively contract. By using an isometric force transducer we found the $\alpha_1$AR agonist PE stimulated sustained tonic contractions of a 7 day bone fracture callus, signifying the likely existence of $\alpha_1$ARs on contractile cells of callus. The presence of these receptors was supported by their immunohistochemical localization in osteoprogenitor cells of early callus. Our findings suggest that $\beta_2$ARs may not be present in callus osteoprogenitor cells and perhaps contraction (rather than relaxation) is the preferential mechanism to promote fracture healing.

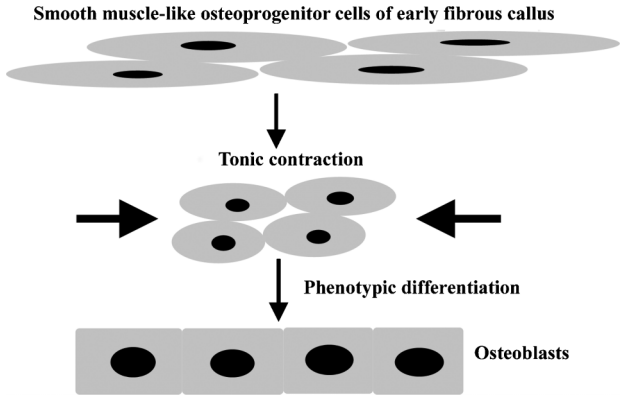


**Figure 5.** Hypothesized role of how $\alpha_1$AR agonists may promote osteoprogenitor cell contraction and differentiation to osteoblasts during early bone fracture healing in vivo.

It remains to be seen whether this pharmacologically induced contraction is unique to the healing rat rib or whether it may occur in other bone fracture models. We propose that, $\alpha_1$AR agonists such as noradrenaline may play a role in the healing process in vivo, at least in the rat rib, by actively contracting callus tissue to provide strain that favors osteogenesis. Therefore, future studies should investigate whether administration of $\alpha_1$AR agonists during the early callus formation improves fracture healing in vivo.

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